





The Science and Applications of Synthetic and Systems Biology: Workshop Summary

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Eileen R. Choffnes, David A. Relman, and Leslie Pray, Rapporteurs;
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THE SCIENCE AND APPLICATIONS OF SYNTHETIC AND SYSTEMS BIOLOGY

Workshop Summary

Eileen R. Choffnes, David A. Relman, and Leslie Pray, *Rapporteurs*

Forum on Microbial Threats

Board on Global Health

INSTITUTE OF MEDICINE
OF THE NATIONAL ACADEMIES

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Willing is not enough; we must do.”*

—Goethe



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Although the reviewers listed above have provided many constructive comments and suggestions, they were not asked to endorse the final draft of the report before its release. The review of this report was overseen by **Dr. Melvin Worth**. Appointed by the Institute of Medicine, Dr. Worth was responsible for ensuring that an independent examination of this report was carried out in accordance with institutional procedures and that all review comments were carefully considered. Responsibility for the final content of this report rests entirely with the authoring committee and the institution.

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The Forum on Emerging Infections was created by the Institute of Medicine (IOM) in 1996 in response to a request from the Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH). The purpose of the Forum is to provide structured opportunities for leaders from government, academia, and industry to regularly meet and examine issues of shared concern regarding research, prevention, detection, and management of emerging, reemerging, and novel infectious diseases in humans, plants, and animals. In pursuing this task, the Forum provides a venue to foster the exchange of information and ideas, identify areas in need of greater attention, clarify policy issues by enhancing knowledge and identifying points of agreement, and inform decision makers about science and policy issues. The Forum seeks to illuminate issues rather than resolve them. For this reason, it does not provide advice or recommendations on any specific policy initiative pending before any agency or organization. Its value derives instead from the diversity of its membership and from the contributions that individual members make throughout the activities of the Forum. In September 2003, the Forum changed its name to the Forum on Microbial Threats.

The Forum on Microbial Threats and the IOM wish to express their warmest appreciation to the individuals and organizations who gave their valuable time to provide information and advice to the Forum through their participation in the planning and execution of this workshop. A full list of presenters, and their biographical information, may be found in Appendixes B and F, respectively.

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Workshop Overview

THE SCIENCE AND APPLICATIONS OF SYNTHETIC AND SYSTEMS BIOLOGY

Introduction

Humans have been modifying the genetic characteristics of plants and animals for millennia by controlling the breeding of species in order to select for certain traits or characteristics and to reduce or eliminate others. The discovery of the structure of DNA in 1953, the recognition of its importance as the carrier of heritable genetic information, followed by the development of recombinant DNA technology two decades later, paved the way for powerful technologies to manipulate genes directly and in such a way that the genotype and phenotype of an organism can be altered with utmost precision in a single generation.

The realization of viral and microbial genomics, in the last few decades of the 20th century, coupled with the completion of the initial draft of the human genome sequence in 2001, reflect a fundamental shift in the way biology is studied, and has opened a portal to vast postgenomic possibilities. Because of the Human Genome Project, scientists have already identified more than 1,800 genes associated with particular diseases. More recently, scientists have developed techniques to more efficiently synthesize or modify larger segments of DNA, marking a significant change in the way people study biological systems and a growing capacity for both experts and amateurs to manipulate such systems.

Until the past decade, the work was often painstakingly slow, and able to address only relatively straightforward challenges such as the manipulation of one gene at a time. More novel or complex genetic modifications would be difficult

to construct using the more conventional recombinant DNA¹ (rDNA) techniques of the 1970s and 1980s. In the past decade or two newer approaches—combining engineering and biological techniques—have enhanced researchers’ abilities to manipulate DNA. These new synthetic techniques allow for genes and long chains of DNA to be designed and constructed from scratch using a computer and relevant chemical compounds, rather than by employing a “trial-and-error” approach to the identification and insertion of pieces of existing genes from living cells into a novel host environment.

In May 2010, researchers at the J. Craig Venter Institute announced that they had produced the first functional, self-replicating, bacterium whose entire nuclear genome had been synthesized artificially in the laboratory, albeit using a naturally occurring genome sequence as a template (Gibson et al., 2010). While the achievement did not, as some media reports at the time suggested, represent the “creation of life,” it did propel the nascent field of synthetic biology into the mainstream, and generated a number of questions and much speculation about the potential power, utility and risks associated with work in this field.

Although biologists may have a long way to go before they have enough knowledge and the tools necessary to design and build life, the emerging field of synthetic biology has already reduced several novel products and lead compounds for drugs and vaccines, fuel, biofabrication of materials, and other industrial applications. Most, if not all, of these products and compounds are being generated via the type of top-down approach, with scientists reengineering existing cells to do things that they do not normally do. By inserting the genetic machinery for metabolic pathways into *Escherichia coli* and other host organisms, scientists are attempting to create microbial bio-factories for the production of pharmaceutical ingredients, flavors, fragrances, and other chemical products (Ro et al., 2006). The goals also include compounds and cells with new phenotypes and functionalities, such as cells that can produce carbon-neutral biological fuels with properties that are similar to those of petroleum-based fuels (Fortman et al., 2008; Keasling, 2010) and novel drugs (Li and Vederas, 2009).

The United Kingdom’s Royal Academy of Engineering observed that “[s]ystems biology aims to study natural biological systems as a whole, often with a biomedical focus, and uses simulation and modeling tools in comparisons with experimental information. *Synthetic biology* aims to build novel and artificial biological parts, devices and systems. Many of the same methods are used and as such there is a close relationship between synthetic biology and systems biology. But in synthetic biology, the methods are used as the basis for engineering applications” (Royal Academy of Engineering, 2009, *emphasis added*). While both disciplines use similar approaches, systems biology uses these approaches to better understand the inner-workings of life, whereas synthetic biology emphasizes

¹ Recombinant DNA: DNA that is created in the laboratory by splicing together DNA molecules from different sources, usually for replication in a host organism.

the application of the lessons learned from systems biology for the purpose of engineering (or reengineering) living systems to behave in specified ways.

Many potential applications of synthetic and systems biology are relevant to the challenges associated with the detection, surveillance, and responses to emerging and re-emerging infectious diseases. On March 14 and 15, 2011, the Institute of Medicine's (IOM's) Forum on Microbial Threats convened a public workshop in Washington, DC, to explore the current state of the science of synthetic biology, including its dependency on systems biology; discussed the different approaches that scientists are taking to engineer, or reengineer, biological systems; and discussed how the tools and approaches of synthetic and systems biology were being applied to mitigate the risks associated with emerging infectious diseases. Through invited presentations and discussion, participants explored the ways in which synthetic and systems biology are contributing to drug discovery, development, and production; vaccine design and development; and infectious disease detection and diagnostics. In addition, workshop participants considered how synthetic biology could be used to engineer, or reengineer, microbial host cells to detect environmental toxins, produce carbon-neutral fuels, and produce novel raw materials.

Organization of the Workshop Summary

This workshop summary was prepared by the rapporteurs for the Forum's members and includes a collection of individually authored papers and commentary. Sections of the workshop summary not specifically attributed to an individual reflect the views of the rapporteurs and not those of the members of the Forum on Microbial Threats, its sponsors, or the IOM. The contents of the unattributed sections are based on presentations and discussions at the workshop.

The summary is organized into sections as a topic-by-topic distillation of the presentations and discussions that took place at the workshop. Its purpose is to present information from relevant experience, to delineate a range of pivotal issues and their respective challenges, and to offer differing perspectives on the topic as discussed and described by the workshop participants. Manuscripts and reprinted articles submitted by some but not all of the workshop's participants may be found, in alphabetical order, in Appendix A.

Although this workshop summary provides a description of the individual presentations, it also reflects an important aspect of the Forum's philosophy. The workshop functions as a dialogue among representatives from different sectors and allows them to present *their* views about which areas, in their opinion, merit further study. This report only summarizes the statements of participants at the workshop over the course of two consecutive days. This workshop summary report is not intended to be an exhaustive exploration of the subject matter nor does it represent the findings, conclusions, or recommendations of a consensus committee process.

What Is Synthetic Biology?

The idea of managing or manipulating biology to identify or develop specific characteristics is not new. Scientists have used DNA to create genetically engineered cells and organisms for many years; the entire biotechnology industry has grown around our expanding abilities in this area.

—Presidential Commission for the Study of Bioethical Issues (2010)

Synthetic biology is not an entirely new science. Rather, aspects of it are an outgrowth of what plant and animal breeders have been doing for thousands of years and genetic engineers have been doing for decades—mixing and matching genetic material with the goal of “creating” novel plants and animals with desirable traits. What differentiates synthetic biology from genetic engineering is its goal of designing new genetic systems and organisms using standardized parts from the “ground up.”

Although the term “synthetic biology” has been used in various ways, it is generally understood to describe research that combines biology with the principles of engineering to design, construct, or adapt existing DNA, or other biological structures into standardized, interchangeable, building blocks for use in creating genetic systems that carry out desired functions. The vision behind this science is that these biological “parts” can be joined to create engineered cells, organisms, or biological systems that reliably behave in predictable ways to perform specific tasks (Khalil and Collins, 2010; NSABB, 2010; Presidential Commission for the Study of Bioethical Issues, 2010; Royal Academy of Engineering, 2009). Synthetic biologists eventually hope to be able to program cells, cell systems, or organisms to perform specific tasks and functions (see Figure WO-1).

Synthetic biology may also involve modifying naturally occurring genomes² to allow these modified genomes to function in new contexts or to create entirely novel organisms. In 2010, when scientists at the J. Craig Venter Institute, Rockville, Maryland, reported having designed, synthesized, and assembled a complete *Mycoplasma mycoides* genome which they then transplanted into an *M. capricolum* recipient cell, creating a continuously self-replicating cell controlled by an artificial genome, much of the media proclaimed that the scientists had created “artificial” life (Gibson et al., 2010). Despite the “hype” surrounding this experiment the Venter Institute scientists did not create artificial, or even new, life.

In fact, Venter’s team did what breeders have been doing for millennia—they facilitated and helped to direct the transfer of genetic material from one organism into another. But they did it in a way that had never been done before. They developed new methods that allowed them to assemble an entire synthetic genome and fabricate its parts faster and with fewer errors than investigators have done in the past with other large pieces of DNA (Bedau et al., 2010). So while synthetic

² An organism’s entire hereditary information usually encoded in DNA.

WORKSHOP OVERVIEW

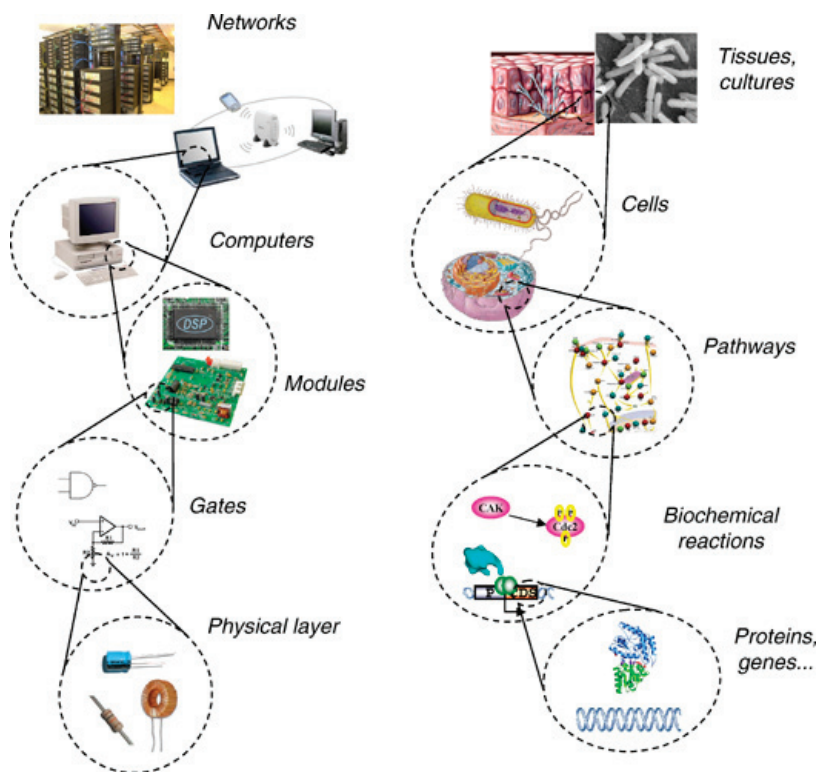


FIGURE WO-1 This figure illustrates the synthetic biology concept that complex biological systems can be broken down into their component parts in a similar way as more traditional engineering disciplines.

SOURCE: Andrianantoandro et al. (2006).

biology represents a revolutionary change in the way people interact with life, as James Collins of Boston University observed, “Synthetic biology is in its very early stages. Don’t believe the hype.” (Dr. Collins’ contribution to the workshop summary report can be found in Appendix A, pages 117-150.)

Speaker Christopher Voigt of the University of California, San Francisco (UCSF)³ noted that one goal of synthetic biology was to be able to “mix and match” functions from the natural world in order to create organisms that carry out these functions in ways that a single, naturally occurring organism cannot. (Dr. Voigt’s contribution to the workshop summary report can be found in Appendix A, pages 429-449.) Some researchers seek to exploit systems biology–derived

³ At the time of this workshop, Dr. Voigt was at the University of California, San Francisco. He is now at the Massachusetts Institute of Technology.

modeling tools to guide the design of synthetic gene networks. In fact, because of this element of design, some have compared synthetic biology to engineering (Khalil and Collins, 2010; NSABB, 2010; Presidential Commission for the Study of Bioethical Issues, 2010; Royal Academy of Engineering, 2009). Yet, naturally occurring living cells and organisms are complex adaptive systems, whose behavior lies far beyond our ability to re-create with currently available principles and components.

Biological Systems

One important driver of the rapid growth of synthetic biology has been a shift away from the traditional reductionist method for understanding biological processes to one that favors a more holistic “systems” approach. Historically, the typical way one examined biological systems had been to isolate a small subset of biological components, which could then be interrogated individually to better understand their structures and functions. This approach assumes that the interactions of biochemical components occur in isolation, resulting in discrete, cause-and-effect, relationships.

As our understanding of biology at the level of DNA, RNA, and proteins has increased, it has become clear that biological processes occur not in isolation but rather within the context of complex systems of components, regulated by intricate networks of feedback loops. These systems operate on a variety of levels: from that of RNA polymerase interacting with a DNA strand to start the process of DNA transcription, to a signal-transduction pathway within a cell, to complex interactions between systems of organisms. While our appreciation of the complexity of interactions within and between these systems has grown, there has been a corresponding recognition that the traditional, reductionistic, scientific approach severely limits our ability to understand complex biological phenomena and interactions within and between cells. Investigators have increasingly embraced systems approaches in their efforts to understand biological interactions, taking advantage of the power of mathematical and computer modeling to examine the complex interactions between components of a biological system (Royal Academy of Engineering, 2009).

What Is Systems Biology?⁴

The increased emphasis on understanding biological phenomena in the context of the system within which it occurs gave rise to systems or integrative biology—which is the “study of the behavior of complex biological organization and processes in terms of the molecular constituents” (Kirschner, 2005). It takes

⁴This section was adapted from p. 173 of the 2006 Institute of Medicine report, *Globalization, Biosecurity, and the Future of the Life Sciences*. Washington, DC: The National Academies Press.

advantage of high-throughput, genome-wide tools—such as microarrays—for the simultaneous study of complex interactions involving molecular networks, including DNA, RNA, and proteins. It is, in a sense, classical physiology taken to a new level of complexity and detail.

The term “systems” comes from systems theory or dynamic systems theory: systems biology involves the application of systems- and signal-oriented approaches to the understanding of inter- and intracellular dynamic processes (Wolkenhauer et al., 2005). Systems-level problem solving in living systems is based on the observation that cellular behavior involves a complex coordination of dynamically interacting biomolecular entities. Systems biologists seek to quantify all of the molecular elements that make up a biological system and then integrate that information into network models that can serve to generate predictive hypotheses.

A growing number of investigators within the life sciences community are recognizing the utility of systems biology tools and approaches for studying complex regulatory networks—both inside the cell, as well as the regulatory networks that integrate and control function of distinctly different cell types in multi-cellular organisms like humans—and for making sense of the vast, and rapidly accumulating, genomic and proteomic data sets (Aloy and Russell, 2005; Goldbeter, 2004; Rousseau and Schymkowitz, 2005; Uetz et al., 2005). These efforts draw heavily on computational methods to model the biological systems.

Systems biology has become a valuable approach for drug discovery (Apic et al., 2005; Young and Winzeler, 2005). In medicine, disease is often viewed as an observable change of the normal network structure of a system resulting in damage to the system.⁵ A systems biology approach can provide insights into how disease-related processes interact and are controlled; guide new diagnostic and therapeutic approaches; and enable a more predictive, preventive, personalized medicine (Hood et al., 2004).

Relationship Between Synthetic and Systems Biology

Advances in synthetic biology are closely dependent on, and interactive with, advances in systems biology. When assembling new structures, whether single genes or more complex whole cells or organisms, the complexity imbued by interactions among components is vitally important to the proper functioning of the system-to-be. The key to successfully engineering, or reengineering, biological systems, is through understanding their complexity.

Synthetic biologists are making headway toward handling more complex structures in more efficient ways. Chris Voigt’s work with code refactoring is an example of how advances in synthetic biology are paving the way for more re-

⁵ Disease-perturbed proteins and gene regulatory networks differ from their healthy counterparts, because of genetic or environmental influences.

financed biological synthesis. Code refactoring is a reordering of the DNA sequence of a gene or gene cluster in order to eliminate inefficiencies—such as overlapping sequences—and otherwise alter the genetic code so that it can more readily be integrated into multiple genetic backgrounds.

Because synthetic and systems biology both employ similarly complex computational modeling and emphasize the role of the biological systems to contextualize and make sense of biological phenomena, the distinctions between these two disciplines sometimes seem blurred. Nevertheless, there is an important distinction between these two approaches. “While [s]ystems [b]iology attempts to obtain a quantitative understanding of existing biological systems, [s]ynthetic [b]iology is focused on the rational engineering of these systems” (Serrano, 2007).

Is Synthetic Biology a True Engineering Discipline?

DNA synthesis enables the *de novo* generation of genetic sequences that specifically program cells for any of a wide range of purposes, including the expression of a given protein. Technical developments continue to increase the speed, ease, and accuracy with which larger and larger sequences may be chemically generated. By the early 1970s, scientists had demonstrated that they could produce synthetic genes (Agarwal et al., 1974). Yet, it was the automation of *de novo* DNA synthesis and the development of the polymerase chain reaction (PCR) in the early 1980s that catalyzed the development of a series of cascading methodologies for the analysis of gene expression, structure, and function. Our ability to synthesize short oligonucleotides (typically 10 to 80 base pairs in length) rapidly and accurately has been an essential enabling technology for countless advances, not the least of which has been the sequencing of the human genome. The past few years have seen remarkable technological advances in this field, particularly with respect to the *de novo* synthesis of increasingly longer DNA constructs.

The field of synthetic biology is driven by this increasing capacity to make long, accurate, DNA molecules of pre-specified sequence. Indeed, DNA synthesis is arguably the most important tool in the synthetic biologist’s toolbox. Keynote speaker Andrew Ellington of the University of Texas emphasized that sequence information is the basic commodity of synthetic biology—it is fungible, digital, and portable. (Dr. Ellington’s contribution to the workshop summary report can be found in Appendix A, pages 150-159.) Moreover, sequence information has the potential to be standardized, recoded, or programmed. Genome sequences, moreover, provide a huge amount of information about model organisms that synthetic biologists use as platforms for their gene circuit designs.

Ellington observed that this growing capacity for *de novo* synthesis is due, in large part, to the large number of participants in the commercial sector, as well as funding from the public sector. The chemical synthesis and ligation of large segments of a DNA template, followed by enzymatic transcription of RNA led to the *de novo* creation of the poliovirus genome in 2002 (about 7,500

nucleotides in length), from which the infectious, virulent virus was rescued following its transfection into permissive cells (Cello et al., 2002). The following year, scientists announced the successful assembly of a bacterial virus genome (Smith et al., 2003). Parallel efforts in industry and academia led to the synthesis and assembly of large segments of the hepatitis C virus genome, from which replication-competent RNA molecules were rescued. Ellington cautioned that even these systems would perform differently in different host “contexts” and in ways that are not entirely predictable.

Ellington went on to remark that, while scientists clearly are building synthetic biological systems that work, the “ability to model such systems—not make them work but model them in a true engineering way—is somewhat limited.” He pointed to the photographic bacteria built by Levskaya et al. (2005) and the “edge detector” *E. coli* built by Tabor et al. (2009)—illustrated in Figure WO-2—as examples of synthetic systems that clearly work.

Even those parts that are well characterized, quantitatively or otherwise, do not always behave as expected. As Ellington observed, “The construction of very large fragments of DNA is no longer a limitation in the engineering of biological systems. Predicting the behavior of complex genetic programs *de novo* is now the limiting step in the programming of cellular behavior” (Tabor et al. 2009). Ellington reported that the reengineered viruses that he and his team built did not function once they were actually inserted into host cells. Therefore, successfully building, or rebuilding, a virus or other biological system according to Ellington does not necessarily mean that the system is actually going to work.

Ellington and his team have applied a so-called “supercharging technology” to the anti-MS2 antibody (Lawrence et al., 2007). They synthesized a large number of genes that conferred either large negative or large positive charges on the antibodies, overexpressed them, and then tested them for functionality. In the end, only a few candidate antibodies were able to bind to MS2. Despite using the best predictive modeling available, the success rate was low enough that only by rapid prototyping of multiple, different antibody variants could they find ones that had both improved thermal resistance as well as improved affinity. Ellington concluded, “You can make a lot of DNA, but that doesn’t mean it is going to function the way you want it to.”

Added to this systems-level complexity is the challenge of evolution and the reality that genetically-based biological systems gain, and lose, functionality over time (Tabor et al, 2009). According to Ellington and others at this workshop, organisms are “evolutionary machines.” Not only does evolution happen, making it extraordinarily difficult to predict how an engineered biological system will behave over time but, as Ellington observed, a device that works in one host organism does not necessarily function the same way in another host organism.

Ellington commented that while the development of standardized parts was not impossible, our present capacity to accurately predict how these “parts” would function in a particular system is still “somewhat limited.” He referred to

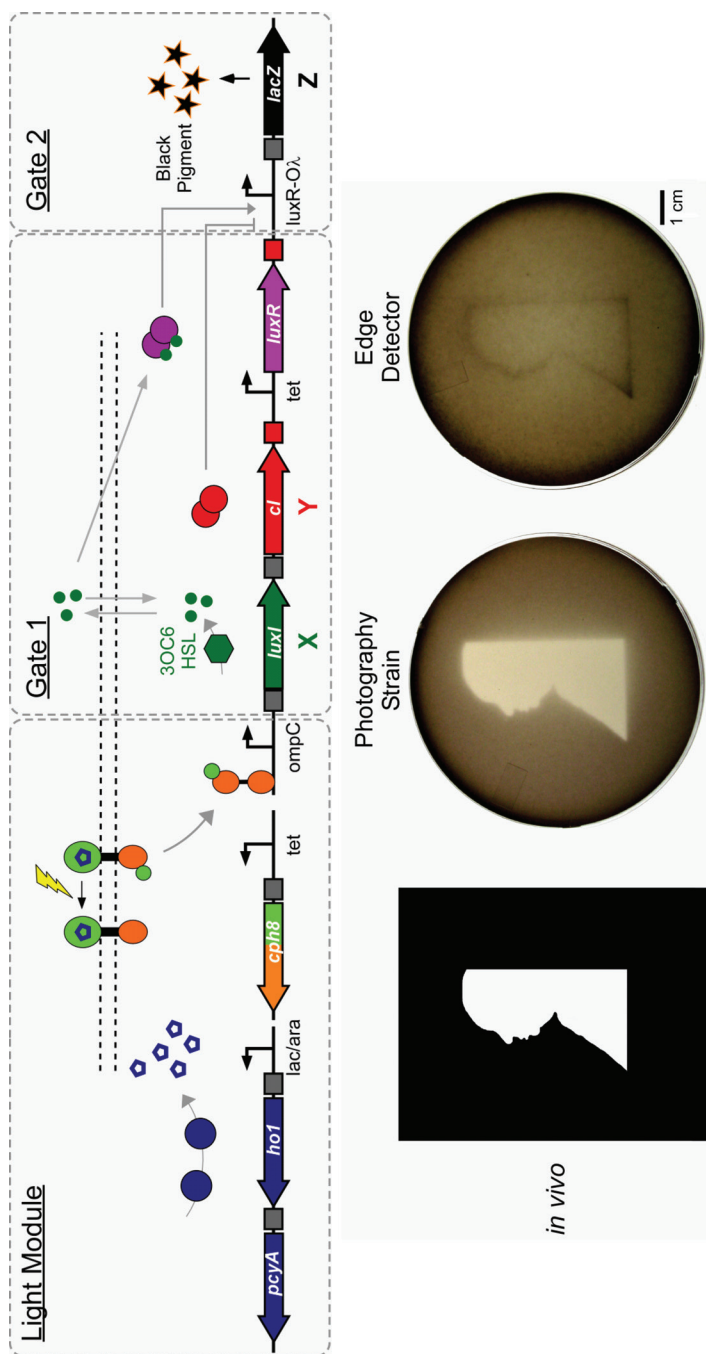


FIGURE WO-2 Construction of bacteria that are capable of light-dark edge detection. SOURCE: Ellington (2011; adapted from *Cell*).

the “unrealized promise of BioBricks,”—“that we are going to be able to say that a given part works a given way in a system not yet determined.” He went on to observe that unfortunately, the complexity of organisms—and this is the systems biology part of things—dwarfs our ability to accurately model function.

The same technologies employed by synthetic biologists for “good” could also be exploited for malevolent purposes—a classical dual-use dilemma. Ellington pointed to DNA synthesis and the growing capacity to make larger pieces of DNA quickly and affordably as the greatest cause for concern. While the dual-use dilemma is real, Ellington observed that the threats posed by synthetic biology are dwarfed by the expansive realm of real microbial threats that already exist in nature.

Design and Complexity

Speaker Herbert Sauro, of the University of Washington, observed that the level of complexity in a synthetic biological system expands very quickly as the number of feedbacks in a system increases. (Dr. Sauro’s contribution to the workshop summary report can be found in Appendix A, pages 394-417.) Simple linear pathways, whereby a perturbation of a single enzyme affects another enzyme in a predictable manner, according to Sauro, is largely understandable and engineerable; the greater the complexity, the less “engineerable” the system is. As depicted in Figure WO-3, if the pathway has any sort of feedback in it, understanding—and engineering—that pathway becomes slightly more complicated, requiring some simulation. Sauro observed that pathways with two or more feedbacks are virtually impossible to understand analytically and require a considerable amount of simulation.

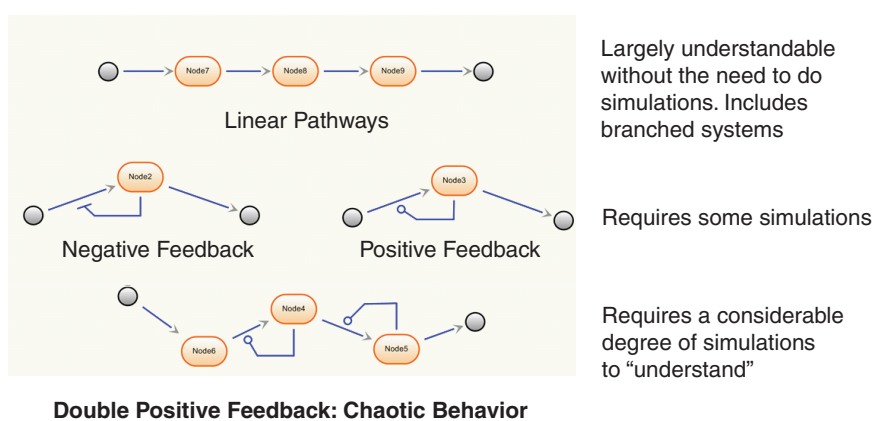
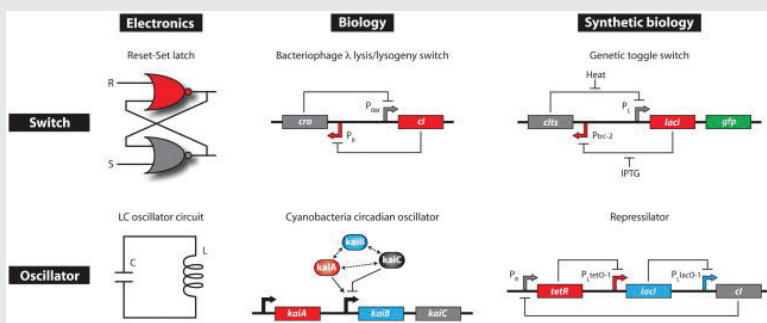


FIGURE WO-3 Biosynthetic pathways with increasing complexity.
SOURCE: Sauro (2011).

BOX WO-1 Early Synthetic Biology Designs: Switches and Oscillators



Switches and oscillators that occur in electronic systems are also seen in biology and have been engineered into synthetic biological systems.

Switches

In electronics, one of the most basic elements for storing memory is the reset–set (RS) latch based on logical NOR gates. This device is bistable in that it possesses two stable states that can be toggled with the delivery of specified inputs. Upon removal of the input, the circuit retains memory of its current state indefinitely. These forms of memory and state switching have important functions in biology, such as in the differentiation of cells from an initially undifferentiated state. One means by which cellular systems can achieve bistability is through genetic mutual repression. The natural $P_{R}-P_{RM}$ genetic switch from bacteriophage λ , which uses this network architecture to govern the lysis–lysogeny decision, consists of two promoters that are each repressed by the gene product of the other (that is, by the Cro and C1 repressor proteins). The genetic toggle switch constructed by Dr. Collins' research group is a synthetically en-

James J. Collins, from Boston University, agreed with Sauro that it is very difficult to build biological systems that function in a predictable manner. Nonetheless, synthetic biology, according to Collins, is “taking inspiration” from engineering, electrical engineering in particular. As illustrated in Box WO-1, many of the earliest synthetic biology devices were the biological equivalents of electronic toggle switches, latches, oscillators, and other similar devices (Elowitz and Leibler, 2000; Gardner et al., 2001; Khalil and Collins, 2010).

Collins observed that many biologists have a misperception of engineering with respect to the importance of mathematical modeling. According to Collins, most practicing engineers use mathematical modeling as a guide only—in much the same way that synthetic biologists do. When actually assembling components, engineers rely on intuition and “tinkering,” often without even understanding how a system works.

gineered version of this co-repressed gene regulation scheme (Gardner et al., 2000). In one version of the genetic toggle, the P_L promoter from λ phage was used to drive transcription of *lacI*, the product of which represses a second promoter, *P_{trc2}* (a *lac* promoter variant). Conversely, *P_{trc2}* drives expression of a gene (*cl-ts*) encoding the temperature-sensitive (*ts*) λ CI repressor protein, which inhibits the P_L promoter. The activity of the circuit is monitored through the expression of a green fluorescent protein (GFP promoter). The system can be toggled in one direction with the exogenous addition of the chemical inducer isopropyl- β -D-thiogalactopyranoside (IPTG) or in the other direction with a transient increase in temperature. Importantly, upon removal of these exogenous signals, the system retains its current state, creating a cellular form of memory.

Oscillators

Timing mechanisms, much like memory, are fundamental to many electronic and biological systems. Electronic timekeeping can be achieved with basic oscillator circuits—such as the LC circuit (inductor L and capacitor C)—which act as resonators for producing periodic electronic signals. Biological timekeeping is achieved with circadian clocks and similar oscillator circuits, such as the one responsible for synchronizing the crucial processes of photosynthesis and nitrogen fixation in cyanobacteria. The circadian clock of cyanobacteria is based on, among other regulatory mechanisms, intertwined positive and negative feedback loops on the clock genes *kaiA*, *kaiB*, and *kaiC*. Elowitz and Leibler constructed a synthetic genetic oscillator based not on clock genes but on standard transcriptional repressors (the repressilator) (Elowitz and Leibler, 2000). Here, a cyclic negative feedback loop composed of three promoter–gene pairs, in which the “first” promoter in the cascade drives expression of the “second” promoter’s repressor, and so on, was used to drive oscillatory output in gene expression.

SOURCE: Image and text: Khalil, A. S., and J. J. Collins. 2010. Synthetic biology: applications come of age. *Nature Reviews Genetics* 11:367-379. Reprinted with permission from Nature Publishing Group.

As discussed by Ellington and Sauro, evolution can, for example, change the dynamics of a synthetic biological system. As Sauro explained, the unpredictable nature of, and responses to, selection makes it very difficult for synthetic biologists to control the behavior of their engineered systems. In 2008, a group of investigators built a genetic circuit containing a luminescence gene *luxR*. As illustrated in Figure WO-4, the Lux-R protein was activated when the bacterial signaling molecule acyl homoserine lactone (AHL) was present in the environment, triggering production of green fluorescent protein; GFP production was observed to degrade and terminate after 30-40 generations (Canton et al., 2008).

Sauro and colleagues subsequently constructed a variety of circuits derived from the Canton et al. (2008) circuit and demonstrated that the system loses functionality over time due to the evolution of nonluminescent mutant cells with smaller metabolic loads that grow faster than the bioluminescent cell lines

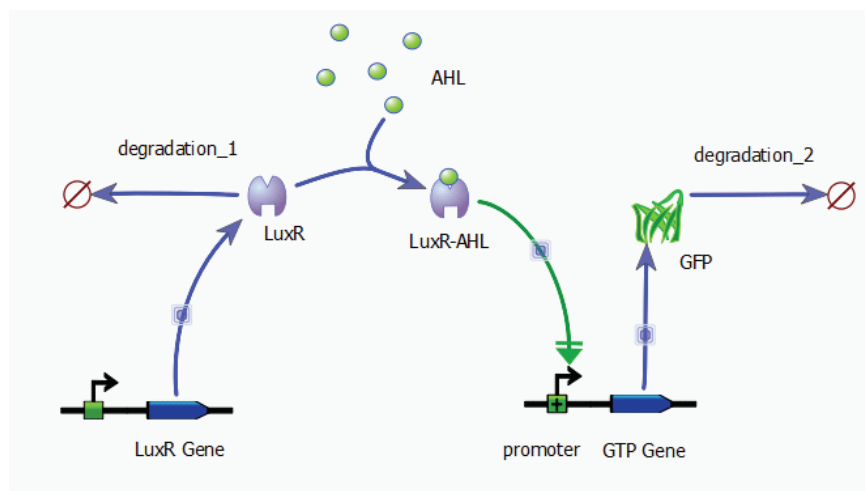


FIGURE WO-4 Improving mutational robustness in a genetic circuit.
SOURCE: Sauro (2011; adapted from Canton et al. [2008]).

(Sleight et al., 2010). Sauro went on to explain that, “every time you put a circuit into a cell, you are overloading it. The minute you overload it, the growth rate goes down a little bit.” If any mutants emerge that can grow faster than the synthetic cell type, they will eventually become the predominant cell type in the population.

Sauro and his team have demonstrated how reengineering components of genetic circuits, by using different transcript terminator, can prolong the evolutionary time over which a device is effective (Sleight et al., 2010). Sauro is also exploring the possibility of actually using directed evolution, instead of engineering, to build more robust and stable circuits. On a more practical level, Jay Keasling of the University of California, Berkeley, and his team of investigators engineered a *Saccharomyces cerevisiae* yeast to produce artemisinic acid by modifying an existing metabolic pathway in the yeast and adding in a gene from *Artemisa annua* to convert the product into the drug precursor (Ro et al., 2006). (Dr. Keasling’s contribution to the workshop summary report can be found in Appendix A, pages 243-254.)

Synthetic Biology: Top-Down Versus Bottom-Up Approaches

Over the past 10 years, two different experimental approaches have emerged in synthetic biology. Both seek novel biological structures or systems with predictable properties and functions. The first, known as the “top-down” approach, involves modifying or reengineering an existing, functioning organism, biologi-

cal system, or genome to perform new tasks. Top-down synthetic biology experiments have predominated in the first decade of the field, allowing researchers to test designed biological circuits in a “chassis”⁶—such as *E. coli*—that is already functioning and self-replicating. The “bottom-up” approach, involves synthesizing functioning circuits and systems entirely from “scratch” using nonliving materials such as DNA nucleotides and lipid monomers. Bottom-up synthetic biology is still in its infancy—the assembly of standardized components into a functioning system is significantly more challenging than modifying an existing system to perform a new task (Bedau et al., 2010; Benner and Sismour, 2005; Fritz et al., 2010; Purnick and Weiss, 2009; Royal Academy of Engineering, 2009). Figure WO-5 provides a simplified conceptual framework for top-down versus bottom-up perspectives in the synthetic biology design process.

Most top-down systems biology experiments fall into one of two categories:

- attempts to combine useful elements from several different living systems to create a modified organism that can perform a desired task, and
- attempts to simplify existing organisms down to only those parts that are essential for life.

Examples of experiments in the first category include the engineering of a *Saccharomyces cerevisiae* yeast, allowing it to synthesize a precursor of the antimalarial drug artemisinin (Ro et al., 2006), and the modification of *Salmonella spp.* to produce spider silk (Widmaier et al., 2009). An example of the second category of top-down experiments is the attempt to create a minimal genome (Glass et al., 2006). The goal behind this effort is to develop a simplified “chassis organism” into which synthesized parts could be added with fewer complications than investigators currently confront when working with wild-type organisms (Glass et al., 2006; NSABB, 2010; Presidential Commission for the Study of Bioethical Issues, 2010).

The practitioners of bottom-up synthetic biology believe that it represents the future of the field, potentially allowing researchers to design entirely new forms of life. One of the key milestones for bottom-up synthetic biologists is the development of libraries of diverse, well-characterized biological components that can be assembled to form new systems—analogueous to how one assembles component parts on a computer motherboard. The aspirational goal of this effort is to one day be able to select these “parts” from a catalogue and use them to create completely synthetic “novel” self-replicating life forms that are purpose-built rather than derived from a preexisting organism.

⁶ In the context of synthetic biology, chassis refers to the cell or organism in which the engineered DNA or biopart is embedded in order to produce the desired device or system (Royal Academy of Engineering, 2009).

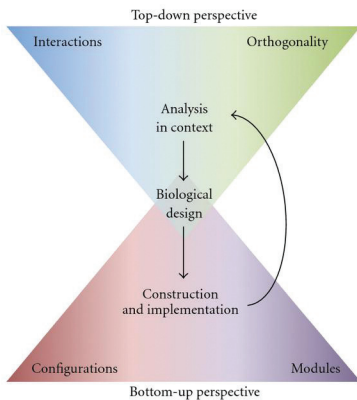


FIGURE WO-5 General conceptual framework for incorporating top-down and bottom-up perspectives in the synthetic biology design process. Due to our incomplete knowledge of biology, the design of biological systems through synthetic biology is currently an iterative process that incorporates both top-down and bottom-up design considerations. First, a design objective is identified. Next, a suitable synthetic biological system is designed given the known properties of well-characterized components (bottom-up). The synthetic system is then constructed and inserted into a larger biological context with which the synthetic system may interact (top-down), and performance of the combined system is assessed. If the system fails to meet performance requirements, this new information can be used to refine the design and repeat the cycle. Our ever-improving understanding of biology should reduce the number of iterations necessary to achieve a specific design objective.

SOURCE: Fritz et al. (2010).

Applications of Systems Biology

Mathematical methods that enable quantitative descriptions of the dynamic interplay between the molecules in living cells are being developed and, for the first time, it is possible to envisage a comprehensive molecular description of the functional circuitry of cellular systems.

—Bakker et al. (2010)

The grand challenge for biology and medicine at the beginning of the 21st century is to understand the biological complexity that emerges from interactions between our genomes and the environment. As speaker Bali Pulendran of Emory University observed, we are uniquely poised to tackle this challenge of biological complexity by the convergence of a new intellectual framework (a systems rather than a reductionistic view) and new technologies (for measuring and visualizing the behavior of genes, molecules, cells, organs, and organisms), coupled with the

innovation of computational and mathematical tools for dealing with complex data sets. (Dr. Pulendran's contribution to the workshop summary report can be found in Appendix A, pages 336-364.) The convergence of these disparate threads offers us an unprecedented opportunity to understand the fundamental features of life—from a holistic rather than solely reductionistic viewpoint; from a predictive rather than descriptive viewpoint; and, in short, from a systems biological viewpoint (Pulendran et al., 2010).

Systems biologists seek to quantify all of the molecular elements that make up a biological system and then integrate that information into network models that can serve to generate predictive hypotheses (Herrgård et al., 2008). A growing number of investigators are appreciating the utility of a “systems” approach for studying complex regulatory networks—both inside the cell, as well as among distinct cell types in multi-cellular organisms—and for making sense of the rapidly accumulating genomic and proteomic data sets (Aloy and Russell, 2005; Goldbeter, 2004; Rousseau and Schymkowitz, 2005; Uetz and Finley, 2005).

Despite the fact that this is still a young science—arguably in need of a “clear methodology”—scientists are relying on the tools and approaches of systems biology to make tremendous leaps in their understanding of various physiological phenomena, such as the mammalian immune response, and creating the potential for synthetic biologists to translate that knowledge into practice (Westerhoff et al., 2009). Speaker Bernhard Palsson, of the University of California, San Diego, observed that systems biology is being applied “more and more” to the study of infectious diseases. (Dr. Palsson's contribution to the workshop summary report can be found in Appendix A, pages 325-336.) Speakers Stephen Johnston of Arizona State University and Bali Pulendran remarked that the field of vaccinology may have reached a point where, with the appropriate support, it could become a more predictive science, guided by rational design rather than by Pasteurian trial and error.

Synthetic Biology Tools, Technologies, and Approaches

The rapid growth of bio- and other relevant technologies over the past 30 years has been driven by two processes working together: a quantitative increase in performance and decrease in cost of existing technologies and instruments, and qualitative changes resulting from unplanned new inventions, unexpected discoveries, and unexpected historical events. Synthetic biology would not be possible without a series of key technologies that have enabled investigators to design, fabricate, and manipulate DNA.

The following section briefly discusses five key sets of tools and technologies that drive synthetic biology: *DNA sequencing*, which has led to over 100 million gene sequences from approximately 260,000 different species being stored in public databases; *DNA synthesis*, which enables researchers to “reprint” whichever sequences they choose; *directed evolution*, which enables the rapid modifi-

cation of preexisting proteins and chemical pathways so that they perform new functions; *high throughput screening* (HTS), which provides scientists with the means to perform and measure large numbers of biochemical reactions rapidly; and *computational modeling*, which allows researchers to make qualitative or quantitative predictions about how their engineered systems are likely to function.

DNA Sequencing

DNA sequencing allows one to map an organism's genetic composition. Sequencing advances were instrumental to the success of the Human Genome Project and have allowed complete and large-scale DNA sequencing of many bacterial, and several plant and animal genomes. These genome sequences have provided a huge amount of information about many of the model organisms, such as *E. coli*, into which synthetic biologists often place their design circuits. Sequencing also allows synthetic biologists to verify whether their designed DNA circuits and parts have been correctly fabricated (Royal Academy of Engineering, 2009).

Significant reductions in the cost of DNA sequencing have also allowed the technology to proliferate (see illustration of this point in Figure WO-6). While it took years and cost approximately US\$300 million to sequence the first human genome a decade ago, second-generation sequencers now accomplish the same feat in a matter of days for approximately US\$20,000. It is anticipated that with the next generation of DNA sequencers the cost to sequence an entire human genome will drop to several hundred dollars (Metzker, 2010). Similarly, sequencers that once cost millions of dollars can now be purchased on eBay® for less than one thousand dollars.

DNA Synthesis

DNA synthesis is a technology that enables the *de novo* generation of genetic sequences that specifically program cells for the expression of a given protein. It is not new, but technical enhancements continue to increase the speed, ease, and accuracy with which larger and larger sequences may be chemically generated. DNA synthesis is arguably the most important tool in the synthetic biologist's toolbox.

By the early 1970s, scientists had demonstrated that they could produce synthetic genes (Agarwal et al., 1974). However, it was the automation of *de novo* DNA synthesis and the development of the polymerase chain reaction PCR in the early 1980s that catalyzed the development of a series of cascading methodologies for the analysis of gene expression, structure, and function. Our ability to synthesize short oligonucleotides (typically 10 to 80 base pairs in length) rapidly and accurately has been an essential enabling technology for countless advances, not the least of which has been the sequencing of the human genome.

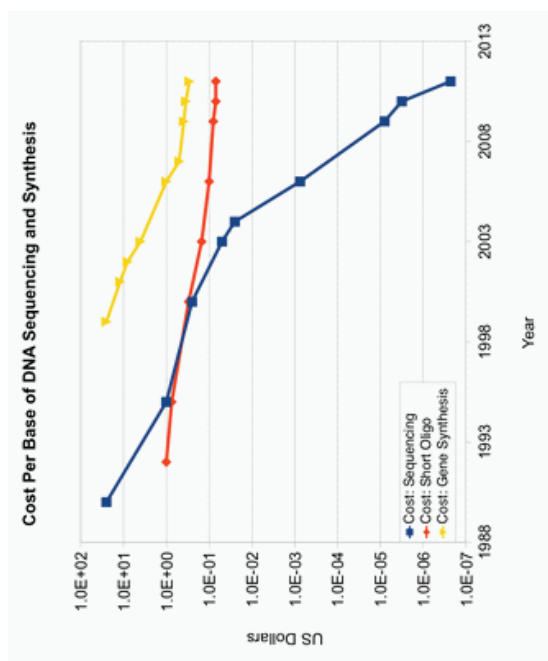
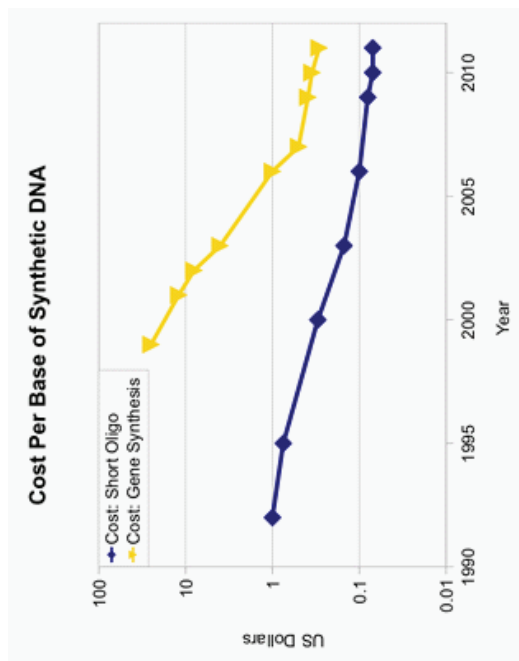


FIGURE WO-6 Cost per base of DNA synthesis and sequencing.
SOURCE: Carlson (2011).

DNA synthesis technology is currently limited by its high error rate coupled with the cost and time involved to create long DNA constructs of high fidelity; yet, even this constraint is rapidly changing. As discussed earlier in this chapter, the J. Craig Venter Institute announced last year that they had synthesized a modified version of the entire 1 million base pair genome of *Mycoplasma mycoides*. This technological feat marked a new milestone in the length of a DNA molecule that could be accurately synthesized and assembled (Gibson et al., 2010). Current estimates for generating simple oligonucleotides on chips are approximately \$0.50 per base pair—including synthesis of the oligonucleotides plus error correction (Carlson, 2010; Figure WO-6). While chip technology reduces the cost of DNA synthesis, scale-up has been limited by high error rates and other technical challenges. A novel method for highly parallel gene synthesis was used to assemble a DNA construct 50 times larger than previously published attempts (Kosuri et al., 2010).

Speaker George Church of Harvard University remarked that the current standard for producing DNA constructs is to sequence multiple configurations and then discard those that are incorrect as the sequencing error rate is significantly lower—around 10^{-5} - 10^{-7} —than the synthesis error rate of 1 in 500. In his prepared remarks, Church discussed four commercially available, homology-directed technologies for synthesizing DNA on chips. One of the most commonly used methods for large-scale genome engineering is doubled-strand break repair, which Church and colleagues have optimized to a point where large amounts of DNA, including multiple oligonucleotides, can be integrated into a host genome with nearly 100 percent efficiency (Mosberg et al., 2010). An entirely automated method called Multiplex Automated Genome Engineering has now been developed that inserts thousands of unique constructs into *E. coli* host cells in a combinatorial fashion and then uses accelerated directed evolution to select for cells with the desired properties (Mosberg et al., 2010).

The greater challenge, according to Church, is integrating a synthesized piece of DNA into a host genome so that the DNA functions efficiently. Several protein- or RNA-directed recombination strategies have been developed for inserting DNA into defined places, rather than randomly, all of which have been tested in mammalian genomes (Muñoz et al., 2011; Urnov et al., 2005). Biologists have also developed several DNA homology-directed strategies that have been tested in bacterial genomes and are beginning to show promise in mammalian genomes (e.g., Costantino and Court, 2003; Link et al., 1997; Wang et al., 2009; Yu et al., 2000; Zhang et al., 1998).

Directed Evolution

First described in the literature almost 20 years ago, directed evolution is an accelerated mutagenesis technology that allows biologists to generate novel proteins and chemical pathways, as well as whole organisms, with desired properties

in a more cost-effective manner than conventional breeding and in a fraction of the time (Chen and Arnold, 1993). Synthetic biologists use directed evolution to rapidly modify preexisting proteins and chemical pathways so that they perform new functions, and to develop new parts without needing to understand the mechanistic properties of a pathway or system at the level that would be necessary to design the parts from scratch (Dougherty and Arnold, 2009; Forster and Church, 2010).

Classical genetic breeding starts with a parental pool of related sequences (e.g., genes, proteins), sometimes created by random mutagenesis, that then gives rise, through a single round of replication, to an offspring pool of molecules. The “best” offspring—as determined by the investigators under selective conditions—are identified and used as the parental pool for the next generation. This iterative selection process is repeated for several generations. Directed evolution is used to improve and optimize biological molecules, pathways, networks, and even whole organisms by mimicking Darwinian evolutionary adaptation in the laboratory. Conceptually, directed evolution is closely related to artificial selection, performed previously on microorganisms by selecting for new traits during growth under specific conditions that favor these traits. The “novel” aspect of directed evolution is that instead of relying on natural or random mutations induced in a whole genome by mutational events, the rate, general location and nature of the mutations are specified and controlled, usually by performing the mutagenesis *in vitro*. One introduces mutations into the DNA segment of interest, and expresses the DNA either in a whole microorganism, or *in vitro*. The next step is “selection,” the goal of which is to identify improved sequences. This may be carried out by either screening the resulting clones for the desired properties or by artificial selection. Important for an evolutionary process, the improved sequence(s) are fed back into the process, which is iterative. Mutations are accumulated in an evolutionary fashion, until the desired phenotype is reached (or not).

With directed evolution, sequence diversity is generated by mutating or fragmenting and recombining (“shuffling”) the DNA sequence or gene of interest and creating a library of genetic variants. A schematic of the steps used in directed evolution studies is presented in Figure WO-7. The reassortment that occurs in DNA shuffling yields a higher diversity of functional progeny sequences than can be generated by a sequential single-gene approach.

One of the earliest demonstrations of DNA shuffling involved four separately evolved members of a single gene family from four different microbial species. Some of the selected, shuffled “hybrids,” illustrated in Figure WO-8, encoded proteins with 270 to 540 times greater enzymatic activity than the most active parental sequence (Cramer et al., 1998). Even if that same recombined enzyme could have been evolved through single-gene mutagenesis (and this is unlikely), the process would have been dramatically slower. Evidence from at least one study demonstrates that the best parent is not necessarily the one most similar in sequence to the best chimeric offspring and therefore might not represent the optimal starting point for single-gene evolution (Ness, 1999).

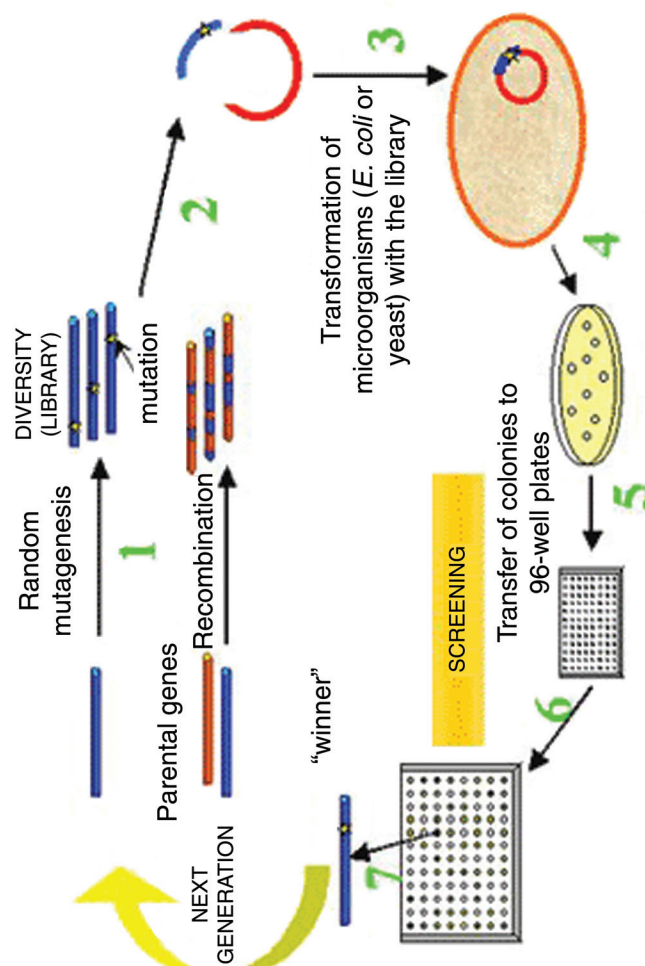
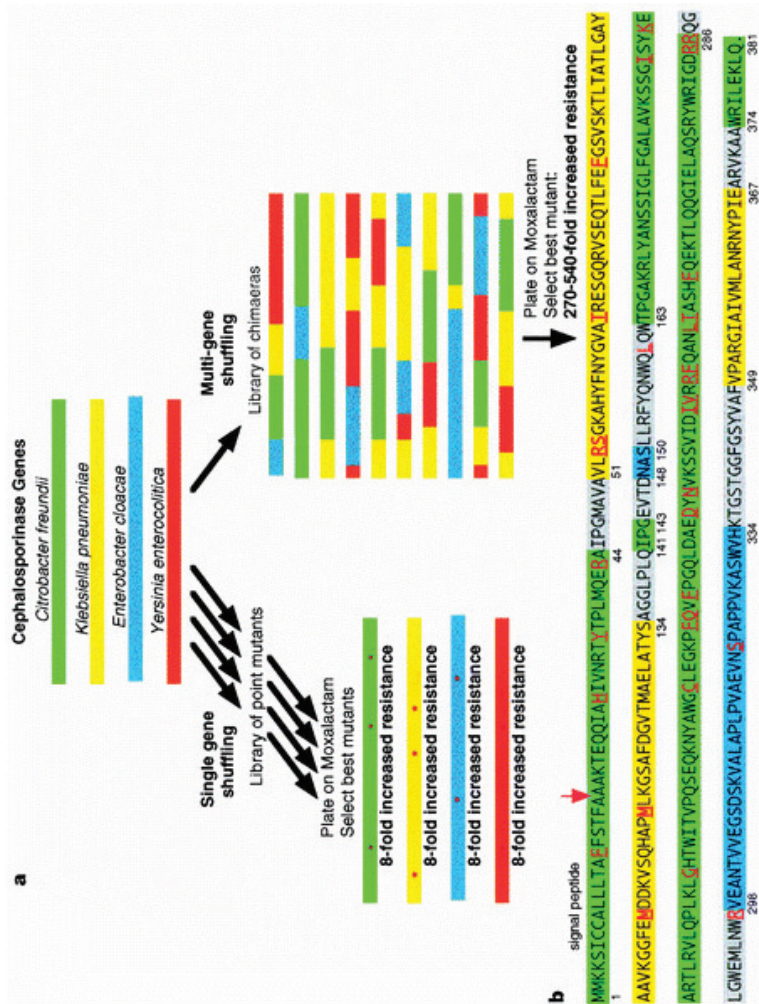


FIGURE WO-7 Schematic presentation of directed evolution studies. In place of screening, one can exploit selection using conditions that favor the growth of mutants with desired properties.
 SOURCE: Institute of Molecular Biology and Biotechnology (www.imbb.forth.gr).

FIGURE WO-8 Breeding by DNA shuffling. (a), Comparison of single sequence shuffling versus sequence family shuffling. (b), Sequence of a chimeric mutant obtained by family shuffling. The segments derived from *Enterobacter* are shown in blue, those from *Klebsiella* are shown in yellow, and those from *Citrobacter* are shown in green. The grey segments are where the crossovers have taken place. Because of DNA homology in the grey segments, the exact location of the crossover cannot be determined more exactly. The amino acid point mutations are shown with underlined red letters. The numbers at the beginning and end of each segment are the numbers from the GenBank protein files of the wild-type enzymes and differ from those used for the *Enterobacter cloacae* enzyme. SOURCE: Reprinted by permission from Macmillan Publishers Ltd; *Nature*. Cramer, A., S-A. Raullard, E. Bermudez, and W. P. C. Stemmer. 1998. DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature* 391:288-291, copyright 1998.



Directed evolution has advanced to the point where scientists are not just evolving single genes—they are evolving entire genomes. In 2002, biologists used whole-genome DNA shuffling to improve the production of tylosin (an antibiotic) in the bacterium *Streptomyces fradiae*. After only two rounds of shuffling, a bacterial strain was generated that produced tylosin at a rate comparable to strains that had gone through 20 generations of sequential selection (Zhang et al., 2002). That same year (2002), a report was published describing the shuffling of a portion of the HIV genome to create a new strain of HIV that was able to replicate in a monkey cell line that had previously been resistant to viral infection (Pekrun et al., 2002). By 2003, reports described the shuffling of many mammalian DNA sequences together into a single bacterial cell line. In one study, scientists shuffled the gene of a cytokine from seven genetically similar mammalian species (including human) to generate an “evolved” cytokine that demonstrated a 10-fold increase in activity compared to the human cytokine alone (Leong et al., 2003). The DNA shuffling approach allows researchers to rapidly modify preexisting proteins and chemical pathways to perform new functions. This enables investigators to develop new circuitry components without the need to understand the underlying mechanistic properties of a pathway or system at the level that would be required to design the part from scratch (Dougherty and Arnold, 2009).

Directed evolution allows investigators to identify a suboptimal or even nonfunctional design and improve it in a fairly reliable fashion. This is critical for synthetic biology. With this technique, investigators can now take suboptimal human designs and fine-tune them. There’s no counterpart in any other engineering/design field—it is a great advantage of biological engineering.

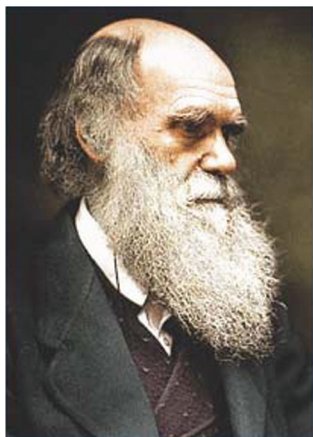
Using Directed Evolution to Create Life from Scratch: Is It Possible? Speaker Gerald Joyce, of The Scripps Research Institute, focused his remarks on whether one could create artificial “life” from scratch using the tools and approaches of bottom-up synthetic biology. (Dr. Joyce’s contribution to the workshop summary report can be found in Appendix A, pages 236-243.) A relevant question, then, is “what is life” and what are the essential characteristics of life? A working definition of life, according to Joyce, is “a self-sustained chemical system that is capable of undergoing Darwinian evolution” (Joyce, 1994). The key principles of Darwinian evolution are, first, heritable variation of form and function among a population of individuals; second, competition for finite resources by those individuals; and third, preferential reproduction of variants that operate most effectively in the competitive environment (Joyce, 2011).

In considering synthetic biology from scratch, the focus is on the evolution of functional molecules rather than organisms (Joyce, 2011). Joyce further observed that the principles of directed molecular evolution are the same as the principles of Darwinian evolution, namely *inherited profitable variation*. In chemical terms, Darwinian evolution involves three processes: (1) reproduction of information-carrying molecules (*inherit*); (2) selection of molecules that meet some fitness cri-

teria (*profitable*); and (3) maintenance of chemical diversity among the population of molecules (*variation*) (Joyce, 2011). As highlighted in Figure WO-9, Darwin observed, in *The Origin of Species* (1859), that “any variation if it be profitable . . . to an individual of any species . . . will generally be inherited by its offspring.”

Over the past 20 years the tools and approaches of directed molecular evolution have become very powerful but also routine. In Joyce’s view, investigators are no longer discussing this approach as a technology but are, instead, using it as a technique. There are many methods available for introducing molecular variation, both for generating initial combinatorial libraries and for maintaining variation in a population (Joyce, 2011). Additional approaches have been developed to select molecules on the basis of their inherent physical properties, capacity for binding to a target, ability to serve as a substrate for a reaction, ability to form a chemical bond, or ability to cleave a chemical bond. Joyce further observed that there are various methods for reproducing the profitable molecules in order to bring about the inheritance of selectively advantageous traits. If the selected molecules are DNA or RNA, then it is relatively straightforward to achieve their amplification by using the appropriate polymerase enzyme(s), resulting in large numbers of progeny. If the molecules selected are proteins, which cannot be amplified directly, then one must amplify nucleic acid molecules that encode, and are physically linked to, the corresponding proteins (Joyce, 2011).

Arguably, the ultimate bottom-up synthetic biology achievement would be to build life from scratch. Joyce explained that none of the technologies available



Charles Darwin
1809-1882

“Owing to this struggle for life, any variation...if it be in any degree profitable to an individual of any species, in its infinitely complex relations to other organic beings and to external nature...will generally be inherited by its offspring.”

The Origin of Species, 1859

FIGURE WO-9 Principles of evolution.
SOURCE: Darwin (1859).

for amplifying molecules are self-sustaining. The informational molecules that keep systems running, such as T7 RNA polymerase, reverse transcriptase, etc., are evolved outside the systems. None of the known directed evolution technologies, at the moment, meet generally agreed-upon criteria for a working definition of life: “a self-sustained chemical system capable of undergoing Darwinian evolution.”

As Joyce observed, the pioneering Miller-Urey experiments of the 1950s did not meet the criteria of “life” either (Miller, 1953). Although Miller and Urey created the necessary preconditions for the spontaneous formation of amino acids from scratch, their synthesized “prebiotic soup”—or “prebiotic consommé” as Joyce described it—contained no informational components and therefore had no self-sustaining capacity, let alone a capacity for Darwinian evolution. Joyce stated that, in the 60 years since the famous Miller and Urey experiment, the field has advanced to a point where scientists are “getting close” to creating life, with a great deal of focus on RNA molecules and their likely critical role in the early history of life on Earth (Cheng and Unrau, 2010; Joyce, 2002; Lincoln and Joyce, 2009; Ricardo and Szostak, 2009). Some RNA molecules have the capacity to catalyze their own replication and, therefore, the potential to evolve—with replication copying errors giving rise to the genetic variation upon which natural selection acts. In fact, as Joyce remarked, many scientists suspect that DNA and protein-based life evolved from an “RNA world” when genetic information was encoded in RNA, approximately 4.2 to 3.6 billion years ago (Atkins et al., 2011; Joyce, 2002).

Self-Sustained Darwinian Evolution⁷ An explicit goal of Joyce’s research is to construct a system of RNA molecules that undergo self-sustained Darwinian evolution (Joyce, 2011). According to Joyce, this goal was recently achieved, although the system still lacks the complexity and inventiveness of what one might regard as life. The self-sustained evolving system employs populations of RNA enzymes that catalyze the RNA-template joining of RNA substrates. The enzymes contain about 55 essential nucleotides and can be made to join pairs of RNA substrates of almost any sequence (Rogers and Joyce, 2001). If the substrates, once joined, form additional copies of the enzymes, then self-replication can be achieved. The newly formed enzymes behave similarly, resulting in exponential growth (Paul and Joyce, 2002). Initially the process could not be sustained indefinitely and was informationally restricted by the requirement that the original and newly formed enzymes must have the same sequence.

Joyce and his team then developed an improved version of the replication system that employs two different RNA enzymes that catalyze each other’s synthesis, enabling their cross-replication and sustained exponential growth (Kim and Joyce, 2004; Lincoln and Joyce, 2009). Each enzyme of the cross-replicating

⁷The following section is adapted from the contributed manuscript by Joyce in Appendix A, pages 236-243.

pair contains two substrate-binding domains that recognize corresponding oligonucleotide substrates through Watson-Crick pairing.⁸ During cross-replication, the “Watson” enzyme joins two pieces of RNA to form the “Crick” enzyme, while the “Crick” enzyme joins two pieces of RNA to form the “Watson” enzyme. Information is passed back and forth between these two enzymes in the form of particular sequences within the two substrate-binding domains.

Joyce observed that it was now possible, following optimization of the cross-replication system, to achieve a 100-fold amplification in the absence of any biological materials in just a few hours at a constant temperature (Lincoln and Joyce, 2009). The only informational macromolecules in the system are the enzymes and their components, which themselves are subject to Darwinian evolution within the system. The only other components are $MgCl_2$, a buffer to maintain pH, and H_2O . Evolution can occur because there are many potential variants of the cross-replicating enzymes that must compete for a finite supply of substrates and can undergo mutation through recombination of the two substrate-binding domains.

Beginning with a small seed of the cross-replicating enzymes, Joyce explained that amplification occurred with exponential growth and was only limited by the amount of substrate available. The amplification profile follows the logistic growth equation:

$$[\text{enzyme}]_t = a / (1 + be^{-ct}),$$

where a is the maximum extent of amplification, b is the degree of sigmoidicity, and c is the exponential growth rate. This equation also describes population growth for biological organisms constrained by the carrying capacity of their local environment.

Joyce remarked that cross-replication of the RNA enzymes could be sustained indefinitely by continuing to supply the necessary substrates to the culture. This is most conveniently achieved through a serial transfer procedure, whereby a small aliquot of material is taken from a spent reaction mixture and transferred to a new reaction vessel that contains a fresh supply of substrates. The new reaction mixture contained only those enzymes that were carried over in the aliquot, and these enzymes immediately resume exponential amplification in the new mixture. Within a period of 24 hours, according to Joyce, an overall amplification factor of $>10^9$ can be achieved (Lincoln and Joyce, 2009).

As illustrated in Figure WO-10 and discussed by Joyce, over a period of 100 hours, the two starter cultures effectively disappeared as new variants emerged and were selected for through recombination. More recently, Joyce and colleagues started a culture with 64 variants of each ribozyme (M. Robertson and G. F. Joyce, unpublished data).

⁸ In the canonical Watson-Crick DNA base pairing, adenine (A) forms a base pair with thymine (T) and guanine (G) forms a base pair with cytosine (C).

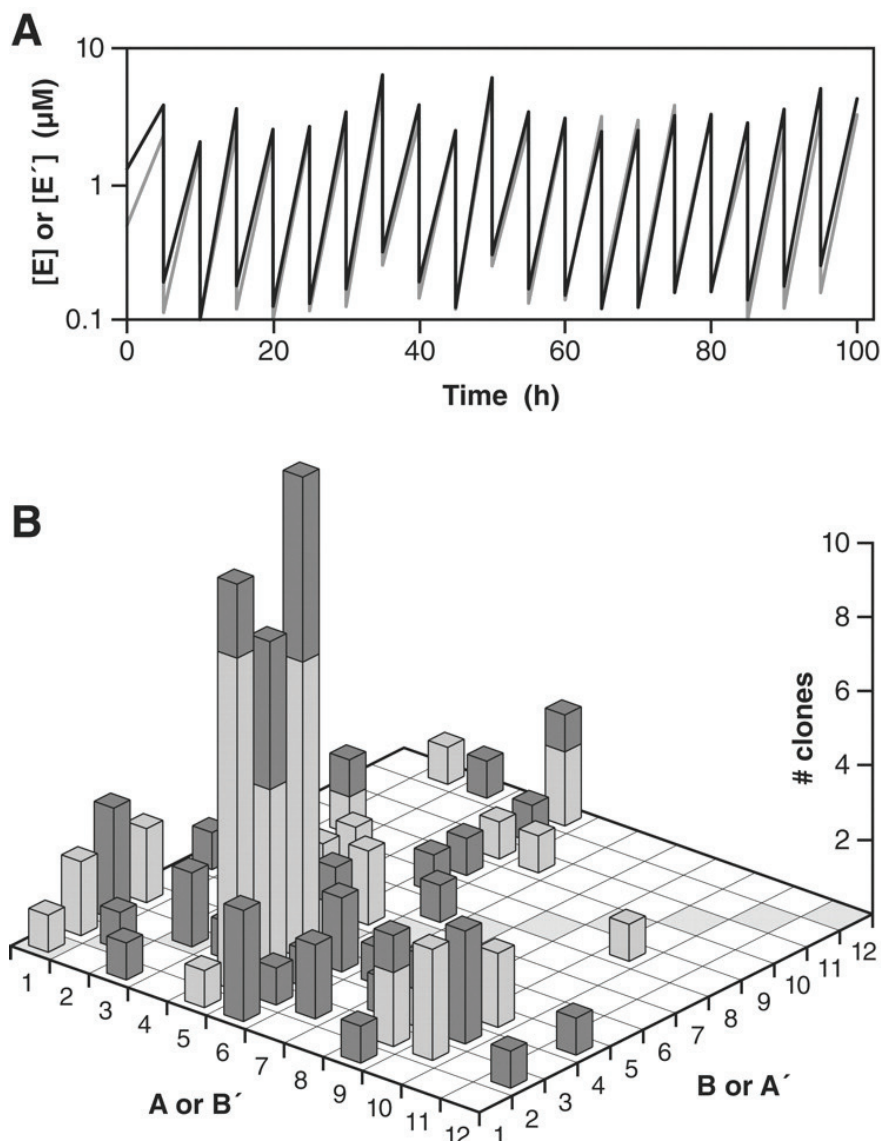
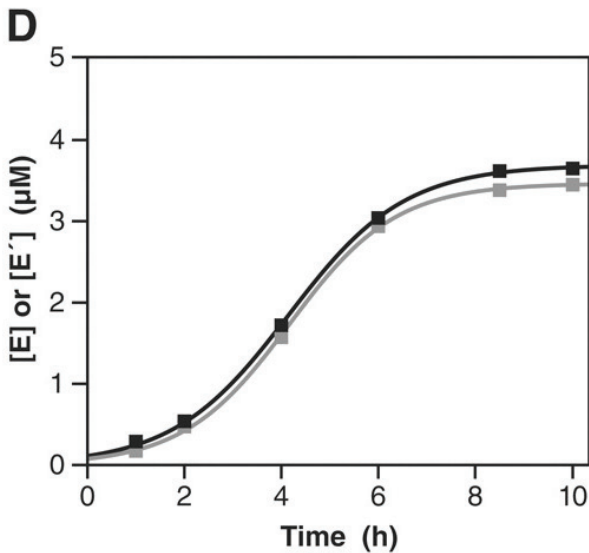
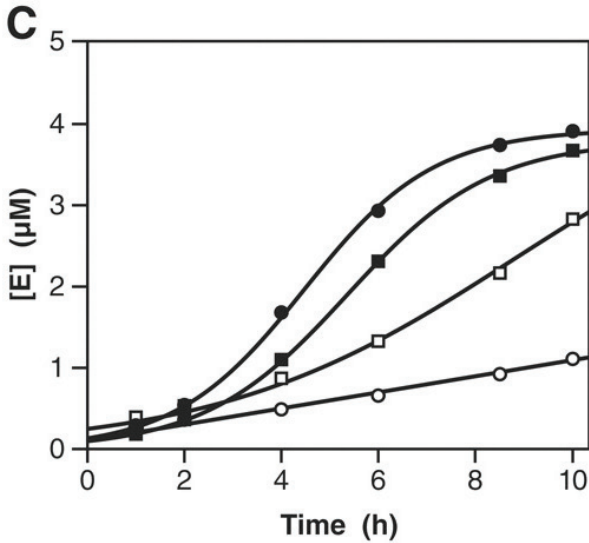


FIGURE WO-10 Self-sustained amplification of a population of cross-replicating RNA enzymes, resulting in selection of the fittest replicators. **(A)** Beginning with 12 pairs of cross-replicating RNA enzymes, amplification was sustained for 20 successive rounds of ~ 20 -fold amplification and 20-fold dilution. The concentrations of all E (black) and E' (gray) molecules were measured after each incubation. **(B)** Graphical representation of 50 E and 50 E' clones (dark and light columns, respectively) that were sequenced after the last incubation. The A and B (or B' and A') components of the various enzymes are shown on the horizontal axes, with nonrecombinant enzymes indicated by shaded boxes



along the diagonal. The number of clones containing each combination of components is shown on the vertical axis. **(C)** Comparative growth of E1 (circles) and A5B3 (squares) in the presence of either their cognate substrates alone (solid symbols) or all substrates that were present during serial transfer (open symbols). **(D)** Growth of A5B3 (black curve) and B5'A3' (gray curve) in the presence of the eight substrates (A5, B2, B3, B4, B5', A2', A3', and A4') that make up the three most abundant cross-replicating enzymes.

SOURCE: Adapted from Lincoln and Joyce (2009).

The next step beyond self-replication with Darwinian evolution, Joyce remarked, is to build a system whose replication is contingent on other functions by, for example, installing an aptamer⁹ on the ribozyme that recognizes a particular substance. When the aptamer recognizes the substance, it changes structure and allows the replicator to replicate (Lam and Joyce, 2009, 2011). There is a wide range of potential diagnostic and environmental sensor applications for such a system.

Joyce emphasized that even though the replicator that he and his colleagues built can do something that no other system outside of biology can do—sustain replication with exponential growth—it is not “alive” in the classic Darwinian definition of “life.” This point is illustrated in Figure WO-11.

Mutation occurs through recombination, and the investigators are exploring increasing the genetic variation available for selection. In an effort to create that system, Joyce and his team are working with starter cultures containing 256 variants of each ribozyme, resulting in the production of 65,536 recombinants. Joyce does not, however, consider the replicator a living system because thus far it lacks the capacity for inventing novel function.

The ultimate application, Joyce said, would be a replicator that invents its own function by evolving over time in response to the constraints of its environment—a feat that would require a significant level of genetic complexity. As Joyce observed, life on Earth, although vulnerable to extreme changes of environmental conditions, has demonstrated extraordinary resiliency and inventiveness in adapting to highly disparate niches.¹⁰ Perhaps the most significant invention of life is a genetic system that has an extensible capacity for inventiveness, something that likely will not be achieved soon for synthetic biological systems (Joyce, 2011).

High-Throughput Screening

High-throughput screening (HTS) is the process of sorting through large numbers of diverse biomolecular or chemical compounds in an efficient manner in order to rapidly identify molecules with properties of interest. Such technologies are essential to achieving any benefit from the construction of large and diverse libraries of compounds, as they are used to select a particular compound having the desired properties. These properties might include desirable biochemical or enzymatic activities for a potential therapeutic agent. Advances in miniaturized screening technologies—bioinformatics, robotics, and a variety of other technologies—have all contributed to the improved biological assay efficiency that characterizes HTS (Gulati et al., 2009). DNA or oligonucleotide microarrays—“DNA chips”—are routinely employed in both basic and applied

⁹ An aptamer is an oligonucleotide or peptide molecule that binds to a specific target molecule.

¹⁰ For a more in-depth discussion see contributed manuscript by Joyce in Appendix A, pages 236-243.

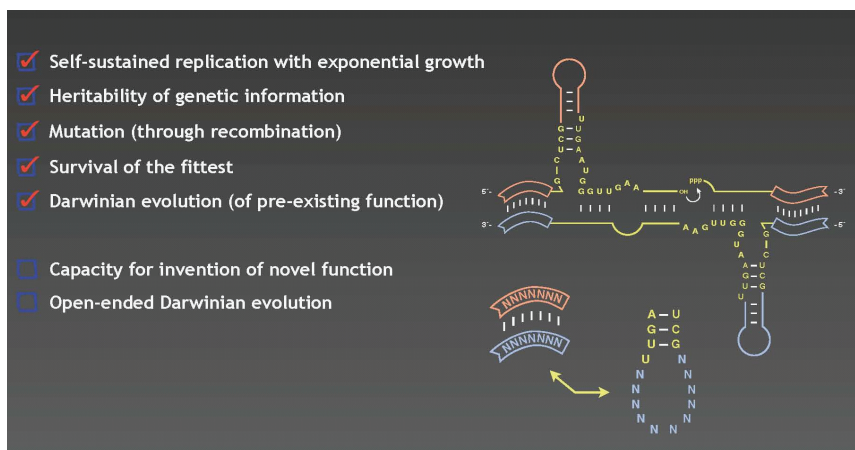


FIGURE WO-11 Is it alive? (no).

SOURCE: Joyce (2011).

research to facilitate the large-scale screening and monitoring of gene expression levels, gene function, and genetic variation in biological samples, and to identify novel drug targets.

The process of screening large numbers of compounds against potential disease targets is characterized by a collection of technologies that strive to increase biological assay efficiency through the application of miniaturized screening formats, and advanced liquid handling, signal detection, robotics, informatics, and a variety of other technologies. Over the past several years, the industry has witnessed a revolution in screening capabilities resulting in the ability of a user to screen more than 100,000 compounds per day for potential biological activity. Evaluating upwards of 1,000,000 compounds for biological (or other) properties in a screening campaign is now commonplace in the pharmaceutical industry.

Metagenomic Mining In addition to screening DNA databases, scientists are screening the natural world for potentially useful DNA sequences. Metagenomic mining involves the extraction of microbial genes from environmental samples without having cultivated the organisms (Rondon et al., 2000). It has been estimated that more than 99 percent of microorganisms in most environments have not been cultured in the laboratory. Because of this, very little is known about their genomes, genes, and encoded enzymatic activities. The isolation, archiving, and analysis of environmental DNA—the so-called metagenomes—have enabled scientists to mine microbial diversity, and has allowed investigators to access the metagenomes of environmental microbial communities, identify protein coding sequences, and even reconstruct biochemical pathways, providing insights into the properties and functions of these organisms. The generation and analysis of

(meta)genomic libraries is thus a powerful approach to harvest and archive environmental genetic resources, leading to the identification of the organisms that are present, what they do, and how their genetic information may have applications to the human condition (Ferrer-Costa et al., 2005). The evaluation of background genetic diversity will enable projections of mutation rates and emergence of new species that could be important for public health applications.

Speaker George Church of Harvard University and his research team used metagenomic mining to identify hundreds of soil bacteria able to subsist on antibiotics as their sole carbon source (Dantas et al., 2008). That such a phylogenetically diverse group of soil bacteria subsists on antibiotics points to a vast unrecognized and unappreciated environmental reservoir of microorganisms that are naturally resistant to antibiotics. Many of the bacteria sampled are closely related to human pathogens (Dantas et al., 2008).

Church discussed how he and others have been using metagenomic mining as a tool for tapping into novel microbial capabilities. Metagenomic mining has been used to extract and express a novel alkane biosynthesis pathway from cyanobacteria in *E. coli* (Schirmer et al., 2010). Synthetic biologists now can use the genetic and enzymatic machinery of this newly discovered pathway to engineer cells to convert renewable raw materials into biofuels (Sommer et al., 2010).

Recycling the “Trash” from HTS: Implications for Emerging Infectious Disease Management

Speaker Stephen Johnston of Arizona State University explored how synthesized sequences, genes, and proteins are creating genomic and proteomic resources for a range of HTS-based applications, such as proteomic screening for vaccines. With the capacity to clone or chemically synthesize entire pathogen genomes, scientists are now able to produce on a single array, or chip, the proteins for all of those genes and screen those proteins for immunoreactivity—such as antibody reactivity and T-cell reactivity. Investigators are also able to probe immunoreactive proteins for their potential to serve as vaccine targets (Borovkov et al., 2009; Stemke-Hale et al., 2005). Johnston observed that some pathogen proteomes are very reactive, whereas others have only a small proportion of immunoreactive proteins. In an HTS technique known as expression library immunization, all of a pathogen’s genes are synthesized and arrayed in groups and used to vaccinate mice that are then challenged with the pathogen of interest. Groups that confer protection are interrogated further in order to identify which gene product(s) are actually protective (Barry et al., 1995; Borovkov et al., 2009; Talaat and Stemke-Hale, 2005).

Expression library immunization is also being used to screen for other types of compounds. Johnston and colleagues have used the technique to identify the gene *B2L* which is believed to be associated with the hyperaccumulation of dendritic cells that occurs following parapox infection. Infected individuals are protected only by a transient period of innate immunity. But that transient protection afforded is so strong that animals vaccinated with Baypamun[®], a parapox

vaccine developed by Bayer Pharma of Leverkusen, Germany, are protected from virtually all pathogens for four to five days. (The vaccine is usually administered to animals being transported under conditions of close confinement.)

In a further application of HTS, Johnston and colleagues are making antibodies much more quickly and less expensively, by using the protein “leftovers” of synthetic biology. Conventional production of antibodies for the entire human genome has been estimated to cost \$750 million and take 10 years. Instead of using proteins as the starting material, Johnston’s group decided to start with synthetic antibodies known as synbodies—random peptide pairs linked together by scaffolds. The technique involves “throwing” the synbodies onto an array containing thousands of human proteins and identifying ligands by observing where on the array the peptides bind (Diehnelt et al., 2010; Greving et al., 2010; Williams et al., 2009).

Compared to conventional antibody production, which takes about three months and involves extracting the antibody from an animal model at a cost of approximately \$3,000 per antibody, generating a high-affinity synbody using this novel synthetic approach takes about five days at one-third of the cost of conventional methods. According to Johnston, the ability to synthesize genes and other molecules, like synbodies, and then screen those compounds for their bioactivity holds great promise for vaccine discovery, antibody production, drug discovery, diagnostics, and other tools for managing emerging infectious diseases.

Rebuilding Complex Functions Encoded by Multiple Genes Speaker Christopher Voigt’s group, at the University of California, San Francisco, has been working with several of these gene clusters as part of an effort to develop a methodology for reengineering entire gene clusters. Several gene clusters have been found to have useful functions with potentially very valuable industrial applications (Fischbach and Voigt, 2010). The challenge, according to Voigt, is that the underlying regulation of many of these gene clusters is very complicated, poorly understood, and highly embedded in the natural regulation of the host organism.

Voigt’s research team is interested in a cluster of genes that *Salmonella typhimurium* uses to build a hypodermic-like needle for the secretion of virulent proteins (Marlovits et al., 2004). The type III secretion system is a common virulence mechanism in Gram-negative bacteria. As illustrated in the cryo-electron microscopy image of Figure WO-12, they form large needle-like appendages that go through both the inner and the outer bacterial membranes and then extend out from the surface. They are normally used to deliver proteins to host cells to do things like rearrange the actin networks to promote invasion or control the trafficking of the bacteriosome inside the cell (Kubori et al., 2000; Marlovits et al., 2004; Sukhan et al., 2003).

In order to harness that needle for other purposes, Voigt and his team are attempting to reengineer the gene cluster in a host organism. *S. typhimurium*, Voigt

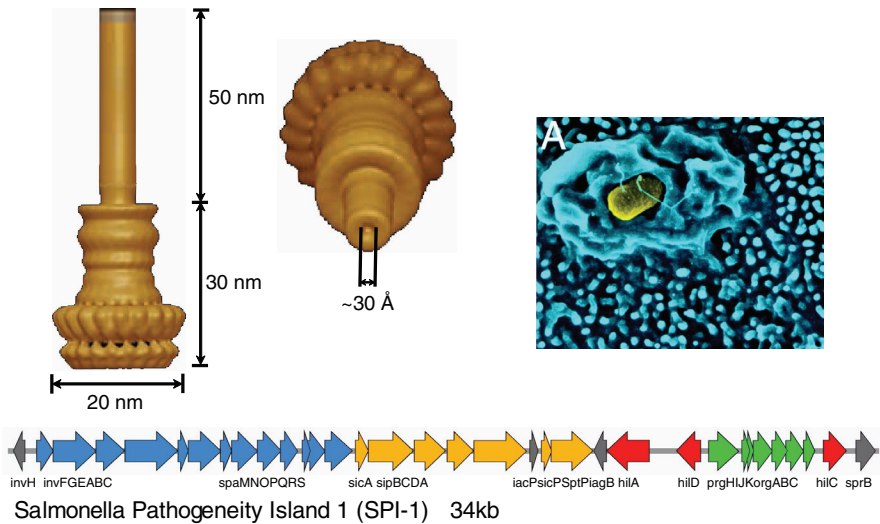


FIGURE WO-12 Type III secretion system.

SOURCE: Adapted from Kubori et al. (2000), Marlovits et al. (2004), and Sukhan et al. (2003).

said, maintains very tight control over when the needle is produced. The conditions must be exactly right for that particular stage of virulence.¹¹ Hijacking the system for another purpose requires breaking through the tight regulation, which Voigt described as a “very nontrivial task.”

Voigt’s group has developed a technology for completely erasing all of the native regulation underlying a single gene cluster in order to have, as Voigt described it, complete control over all of the functions encoded by that gene cluster—when to turn the function on and off—and optimizing the function(s), through a process known as refactoring. “Refactoring” is a term derived by analogy from the software industry. It refers to a modification in the software code without a loss of the basic functionality of that software. When software manufacturers experience a problem with their software, they may fix that problem by rewriting the code in such a way that the underlying software continues to function unchanged. In synthetic biology, refactoring involves rewriting the DNA sequence so that it is easier to engineer but in such a way that the fundamental functionality of that sequence remains the same. A schematic of this approach is illustrated in Figure WO-13.

¹¹ The needle will only be produced (the genes only turned on) for the time required for that stage of virulence to be completed.

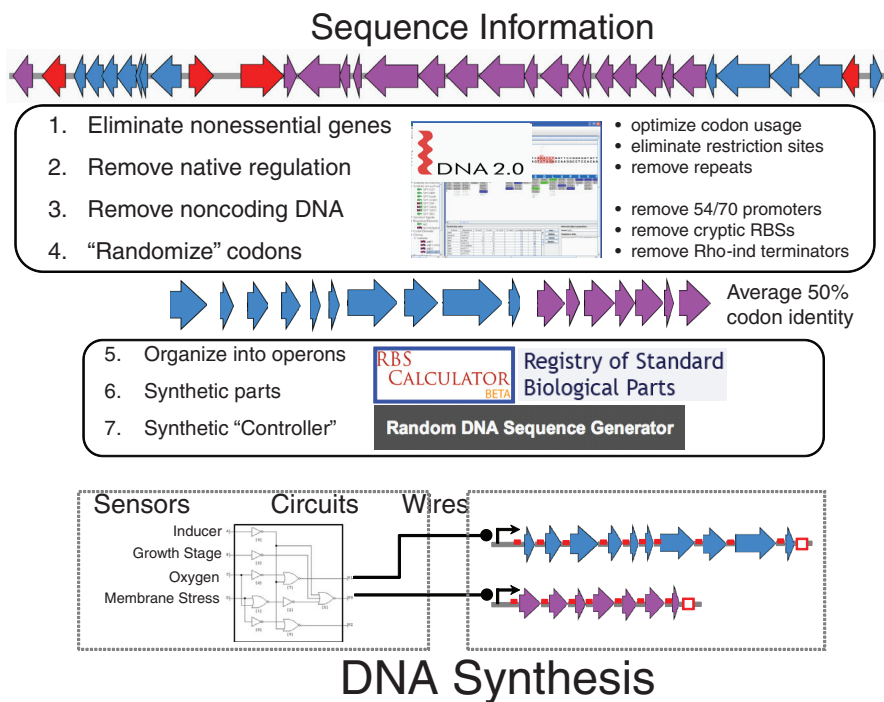


FIGURE WO-13 “Refactoring” gene clusters.

SOURCE: Voigt (2011), DNA2.0 logo kindly provided by DNA2.0 Inc.

As Voigt explained, refactoring gene clusters involves at least six elements:

- eliminating all nonessential genes—everything in the cluster that is not critical to the desired function;
- removing all native regulation within the gene cluster, as well as all noncoding DNA;
- eliminating all of the unknown regulation that might be occurring within each gene (i.e., by removing internal promoters), so that the gene encodes only the amino acid sequence that is being produced;
- organizing the genes into operons that do not necessarily follow the order of genes in the natural system;
- filling in all of the necessary regulation with synthetic parts; and
- building a synthetic controller for the system. The controller contains all of the sensors that feed information into the system (e.g., light, growth stage, oxygen, and membrane stress).

The first refactored system built by Voigt and his team was a nitrogen fixation system in *Klebsiella oxytoca*. The scientists refactored the 20-gene cluster, 25,000 base-pair section of DNA, responsible for reducing atmospheric nitrogen into ammonia (Rubio and Ludden, 2008). The researchers synthesized DNA sequences to build 3,000 to 8,000 base pair units, optimized each unit so that it functioned as close to wild-type levels as possible, and then assembled the refactored cluster in much the same way that Hutchison and his team put together their genomic-size pieces of DNA. Voigt said that the final refactored cluster had only about 0.3 percent of the activity of its wild-type counterpart.

Voigt's research team is also applying the same refactoring process to the previously mentioned gene cluster responsible for type III secretion in *S. typhimurium*—the mechanism that *S. typhimurium* uses to inject virulent proteins into mammalian cells. The cluster is a 34,000 base-pair region of the genome. The encoded “needle” is an 80-nanometer structure that passes through both the inner and outer membranes of the bacterial cell and provides a means of transport for proteins inside the cell to exit. Voigt and his team chose to work with this system because they wanted to harness it to export other, nonvirulent recombinant proteins. Voigt remarked that, while there are a number of scenarios where having such a system in place would be desirable, there are few mechanisms for transporting proteins through the double membranes of Gram-negative bacteria.

Spider silk has a similar tensile strength yet is much lighter than steel. Until recently there has been no way to produce spider silk in sufficient quantities to explore its use in new materials. A significant finding that could have widescale application is the synthetic engineering of *Salmonella* species that can secrete spider silk. Large-scale production of spider silk by engineered *Salmonella* could lead to new, superstrong and light weavable materials, that could have major applications in the aeronautic, automotive, and transportation industries (Royal Academy of Engineering, 2009).

Voigt's team demonstrated that they could modify the type III secretion system of *Salmonella* using synthetically designed genes to, in effect, turn the *Salmonella* into small spider silk factories (Widmaier and Voigt, 2010; Widmaier et al., 2009). Refactoring was exploited by Voigt's team as a means to overcome two key challenges: first, the difficulty in turning the natural gene cluster “on” under conditions that are relevant for the production of proteins, or creating the pathogenic conditions needed for turning it on; and second, the short length of time that the natural system remains turned on, which makes it difficult to exploit for production.

Computational Modeling

It is impossible to discuss the rapid progress in biology and genetics without also acknowledging the concurrent rise in computational ability made possible by the growth in the processing power and storage capacity of computers over

the past 30 years. These technologies have led to an explosion in data accessibility and have matured computing to a point at which computational modeling is being used to analyze systems-level biological complexity. Synthetic biologists routinely use computational models,¹² or computer replicas of living systems (“silicon cells”), to make qualitative or quantitative predictions about how their engineered systems are likely to function, based not just on molecular properties of the components but also on dynamics of the system as a whole (Westerhoff and van Dam, 1987). Computational models provide researchers with a tool for mining the vast and growing wealth of biological data and predicting how their engineered network(s) might function in the context of a larger system. They serve as the basis for the forward-engineering approach that many synthetic biologists aspire to achieve.

As a way to interpret computational modeling results and to guide construction of their engineered systems, many investigators employ computer-aided design (CAD) systems (Goler et al., 2008; MacDonald et al., 2011). According to speaker Paul Freemont of the Imperial College London, CAD-based modeling and biological design are a major focus of activity at the newly formed Centre for Synthetic Biology and Innovation (CSynBI), the United Kingdom’s first national synthetic biology center. (Dr. Freemont’s contribution to the workshop summary report can be found in Appendix A, pages 159-178.)

CAD-built models typically show up on a computer screen as three-dimensional models of the system; the DNA sequences represented in the models can be synthesized and then tested in living systems. Sauro described a CAD software application called TinkerCell (www.tinkercell.com) that allows users to diagram and analyze biological systems using a combination of modeling and experimental results (Chandran et al., 2009). The application’s flexible framework enables the addition of new functions via a flexible plug-in framework; the functions can be written in any of several programming languages (e.g., C, C++, MATLAB). The driving force behind the TinkerCell initiative, Sauro said, was the desire to connect the different types of information emerging from computational modeling results, experimental results, and biological parts data.

Microbial Systems Biology: Building Genome-Scale Metabolic Networks

The field of microbial systems biology is being driven by a growing number of well-curated computational reconstructions of biochemical reactions that underlie cellular processes (Feist et al., 2009). Speaker Bernhard Palsson, of the University of California, San Diego, observed that, over the past decade, microbial systems biologists have reconstructed several organism-specific, genome-scale metabolic networks and have used the networks for a variety of basic and

¹²There are numerous software tools available for computational modeling, many of which are listed on the Systems Biology Markup Language website (www.sbml.org).

applied research activities, including metabolic engineering and the study of bacterial evolution. Palsson explained that the networks differ from biochemical networks diagrammed in textbooks; every reaction has a known genetic basis and is directly linked to a genetic element in the target organism. Moreover, according to Palsson, microbial metabolic systems biology is being applied more and more to infectious disease and speaks to the interesting coupling that takes place between the host and the pathogen, and the many different microenvironmental niches that pathogens find in the human body.

A microbial cell is a very crowded and interconnected space, placing severe constraints on biological functioning. Metabolic network data provide scientists with a tool for understanding pathways in the context of the cell or organism as a whole and for predicting the systems-level flow of metabolites (Orth et al., 2010). One of the greatest attributes of these curated databases that effectively become knowledge bases, Palsson said, is that they can be accessed mathematically in a way that allows investigators to examine the underlying biochemical basis of genotype-phenotype relationships and to make quantitative predictions.

According to Palsson, everyone working with a particular target organism would like a community consensus reconstruction of that target organism (Herrgård et al., 2008). Given the importance of genome-scale reconstruction in microbial systems biology, the technique has rapidly matured to a point at which there are standard operating procedures for genome-scale network reconstructions (Thiele and Palsson, 2010). In fact, some of the procedures have been partially automated (Henry et al., 2010). Still, network reconstruction is a difficult endeavor, as computing phenotypes from genome information requires going through a multistep process of testing many possible functions; the process also requires characterizing the genome metastructure (Cho et al., 2009).¹³

Palsson added that most genome annotations, which serve as the starting point for reconstructions, are incomplete and often have numerous errors, with as much as 25 percent of some genomes needing to be corrected, for poorly characterized organisms, before the reconstruction process can even begin. *H. influenzae* was the first microbial organism to have its reconstructed metabolic network published with over 70 curated networks published, and now a much greater number will become available (Edwards and Palsson, 1999; Singh et al., 2002). Organisms with published metastructures that enable genome-wide metabolic network reconstruction include *Mycoplasma genitalium* (Gibson et al., 2008), *Helicobacter pylori* (Palsson and Zengler, 2010), and *Geobacter sulfurreducens* (Qui et al., 2010). The phases and data requirements needed to generate a metabolic reconstruction are illustrated in Figure WO-14.

More recently, the application of network reconstruction was expanded beyond metabolism by building a genome-wide network representing the complete

¹³ Organization of the genome with respect to where the various structural and functional components are located.

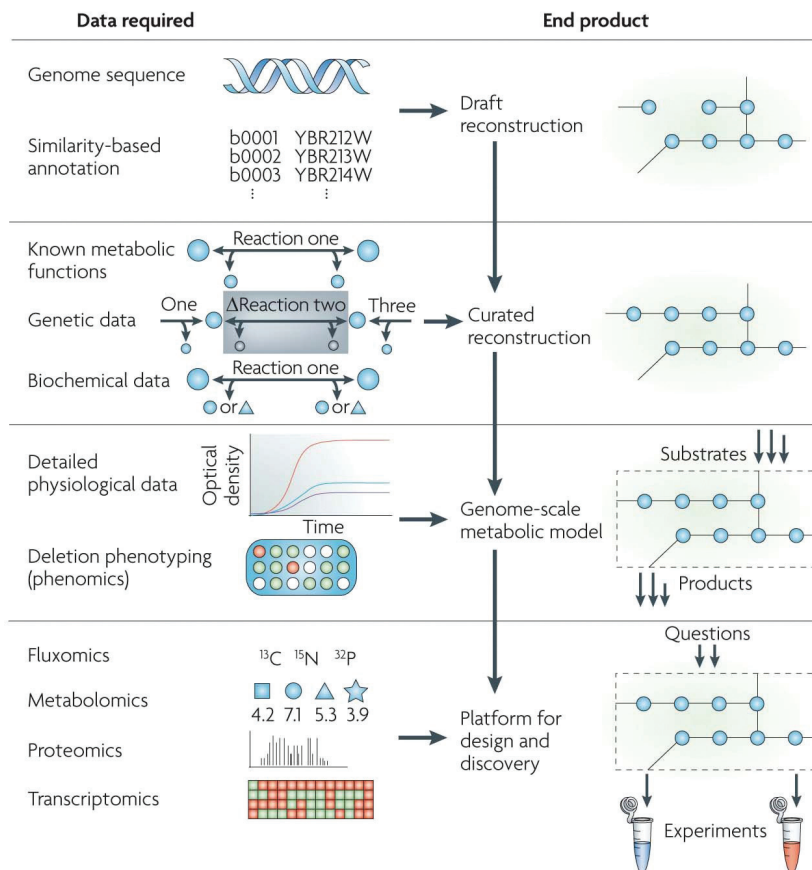


FIGURE WO-14 Phases and data used to generate a metabolic reconstruction. Genome-scale metabolic reconstruction can be divided into four major phases, each of which builds from the previous one. An additional characteristic of the reconstruction process is the iterative refinement of reconstruction content that is driven by experimental data from the three later phases. For each phase, specific data types are necessary that range from high-throughput data types (for example, phenomics and metabolomics) to detailed studies that characterize individual components (for example, biochemical data for a particular reaction). For example, the genome annotation can provide a parts list of a cell, whereas genetic data can provide information about the contribution of each gene product toward a phenotype (for example, when removed or mutated). The product generated from each reconstruction phase can be used and applied to examine a growing number of questions, with the final product having the broadest applications.

SOURCE: Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Microbiology*, copyright 2009.

cellular biochemical machinery for protein and RNA synthesis in *E. coli* (Thiele et al., 2009). Palsson remarked that the RNA modification information contained in the expanded network is especially noteworthy because of the constraints that RNA modifications place on the functioning of metabolic activities. When combined with metabolic networks, Palsson observed, genome-scale reconstructions of transcriptional and translational machinery provide maps of every known antibiotic target with the exception of DNA gyrase. Investigators can use these networks to predict not only how a small molecule will likely impact a particular protein in a network but what type of metabolic response that small molecule will likely induce; one can even examine the likely phenotypic results of off-target binding. Currently, Palsson and his team are reconstructing the *E. coli* operon network, with the goal of integrating all three genome-scale networks—metabolic, transcription/translation, and operon/regulatory. As Palsson and colleagues concluded, this work represents a crucial step toward the important and ambitious goal of whole-cell modeling (Thiele et al., 2009).

While genome-scale reconstructions are driving the rapid growth of microbial systems biology, Palsson emphasized that the curation and integration of high-throughput data remains a significant challenge (Palsson and Zengler, 2010). Palsson and his team are using transcription and translation network reconstructions to better understand how pathogenicity islands in various species are regulated at a genome-scale level, and how pathogenic microbes differ from nonpathogenic organisms. Palsson and his team are doing this by first mapping all of the genes in the reconstructed networks of well-known non-pathogenic organisms—such as *E. coli*—onto the genomes of related pathogenic organisms (uropathogenic *E. coli*) and then conducting what is known as gap-filling analysis to generate hypotheses about pathogenicity (Cho et al., 2009; Orth and Palsson, 2010).

A Systems-Level Approach to Understanding and Developing More Effective Vaccines

Most vaccines have been designed and developed through trial and error, with scientists having no idea how they confer protective immunity. Systems biology has enormous potential to radically transform the field of vaccinology by providing biologists with the means to understand how vaccines “work” when they stimulate the immune response (Pulendran et al., 2010). The major objectives of speaker Bali Pulendran’s research program at Emory University are to take a systems-level approach toward understanding how some of the many existing successful vaccines mediate immune responses. The ultimate goal of his research is to use this knowledge to rationally design new vaccines (Pulendran and Ahmed, 2006) and apply the same systems-level approach to identify biological signatures that predict immunogenicity, adverse vaccine reactions, and vaccine ineffectiveness in certain populations (Nakaya et al., 2011; Pulendran et al., 2010; Querec et al., 2009; Thomas and Doherty, 2009).

The live attenuated yellow fever vaccine 17D (YF-17D) developed over 65 years ago is one of the most effective vaccines ever made, Pulendran remarked. Over 600 million people worldwide have been protected against this vector-borne disease through the administration of this vaccine. Yet, despite the vaccine's "success" scientists know very little about how the vaccine actually works. The first clues to how the vaccine "works" were reported in 2006, and suggest that the vaccine activates multiple Toll-like receptors (TLRs) via multiple subsets of dendritic cells,¹⁴ eliciting a broad spectrum of immune responses (Querec et al., 2006).

More recently, this same team of investigators used a systems biology approach to identify early genetic signatures that predict immunogenicity—either T-cell or antibody responses—in humans so that individuals can be classified as low versus high responders (Querec et al., 2009). Pulendran remarked that he and his colleagues were excited by the feasibility of the systems-level approach, which had not previously been applied to vaccinology, despite its widespread use in predicting cancer therapy response(s). One of the genes expressed early on in the infection process, and that was well correlated with a subsequent CD8+ T-cell response, was *EIF2AK4*, or *GCN2*, a key gene in the integrated stress response. When cells are stressed in response to amino acid starvation, the protein encoded by *EIF2AK4/GCN2* is phosphorylated and the translation of mRNA is shut down. Other genes involved in integrated stress response, all of which have the ultimate effect of shutting down mRNA translation, include

- *PKR* (which senses viral infections),
- *HRI* (senses oxygenative stress), and
- *PEK* (senses endoplasmic reticulum stress).

Pulendran and his colleagues were curious about the link between expression of these various integrated stress-response genes and YF-17D vaccination. For example, does YF-17D vaccination stimulate the stress response? More specifically, does *EIF2AK4/GCN2* "sense" the yellow fever vaccine, and if so, how? They discovered that vaccination leads to the rapid phosphorylation of the *EIF2AK4/GCN2* protein and the robust formation of stress granules. Using *EIF2AK4/GCN2* knockout mice, the investigators demonstrated that immunization with YF-17D leads to a remarkably impaired T-cell response, suggesting that the *EIF2AK4/GCN2* gene plays a critical role in the induction of adaptive immune responses to this virus. Pulendran observed that these results, while confirming the link between the stress response gene(s) and immunogenicity of YF-17D, were also puzzling. Preliminary data suggest that activation of the gene *EIF2AK4/GCN2*, a gene involved in amino acid sensing, triggers a process known as autophagy, whereby dendritic cells—the cells of the immune system activated by the yellow

¹⁴Dendritic cells present antigen to T-cells. TLRs are innate receptors expressed by dendritic and other cells of the immune system. TLRs have evolved to sense highly conserved molecular patterns within microbes and viruses.

fever vaccine—start consuming their own cytosols. This process, in turn, makes the dendritic cells very efficient at processing viral antigens and communicating that information to T-cells (Nimmerjahn et al., 2003).

Pulendran's research team has been conducting clinical trials with both the trivalent inactivated influenza vaccine (TIV) and the live attenuated influenza vaccine (LAIV; or FluMist®) to determine whether a genetic signature approach can be used to predict immunogenicity (Nakaya et al., 2011). Generally, FluMist® was observed to induce a much weaker response (as measured by hemagglutination inhibition [HAI] titers¹⁵) than the inactivated influenza vaccine does, suggesting that the trivalent inactivated influenza vaccine induces a stronger immune response than the live, attenuated, vaccine (Nakaya et al., 2011). Pulendran noted that this finding contradicts the epidemiological data that have demonstrated that LAIV is, in fact, an effective vaccine in some subjects and that the HAI titer is only one of numerous metrics that can be used to measure the immune response.

In addition, individuals immunized with TIV were found to express several B-cell genes that were correlated to the maximum HAI response and that a cluster of about 150 genes appeared to segregate among “low” versus “high” responders (Nakaya et al., 2011). These genes include *TNFRSF17*, which is also one of the best predictors of neutralizing antibody response to the yellow fever vaccine. This conserved expression of immune responsiveness to an antigen suggests that there may be common predictors of immunogenicity among different vaccines with many genes targeted by the transcription factor XPB-1. Taken together, a handful of genes have been correlated with predicting the maximum HAI response with a greater than 90 percent degree of accuracy. This observation is illustrated in Figure WO-15.

Pulendran expressed the hope that the growing effort to identify gene profiles that correlate with different types of immune system responses—both the innate and adaptive immune responses—may eventually lead to development of a generic vaccine chip containing 50 to 100 genes that can be used to predict the immune responsiveness to a broad range of vaccines. Figure WO-16 illustrates a schematic of such a chip.

Currently, Pulendran and his team are exploring the biology of various genetic signatures in order to determine which ones are predictive signatures of immunogenicity. From an immunological perspective, some of the predictive genes, such as *TNFRSF17*, make sense, Pulendran observed. The potential roles of other genes in protective immune responses to vaccines are not as obvious. For example, CaMKIV knockout mice have a defect in short-term memory and are unable to find their way around mazes (Kang et al., 2001). At the same time, CaMKIV (calcium-calmodulin dependent kinase 4) has been found to play a critical role in calcium sensing and signaling. As illustrated in Figure WO-17,

¹⁵ HAI tests measure the amount of serum antibodies directed against a hemagglutinating virus, with higher levels, or titers, being associated with greater protection.

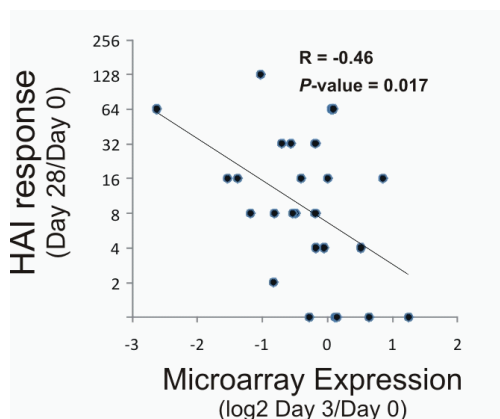


FIGURE WO-17 CAMK4 expression on post-vaccination day 3.
SOURCE: Pulendran (2011; adapted from Nakaya et al. [2011]).

example of where you can mimic a live virus by using some version of synthetic biology. A hypothetical schematic of this “ideal” is illustrated in Figure WO-18.

Systems biology promises to offer a new paradigm in vaccinology. Systems approaches applied to clinical trials may lead to the generation of new hypotheses about the biological mechanisms underlying vaccine-induced immunity. Such hypotheses can then be tested with animal models or in *in vitro* human systems. The insights gained from experimentation can then guide the design and development of new vaccines.

A Systems-Level Approach to Understanding the Immune Response and Managing Immunotherapy

A systems approach to disease is predicated on the idea that the analysis of dynamic, disease perturbed, networks and the detailed mechanistic understanding of disease that it provides can transform every aspect of how we practice medicine—better diagnostics, effective new approaches to therapy and even prevention.

—Heath et al. (2009)

Cells of the immune system are almost always characterized by their functional potential rather than their functional performance. Such a phenotypic classification implies a cellular functional capacity, but it does not describe the actual functions. Because of this naming convention, results from assays that measure immune cell phenotype can be, and often are, misleading. Speaker Jim Heath of the California Institute of Technology explained how a functional analysis, spe-

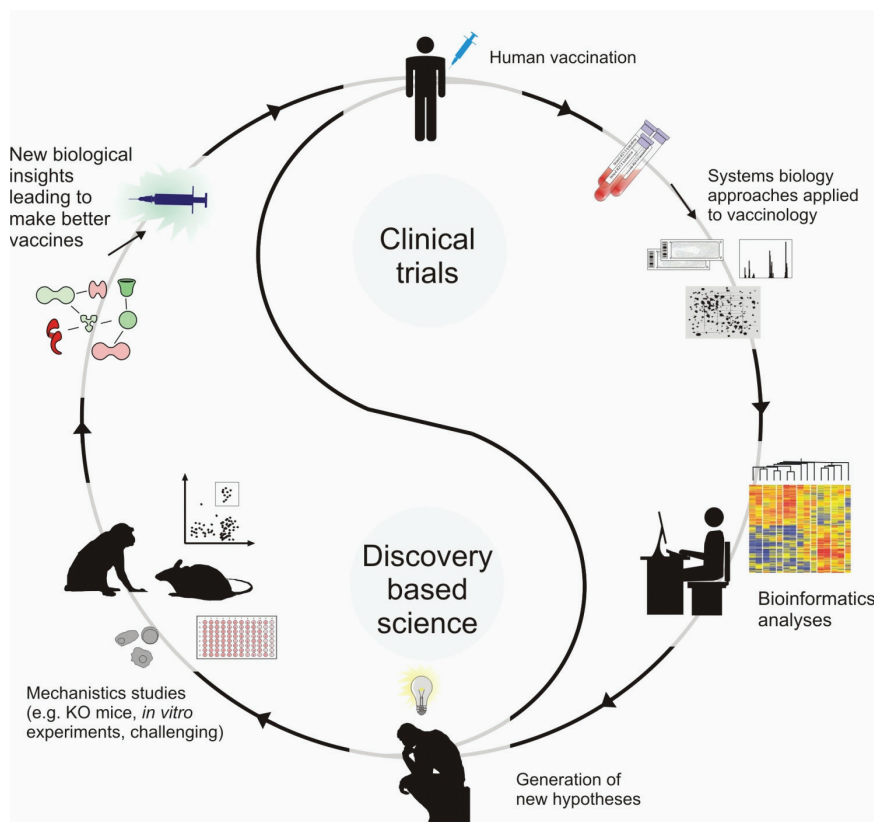


FIGURE WO-18 A framework for systems vaccinology. Systems biology approaches applied to clinical trials can lead to the generation of new hypotheses that can be tested and ultimately lead to developing better vaccines. For example, immune responses to vaccination in clinical trials can be profiled in exquisite depth with technologies such as microarrays, deep sequencing, and proteomics. The high-throughput data generated can be mined using bioinformatics tools and can be used to create hypotheses about the biological mechanisms underlying vaccine-induced immunity. Such hypotheses can then be tested with animal models or *in vitro* human systems. The insights gained from experimentation can then guide the design and development of new vaccines. Such a framework seeks to bridge the so-called gaps between clinical trials and discovery-based science, between human immunology and mouse immunology, and between translational and basic science and offers a seamless continuum of scientific discovery and vaccine invention.

SOURCE: This figure was published in *Immunity*, 33, Pulendran, B., Li, S., Nakaya, H. I., Systems Vaccinology, 516-529, Copyright Elsevier (2010).

cifically a systems-level functional analysis of a network of signaling proteins, might yield more accurate and helpful information about whether and how the immune system is responding to any given therapy. Although he used the example of cancer immunotherapy, and specifically adoptive T-cell immunotherapy for the treatment of melanoma, Heath said that the technologies were general enough that they could also be useful for monitoring T-cell responses to vaccination.

Adoptive T-cell immunotherapy, a technique pioneered by Steven Rosenberg at the National Institutes of Health, involves extracting T-cells from the patient's body, genetically modifying them so that they recognize antigens expressed on the surfaces of the specific cancer cells to be targeted, and then expanding and re-infusing these modified T-cells back into the patient's body (Rosenberg et al., 1988). A timeline for this type of immunotherapy is presented in Figure WO-19. When adoptive T-cell immunotherapy works, Heath said, it is a valuable treatment for advanced cancers; the patients are effectively immunized against their *own* cancers. But getting immunotherapy to work is a nontrivial exercise, made all the more challenging by the fact that it is difficult to even know if the targeted therapy is working until about 1 to 2 months into the treatment, when a positron emission tomography (PET) or computed tomography (CT) scan, or other diagnostic imaging, reveals whether the cancer is shrinking or not.

Within the first few days of the therapy, Heath said, early inflammatory responses on the skin can indicate whether the therapy is working. Following that initial inflammatory skin response, according to Heath, you are “flying blind” until images can be gathered later in the treatment process. In the interim, flow cytometry can be used, but again, the results can be misleading. Flow cytometry can be used to measure the populations of the engineered tumor-antigen-specific T-cells that were infused into the patient. The general idea is that a persistent population of those cell phenotypes is a necessary (but not sufficient) condition for successful therapy. Heath mentioned one patient—Patient 5—whose flow cytometry results suggested that the patient was responding well to therapy when in fact the cancer was returning; the patient ultimately died. Heath and his colleagues hypothesized that “a comprehensive functional analysis of defined T-cell populations, assayed over time, can reveal not just when and how the therapy is working but when and how it fails.” Figure WO-20 summarizes the clinical trial timeline and results for “Patient 5.”

Heath described the engineered T-cells that are used in adoptive T-cell immunotherapy as “tremendously complicated drugs”—that need continuous monitoring. Engineered T-cells are “asked” to carry out more than 20 distinct functions, from finding, targeting, and killing the tumor to replicating and recruiting other cells of the immune system to the tumor to induce phagocytosis of the dead tumor cells. In addition, these cells are being asked to play roles in controlling, mediating, and promoting the inflammatory response in a directed fashion. As Heath observed, if you want to understand the nature of these engineered T-cells, you

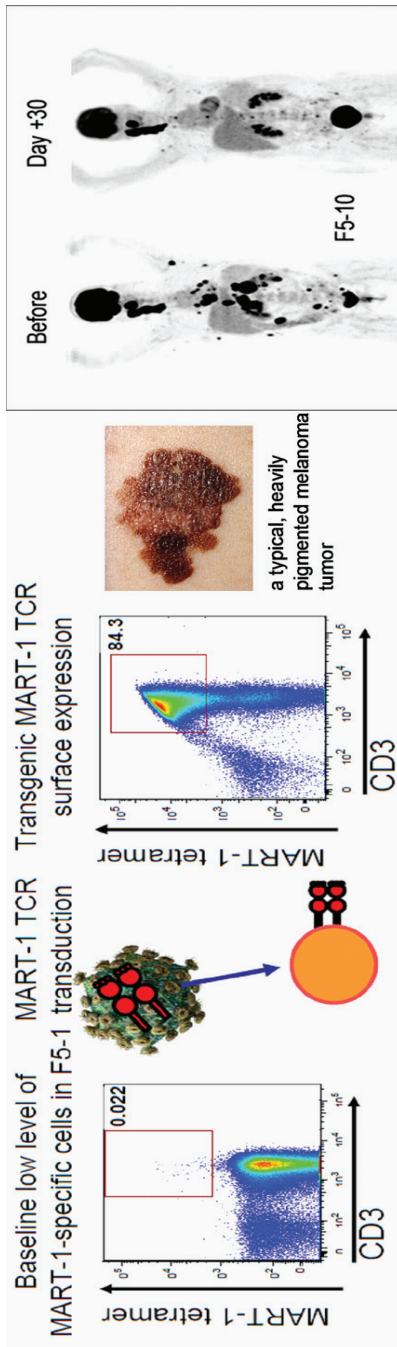


FIGURE WO-19 Adoptive T-cell immunotherapy. Cells are extracted from a cancer patient, and then genetically engineered to express a specific T-cell receptor (TCR) against a specific peptide/major histocompatibility complex (p/MHC) antigen, called the MART-1 antigen, that is on the surface of melanoma tumors. The engineered cells are expanded to $\sim 10^9$ cells and then reintroduced back into the patient. The data shown are flow cytometry data of CD3+ T-cells, from patient F5-1, before and after the genetic engineering process that makes them MART-1 antigen-specific. The MART-1 antigen is associated with the heavy pigmentation of melanoma tumors. At right are presented PET scans from a melanoma cancer patient participating in the immunotherapy trial. The patient exhibits a significant positive response by day 30. For the PET scan corresponding to 30 days after infusion of the engineered T-cells, most of the metabolic activity in the metastatic tumors has decreased. The brain, the bladder, and the kidneys continue to appear dark due to the clearance of fluorodeoxyglucose (FDG) through those organs. SOURCE: Heath (2011).

cannot just look at the T-cell population. One must also look at these functions at the single-cell level, which is where the technology challenge comes.

Monitoring the function of engineered T-cells requires gathering data on individual cell activity via the measurement of the secreted (functional) proteins that describe that activity. Flow cytometry, the established single-cell proteomic technique, is used to measure the membrane proteins that are used to phenotype the cells. For example, a MART-1 antigen-specific CD3+/CD8+ T-cell is identified, using multiplexed flow cytometry, by the MART-1 T-cell receptor, and the CD3 and CD8 membrane proteins. This classification, however, does not characterize the functional performance of those cells.

One way to characterize T-cell functional performance is to measure the secreted proteins that are associated with the specific functions. An overall assessment of T-cell performance can then be estimated by the numbers of functions that each T-cell is performing. An analogy is to consider a highly functioning T-cell as a “Superman®” drug. As functional performance decreases, the T-cells can be considered, successively, as Batman®, Robin®, and finally Homer Simpson® T-cells. This is illustrated in the following cartoon (Figure WO-21).

A simple picture of the effectiveness of a T-cell therapy is to quantitate the numbers of Superman®, Batman®, etc., T-cells. The technical challenge is to measure large numbers of secreted proteins from individual, phenotypically defined T-cells, and to perform that measurement on a statistically representative number of T-cells.

In order to meet this technological challenge, Heath and his colleagues developed two technologies: (1) a nucleic acid cell sorting (NACS) technology for epitope spreading analysis for capturing a large library of tumor antigen-specific T-cells (T-cells associated with the melanoma) (Kwong et al., 2009) and (2) a single-cell barcode chip (SCBC) technology for the T-cell functional analysis (Ma et al., 2011; Shin et al., 2010). Heath remarked that, at its present stage, the SCBC allows for 1,500 individual single-cell experiments to be conducted simul-

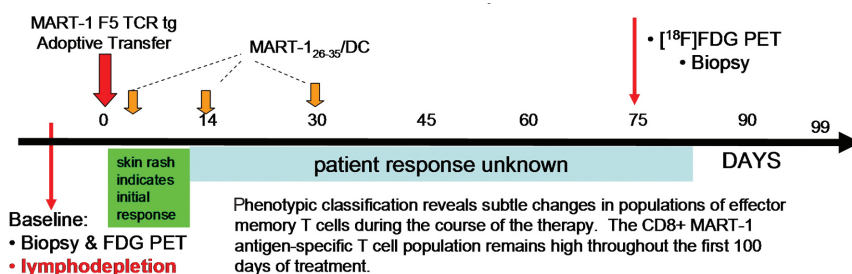


FIGURE WO-20 Clinical trial timeline on patient 5.

SOURCE: Heath (2011).



FIGURE WO-21 The effectiveness of T-cells based upon their functionality is reflected in this analogy. T-cells can do at least 20 to 30 separate key functions, such as replicating and tumor killing. The most effective T-cells have many functions, while the least effective T-cells may not have any. From a perspective of fighting a disease, highly functional (Superman® or Batman®) T-cells are desired.

taneously in a miniaturized array format, with up to 20 proteins quantitatively assayed per single cell. Within the SCBC, individual cells are isolated within ~2 nanoliter volume chambers, and each of these chambers is equipped with a full barcode structured antibody array.

As an initial demonstration of the potential effectiveness and value of this approach, Heath reported that he and his team initially conducted this functional analysis on a single melanoma cancer patient, at a single time point. Heath's team looked at a set of MART-1 antigen-specific CD8+ T-cells. The MART-1 antigen specificity of these T-cells was genetically engineered for the melanoma immunotherapy. By phenotypic analysis, these T-cells were 90 percent homogeneous and could be classified as effective memory T-cells (Ma et al., 2011). By functional analysis, however, they were all over the map. In the top 63 percent of the population, there were approximately 50 different functional phenotypes represented; there were populations of highly functional "Supermans®" at one extreme to populations of "Homer Simpsons®" on the other. These cells were compared against peripheral blood monocytes (PBMCs) collected from healthy individuals. The cells from the healthy donors were, by and large, low-functioning "Homer Simpsons®," exhibiting about one function per cell. The average functional capacity of the MART-1 CD8+ T cells from the melanoma cancer patient was significantly higher—about 5.3 functions per cell.

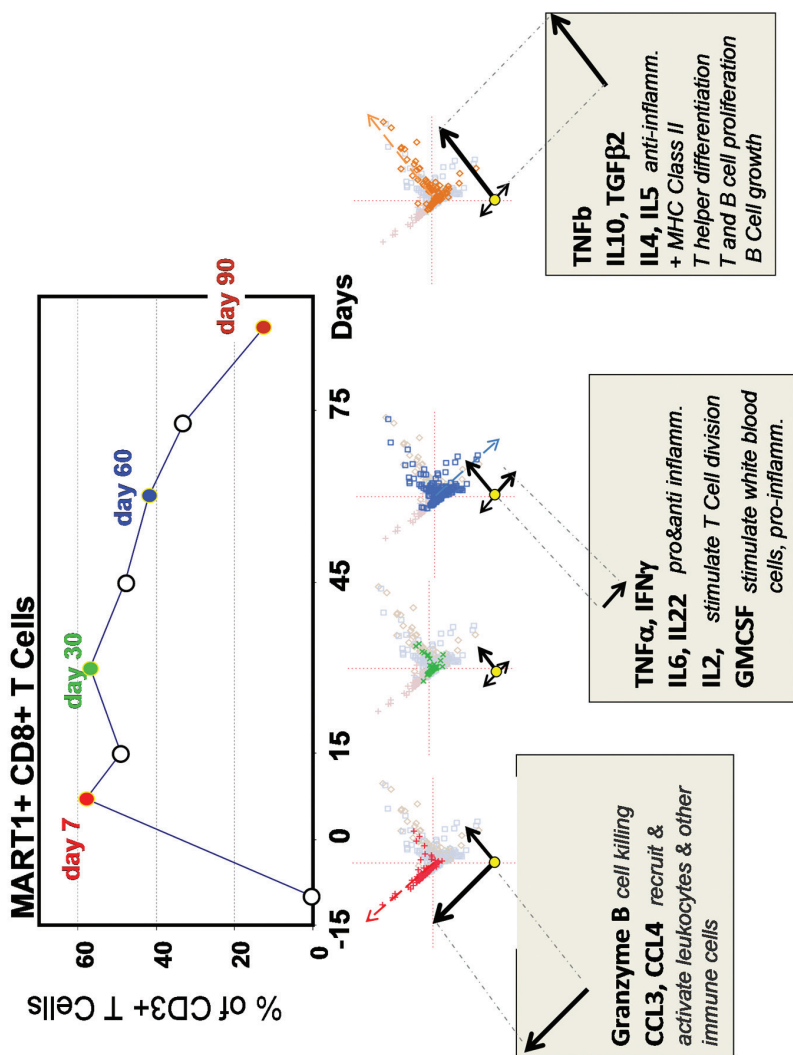
Heath and his team next conducted a longitudinal study in a single patient (unpublished). They quantitatively measured more than 1 million protein functions over the course of therapy. They conducted what is known as principal

component analysis. In such an analysis, the single-cell data are mathematically condensed into vectors, or “functional directions.” If, for example, 20 variables (proteins) are measured for each single cell, then 20 principle components can be extracted. Those principle components can, in certain ways, recapitulate the original data. The top principal component may recapitulate a large percentage (say, 20 percent) of the data, whereas the bottom principle components will only capture a fraction of a percent of the data. Thus, the top couple of principle components can be used to describe the dominant functional directions of the immune cells. By recording how those functional directions changed over time, Heath was able to describe a “movie of how the therapy proceeded.” The researchers separately investigated two phenotypes of engineered cells: MART-1 antigen-specific CD4⁺ T-cells, and MART-1 antigen-specific CD8⁺ T-cells. The MART-1 CD4⁺ exhibited highly coordinated functions, including a small number of anti-tumor functions, but persisted for only a short period of time. By post-infusion day 30, those cells had almost completely disappeared from circulation. By contrast, the engineered MART-1-specific CD8⁺ cells were observed to persist for the duration of the study but were only effective cancer killers for the first couple of weeks, after which their anti-tumor functions were replaced by other functions. As illustrated in Figure WO-22, the disappearance and loss of function of the MART-1-specific CD4⁺ and MART-1-specific CD8⁺ cells, respectively, suggests that this engineered immunity is a short-lived and perhaps ineffective therapy. However, additional measurements indicate that this engineered therapy triggers the development of an acquired immune response. Heath’s group was able to capture that acquired response by measuring the populations of CD8⁺ T-cells that were not MART-1 antigen-specific, but instead were specific for a spectrum of other tumor antigens associated with melanoma. Those cells begin to appear at significant levels around days 20-30 following the start of therapy, and persist with tumor-killing functions for about 2 months. It is possible that this acquired immune response constitutes the majority of tumor killing that occurs during the 90-day period over which the therapy was monitored.

Not only did Heath and his team devise a way to monitor the therapy over time, they found that although the engineered cells kick-started the killing, they were not the dominant killing cells over time. Heath suggested (and has subsequently confirmed) that a similar analysis performed on different melanoma cancer patients participating in the immunotherapy trial would reveal different results that reflect the individual patient’s response to therapy. These types of analyses can provide input for designing improved immunotherapy trials, and perhaps contribute to customizing that therapy in order to optimize individual-patient therapeutic responses.

The technologies that Heath’s research team developed while building a chip for use in immunotherapy monitoring can also be used to examine and describe immune cell function in terms of functional protein signaling networks. Heath emphasized an important distinction between functional protein signaling

FIGURE WO-22 Data showing the persistence of the population of engineered MART-1 antigen-specific CD8+ T-cells and the evolving functional performance of those cells. The graph at top illustrates the population of the engineered cells, as a function of time, over a 90-day period following infusion of the engineered cells into the melanoma cancer patient. Below the graph are plotted the three dominant principal components that describe the functional directions of those T-cells. The data are color encoded to match the day of sampling. A description of the dominant components is provided in the boxes, given as the names of the dominant proteins associated with the component and at least a few of the biological functions that those proteins represent. Note that the strong tumor-killing functions seen on day 7 are reduced by day 30, and day 7 are replaced by non-anti-tumor functions at later dates. **SOURCE:** Heath (2011).



networks and transcriptome networks. As Heath discussed, in a transcriptome network one observes a lot of positive and negative interactions, but it is unclear whether these interactions translate into actual, functional proteins. Functional protein signaling networks, on the other hand, are organized around both positive and inhibitory reactions among proteins and include directional interactions of varying strengths. In a study of macrophages, Heath and colleagues demonstrated the value of functional protein signaling networks. Specifically, Heath's team demonstrated that these networks could be used to predict how perturbations, such as the introduction of antibodies into the system, would likely impact the system (Shin et al., 2011).

In summary, Heath's systems-level approach has led to development of a single-cell proteomic chip (SCPC) platform that allows for hundreds of reactions, or experiments, to be conducted simultaneously. Initial studies have demonstrated that the platform can be used as a way to monitor at least one type of tumor immunotherapy. The technology is general enough that it should be applicable to monitoring vaccination responses as well. Heath's initial results revealed that the actual (functional) immune response was different from what was expected—the dominant tumor-killing cells were not the engineered T-cells but arose from acquired immunity that was triggered by the engineered immunity. This same platform technology might also be used to evaluate how targeted therapies could influence normal cellular programming.

A Systems-Level Approach to Drug Discovery

Systems biology may become a valuable approach for drug discovery (Apic et al., 2005; di Bernardo et al., 2005; Young and Winzeler, 2005). In medicine, disease is often viewed as an observable change of the normal network structure of a system resulting in damage to the system; disease-perturbed proteins and gene regulatory networks differ from their healthy counterparts, due to genetic or environmental influences. A systems biology approach may provide insights into how disease-related processes interact and are controlled, guide new diagnostic and therapeutic approaches, and enable a more predictive, preventive, personalized medicine (Hood et al., 2004).

Speaker Hans Westerhoff, of the University of Manchester and the VU University Amsterdam, discussed how his research team has been using a systems biology approach to develop better drug targets. (Dr. Westerhoff's contribution to the workshop summary report can be found in Appendix A, pages 480-494.) According to Westerhoff, before the utilization of a systems approach to drug discovery, much of the focus of parasitic disease research and drug targeting was through research on the molecular basis of infection. Yet this approach is not quite accurate since, as Westerhoff explained, function depends on network function, on biological function. The viability of a parasite or the effectiveness of a parasite depends not on a molecule but on a network that produces the infection.

Westerhoff described how *in silico* network modeling may be used to identify better drug targets for the treatment of African trypanosomiasis,¹⁶ a vector-borne disease caused by the parasitic *Trypanosoma brucei*, which is transmitted to humans through the bite of a tsetse fly (*Glossina* genus) (IOM, 2011). Westerhoff and his colleagues are using *in silico* modeling to conduct a biochemical network analysis of trypanosome glycolysis and identify novel drug targets (Bakker et al., 2010). Westerhoff remarked that the drug targets identified using a systems-level, network approach are very different than targets identified using a conventional single-molecule approach.

The conventional single-molecule approach pointed to phosphofructokinase (PFK) and other enzymes that control glycolysis in mammals as potential drug targets, while the network approach suggested that, in fact, these enzymes exert very limited control in trypanosomes. Rather, the uptake of glucose across the plasma membrane is a much more important pathway-controlling step and a more promising potential drug target (Bakker et al., 2010). Knock-out experiments confirmed these predictions. Westerhoff went on to state that the “viability of a parasite or effectiveness of a parasite depends not on a molecule but on a network that produces this infection. So one should really look at the network effect. . . . If you look at the network way of doing it, then you see that it is an entirely different target.”

Using Systems Biology to Make Better Antibiotics

Antibiotic resistance in bacteria is neither surprising nor new. However, antibiotic resistance is accumulating and accelerating over time and space, contributing to an ever-increasing global public health crisis. Some strains of bacteria are resistant to all but a single drug, and some may soon have no effective treatments left in the “medicine chest.” The crisis is compounded by the dearth of novel antibiotic compounds in the drug development pipeline (IOM, 2010).

In his prepared remarks, speaker James J. Collins, of Boston University, discussed how he and his research team are using systems biology to study how one particular class of antibiotics, the quinolones, work and how the antimicrobial action of not just quinolones but other existing antibiotic classes could be enhanced by small-molecule inhibitors (Dwyer et al., 2007; Kohanski et al., 2007, 2010a,b). In the past, quinolones were generally thought to kill bacterial cells by inhibiting bacterial DNA gyrase (topoisomerase II or topoisomerase IV), interfering with replication and causing extensive DNA damage (Hancock, 2007). Dwyer et al. (2007) conducted a systems-level analysis of gene expression data to demonstrate that the anticipated DNA-damage response signature, in response to the DNA gyrase inhibitor norfloxacin, represented only a small fraction of the 800 genes with altered expression (see Figure WO-23). Norfloxacin also triggered

¹⁶ Sleeping sickness.

WORKSHOP OVERVIEW

significant up-regulation of additional genes involved in the oxidative damage response, iron uptake and utilization, and iron sulfur cluster synthesis pathways. Through subsequent genetic knockout and phenotype experiments, the scientists discovered that the antibiotic triggers a novel oxidative damage cellular death pathway and that induction of this additional pathway appears to contribute significantly to how quinolones kill.

This systems-level analysis was repeated with eight other antibiotics; norfloxacin, ampicillin, and kanamycin were found to induce similar responses, including lethal hydroxy radical formation (Kohanski et al., 2007) (see Figure WO-24). “These studies . . . show that there is a lot more to understand about antibiotics, a frightening observation when one considers that research on antibiotics has mangled for decades, especially given the enormous importance of these medicines and the growing difficulties with multidrug-resistant ‘Superbugs’” (Hancock, 2007).

On a more practical level these and subsequent studies led Collins and colleagues to wonder if it was possible to enhance the potency of certain antibiotics by blocking the pathways that bacterial cells use to protect themselves against antibiotic-induced oxidative damage (Kohanski et al., 2008, 2010a). By knocking out RecA, a protein that senses DNA damage and activates the bacterial SOS response (a DNA repair pathway), the investigators were able to potentiate

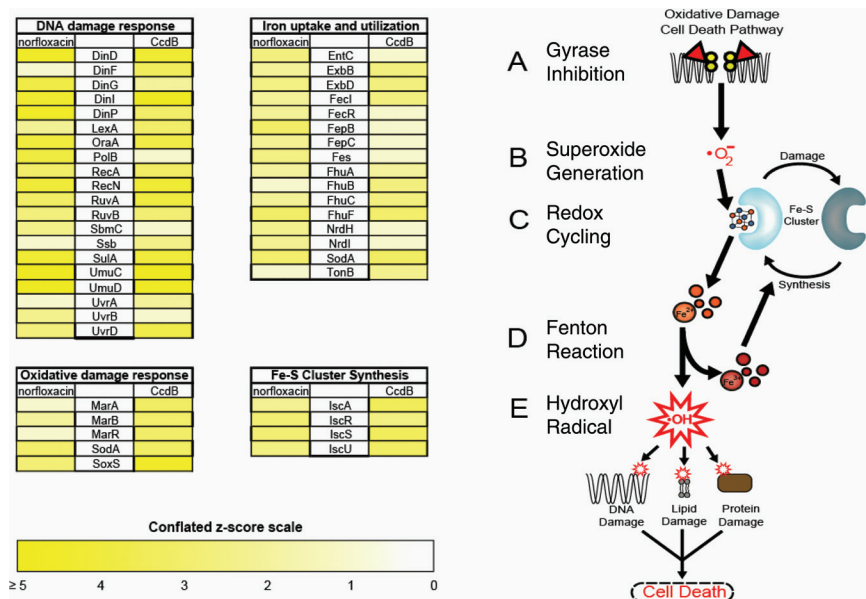


FIGURE WO-23 Gyrase inhibitors induce an oxidative damage cellular death pathway.

SOURCE: Dwyer et al. (2007).

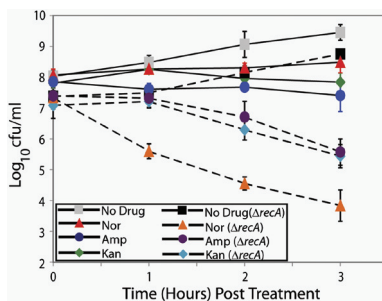
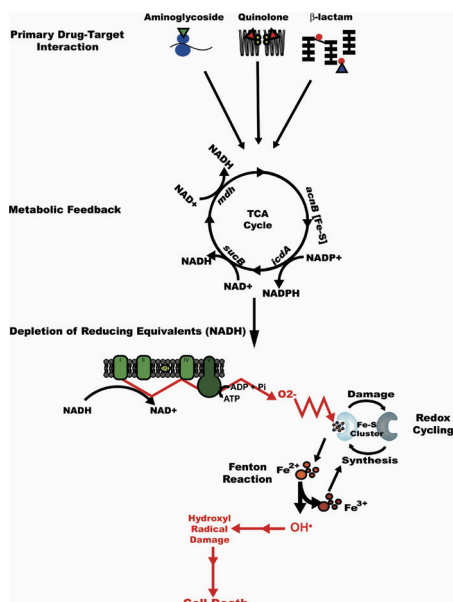


FIGURE WO-24 A common mechanism induced by bactericidal antibiotics.

SOURCE: Reprinted from *Cell*, 130/5, Michael A. Kohanski, Daniel J. Dwyer, Boris Hayete, Carolyn A. Lawrence, James J. Collins, A Common Mechanism of Cellular Death Induced by Bactericidal Antibiotics, 797-810, Copyright (2007), with permission from Elsevier.

several different antibiotics. Using a RecA inhibition assay, the scientists were able to screen over 50,000 bioactive compounds. They were also able to identify a number of small molecules with drug-like properties. Collins noted that their lead candidate compound increased the potency of an aminoglycoside antibiotic, gentamicin, by 1,000-fold.

Currently, these investigators are expanding the search for other small molecules that can be coupled with drugs in other classes of antibiotic. Collins' team is also exploring how silver salts can be used to enhance the killing power of certain antibiotics. According to Collins, they have identified three modes of action for silver salts:

- increase in membrane permeability;
- misregulation of the TCA cycle and electron transport chain, thereby increasing the production of free radicals; and
- breaking down iron-sulphur clusters, further feeding the production of free radicals.

Vancomycin, a glycopeptide antibiotic typically prescribed for Gram-positive bacterial infections, when used in combination with silver salts, can also kill Gram-negative bacteria. Preliminary data demonstrate that 90 percent of infected animals survive when administered silver salts together with vancomycin. When vancomycin is administered alone, only 10 percent of the experimentally-infected animals survive—a nine-fold increase in antibiotic effectiveness.

Collins and his team are also using a systems-wide approach to study the evolution of drug resistance when bacteria are challenged with sublethal levels of antibiotics. Kohanski et al. (2010b) delivered sublethal levels of various antibiotics, selected for drug-resistant bacteria, and screened the genomes of those bacteria. They observed not only an increased mutation rate associated with sublethal antibiotic exposure but also a correlation between the change in mutation rate and the formation of reactive oxygen species (i.e., the same reactive oxygen species identified as the product of a common antibiotic killing pathway by Dwyer et al. [2007] and Kohanski et al. [2007]).

Collins observed that antibiotics are serving as active mutagens—the antibiotics themselves are leading to increased levels of resistance. This observation was supported by a recently published study in the *Proceedings of the National Academy of Sciences* (Read et al., 2011). The investigators reported that while the current practice of aggressive pharmacologic treatment of infections is intended to suppress the appearance of resistant strains, this approach, in fact, *promotes* the evolution of resistance. The authors suggested that current practices should be reevaluated using evidence-based methods (Read et al., 2011).

PROGRESS IN SYNTHETIC BIOLOGY: FROM THE TOGGLE SWITCH TO THE SYNTHETIC CELL

Unlike systems biologists, who adopt a big-picture approach to biology by analyzing troves of data on the simultaneous activity of thousands of genes and proteins, synthetic biologists reduce the very same systems to their simplest unique component parts. They create models of genetic circuits, build the circuits, see if they work, and adjust them if they do not, learning underlying principles of biology in the process.

Because the molecular nature of many cellular reactions is only partially understood, most synthetic genetic circuits require considerable further empirical refinement after the initial computational work. Some scientists use directed evolution to streamline the empirical process. After inserting mutated DNA circuits into cells and selecting for those cells (and the circuits therein) that performed the best, researchers can evolve an effective product in just a couple of generations (Yokobayashi et al., 2002).

One of the goals of the field is to transform bacteria into tiny programmable computers. Just like electronic computers, the living bacterial circuits would use both analog and digital logic circuits to perform simple computations. For

example, researchers are working to develop modular units, such as sensors and actuators, input and output devices, genetic circuits to control cells, and a microbial model organism into which to assemble these pieces. If they are successful, a “registry of biological parts” may enable researchers to go to the freezer, get a part, and hook it up (Registry of Standard Biological Parts). The computing power of programmable cells will likely never rival that of their electronic counterparts. But, the beauty of synthetic biology lies in what living cells can do.

The earliest recognition of a biological “on-off switch” was the discovery of the *lac* operon by Jacob and Monod (Monod and Jacob, 1961). Monod and Jacob observed that the living cell controls its manufacture of proteins through a feedback mechanism analogous to a toggle switch in the presence or absence of a substrate. The discovery that enzyme synthesis was under tight regulatory control earned Jacob, Lwoff, and Monod the Nobel Prize in Physiology or Medicine in 1965.

The earliest synthetic biology devices were the biological equivalents of electrical devices. In 2000, two research teams developed the first synthetic genetic “circuits”—a toggle switch that controls gene expression (Gardner et al., 2000), and an oscillator (“the repressilator”) that periodically induces production of green fluorescent protein (GFP) in *E. coli* (Elowitz and Leibler, 2000). A toggle switch exists in one of two states, alternating between the states in response to a specific stimulus. The Gardner et al. (2000) system was engineered using two promoters, each repressed by the gene product of the other; one of the repressors was temperature sensitive (*cITS*), and the other was sensitive to isopropyl β -D-1-thiogalactopyranoside (IPTG). The state of the system (i.e., position of the switch) was monitored through the expression of a GFP promoter. These investigators demonstrated that the system toggled in one direction when the temperature was raised and toggled in the other direction when exposed to IPTG.

An oscillator is a timing mechanism and oscillator circuits play important roles in many biological systems (e.g., circadian rhythm). The Elowitz and Leibler (2000) system, dubbed “the repressilator,” was engineered with three promoter-gene pairs, with the first promoter driving expression of the second promoter’s repressor, and so on. As with the toggle switch, the state of the system was monitored via expression of a GFP promoter.

Reliance on an engineering-based methodology does not necessarily mean that the prototype is a perfect rendition of its model. As Collins observed, most engineers use modeling as a guide to the design and construction of their systems, relying a great deal on intuition and “tinkering” during the actual assembly of parts. As alluded to earlier, because the molecular nature of many cellular reactions is only partially understood, most synthetic genetic circuits require considerable empirical refinement following the initial computational work. This process has been referred to as the “iterative process of modeling and experiment” that is required to build synthetic genetic systems with desired characteristics (Atkinson et al., 2003).

After inserting mutated DNA circuits into cells and selecting for those cells and circuits that perform the “best,” researchers can evolve an effective product in just a couple of generations (Yokobayashi et al., 2002). Neither approach to modeling biological behaviors is a perfect approximation of what happens in the real world. Systems biologists have a long way to go with respect to developing a comprehensive understanding of how biomolecules interact to yield a healthy, functioning cell and how perturbations interfere with that functioning (Atkinson et al., 2003). Just as advances in systems biology have helped to drive the recent rapid growth of synthetic biology, further advances in systems biology will continue to move the field of synthetic biology forward. It is hoped that better models will lend greater predictability to synthetic biology. In the meantime, synthetic biologists have been using the same basic methodologies to engineer a variety of additional synthetic devices (Atkinson et al., 2003; Elowitz and Leibler, 2000; Gardner et al., 2000).

Synthetic Biology: Building a Better Biosensor

Constructing bioreporter bacteria that detect toxic chemicals can be seen as one of the early accomplishments in the field of synthetic biology. In such bacteria, a rational design for a genetic circuit is produced from cellular sensory and regulatory components that can translate chemical detection by the cell into a quantifiable reporter protein signal. Experience over the past 15 years has yielded a wide range of genetic components for use as sensor elements, DNA switches and reporter proteins, and generated an array of genetic tools for configuring these components into suitable bacterial host cells.

—van der Meer and Belkin (2010)

The early discovery and creation of toggle switches and oscillators led to the development of the first synthetic biosensors—modified bacteria that can detect and “report” the presence of a particular substance or environmental condition. Speaker Christopher French of the University of Edinburgh observed that synthetic biology is especially amenable to novel biosensor development since molecular recognition is a core function of biology, allowing for the creation of biosensors with high sensitivity and specificity. (Dr. French’s contribution to the workshop summary report can be found in Appendix A, pages 178-201.) Unlike the many synthetic biology devices that “count” or turn things on and off—such as counters, oscillators, and switches—that essentially duplicate what computers do, French stated that a practical argument could be made that synthetic biologists “should concentrate on letting the biology do what biology is good at, which is biocatalysis and molecular recognition, and develop good interfaces that allow cells and machines to talk to each other.”

Biosensors have been developed that function at a variety of levels within cells, allowing researchers to alter the transcription of particular genes (transcriptional biosensors), change how expressed genes are translated into proteins



FIGURE WO-25 “Hello World” was the first image taken by the team at the University of Texas at Austin/UCSF with their photosensitive bacterial photographic “film” (this is a later, more polished version).

SOURCE: <http://openwetware.org/wiki/LightCannon>.

(translational biosensors), and trigger signal-transduction pathways in response to a compound (posttranslational biosensors) (Khalil and Collins, 2010). In one of the first synthesized biosensors, researchers modified *E. coli* to detect the presence of the explosive trinitrotoluene (TNT) by designing a protein that binds to TNT molecules (DeGrado, 2003; Looger et al., 2003). Biosensors have also been developed that induce *E. coli* to form biofilms in response to DNA damage and that detect the presence of red light, effectively turning a layer of *E. coli* into a sheet of photographic film (see Figure WO-25) (Kobayashi et al., 2004; Levskaya et al., 2005). These early biosensors have served primarily as “proofs-of-concept” demonstrating their potential for widespread applications in medicine, environmental protection, and environmental remediation (van der Meer and Belkin, 2010).

Christopher French’s work with bioreporters, which are whole-cell biosensors¹⁷ designed to detect arsenic in drinking water, illustrates this potential (Joshi et al., 2009). French explained that whole-cell biosensors are particularly “ideal” for environmental applications because they are self-manufacturing and therefore can be produced at a very low cost. Moreover, at least in principle, they

¹⁷ Whole-cell biosensors are sensors that utilize a component of a living cell as the biological recognition component.

can be very easy to use, with the analyte simply binding to some type of recognition molecule in the device and the device processing an output signal in response to the analyte binding to the recognition molecule—any of a large number of signal-transduction pathways can be hijacked. Despite these advantages, French said, whole-cell environmental biosensors have not made much of an impact on the market. He asked, why not? And what can synthetic biology do to improve the situation?

French explained that arsenic is a popular target for environmental biosensors because there is an enormous need for an inexpensive and simple way to detect arsenic in groundwater. In addition, arsenic biosensors are easy to make. Most bacteria have an arsenic detoxification operon which consists of a single promoter (*ars*). In nature, the operon is expressed in the presence of soluble forms of arsenic. In the laboratory, it is relatively simple, French said, to generate an arsenic biosensor by linking the *ars* operon to a reporter gene. A variety of different reporter systems have been exploited for this purpose, including the use of luminescence, fluorescence, and chromogenic processing (Stocker et al., 2003). Arsenic biosensors are not only relatively simple to make, but are also commercially available. The company Aboatox Oy (Turku, Finland) sells luminescence-based arsenic (and mercury) biosensors based on the work of Marko Virta from the University of Finland (Tauriainen et al., 1999; Turpeinen et al., 2003).

Despite the popularity of arsenic as a target for environmental biosensors and the commercial availability of such devices, arsenic biosensors have not yet made a significant impact on the arsenic screening market. French reviewed how the students in the International Genetically Engineered Machine (iGEM) competition wanted to develop a device that would be less expensive to produce than currently available biosensors and that would be easy for people to use for monitoring their local water supplies. They decided to use pH as the output, rather than luminescence or any of the other output signals devised in the past (Joshi et al., 2009). The use of pH is advantageous because it can be easily visualized using a pH indicator and readily quantified using an inexpensive pH electrode or some other electrical detection device. Basically, the biosensor works via the arsenic promoter's control of β -galactosidase, which catalyzes the first step of lactose fermentation; when arsenic is present the cells ferment lactose and produce large amounts of acid. It has subsequently been improved in numerous ways—from adding bicarbonate to increase the device's sensitivity—to developing a simple webcam-based system that can read and report results in real time (de Mora et al., 2011).

Testing of this device has demonstrated that it works well under real-world environmental conditions, yielding clear results at arsenic concentrations as low as 0.5 ppb (de Mora et al., 2011). French identified several ways that synthetic biology approaches can be used to improve whole-cell environmental biosensors. First, there may be advantages to using an alternative chassis—in addition to or instead of *E. coli*—such as *Bacillus subtilis*. *B. subtilis* is a spore-forming bac-

terium. At a certain stage of its lifecycle, it undergoes a transformation and produces extremely resilient, dormant spores that can be stored at room temperature for decades, even centuries. *B. subtilis* spores can go from dormancy to a vegetative state within 50 minutes after being provided the appropriate growth medium.

French's research team has built a *B. subtilis*-based demonstration biosensor, called the Bacillosensor. The Bacillosensor is a chromogenic system based on the *xylE* reporter gene.¹⁸ French remarked that although the Bacillosensor is not as sensitive as the *E. coli* arsenic biosensor, it has the potential to yield fewer false positives since the dormant spores can be boiled without injury and the samples sterilized before analysis. In addition, while most whole-cell biosensors developed to date require that the analyte enter the cell before it can be detected, it is possible to construct a device that detects an extracellular analyte by building a two-component sensor system with both an extracellular and a transmembrane domain (e.g., Dwyer et al., 2003). French also discussed whether it was possible to build, either by rational design or through random mutagenesis and screening, a universal biosensor platform capable of detecting a wide range of analytes that do not normally enter cells.

Whole-cell biosensors are also “tunable.” According to French, by making very small changes in the configuration of the engineered components—by swapping the order of genes in the operon or placing the repressor gene under the control of another promoter—one can alter sensitivity or increase the range of detection of the biosensor (Stocker et al., 2003). French observed that while you can quite easily generate a wide number of different combinations of binding sites and promoters, putting the genes in different orders, and just screen them for the characteristics that you want. Alternatively, through rational design, it may be possible to develop a transcription amplifier of sorts that can be tuned to provide a desired dynamic range (iGEM, 2009). One can also engineer the system such that different analyte levels produce distinct signals, by incorporating multiple receptors with varying affinities for the analyte or by using multiple synthetic organisms (Wackwitz et al., 2008). One can also engineer novel outputs through using mutant luciferase enzymes or any of a variety of pigment genes. Whole-cell biosensors can also be engineered to produce electrical outputs as well as light signals (e.g., Nivens et al., 2004).

Building a Synthetic Genome from the Top Down—Reverse Engineering

Perhaps the most well-known synthetic biology proof-of-concept study is the J. Craig Venter Institute's “synthetic cell.” In May 2010, the J. Craig Venter Institute (JCVI) announced that they had designed, synthesized, and assembled the complete genome of *Mycoplasma mycoides* and transplanted it into an *M.*

¹⁸The *xylE* gene product converts a colorless catechol substrate into a yellow oxidation product (Zukowski et al., 1983).

capricolum recipient cell to create a continuously self-replicating *M. capricolum* cell controlled by the synthetic genome (Gibson et al., 2010). As Speaker Clyde Hutchison of the JCVI explained, the original intention of this work was to define the minimal set of genes required for cellular life. (Dr. Hutchison's contribution to the workshop summary report can be found in Appendix A, pages 222-235.) By leaving genes out during the assembly process, the scientists hoped to identify which genes were required to generate self-replicating cellular life. Over time, the scientists realized that the approach would have other value as well.

The synthetic genome installed was, for the most part, an artificially (re) constructed naturally occurring genome (Gibson et al., 2010). Starting with two digitized genomic sequences of *Mycoplasma mycoides*, researchers manipulated the genetic sequence on the computer, adding several new “watermark” sequences to further differentiate their new “synthetic” genome from the natural *M. mycoides* genome. The researchers then broke the genome up into smaller pieces, which they then synthesized and reassembled to form a complete 1 million base-pair genome. Finally, the synthetically created genome was transplanted into a recipient *M. capricolum* cell, where it displaced the resident genome to form a new, self-replicating, bacterial cell (Gibson et al., 2010; Kwok, 2010). According to Hutchison, the scientists called the final product a “synthetic cell,” even though the only synthesized component was the nuclear genome. Although the cytoplasmic components of the recipient cell were not synthesized chemically (see Figure WO-26), they were replaced by molecules encoded by the synthetic genome as the new cells grew and divided.

While installing the complete genome into a recipient cell was certainly a technological challenge, Hutchison remarked that building the oligonucleotide starting material was equally challenging. At the time, the largest published DNA synthesis project was 32 kilobases (kb) (Kodumal et al., 2004). The investigators chose a bacterial species with the smallest known genome, *Mycoplasma genitalium*, as their starting point (Gibson et al., 2008). *M. genitalium* has 485 protein-coding genes, with about 100 non-essential proteins. The limitation in using *M. genitalium*, Hutchison said, is its very slow doubling time of 12 to 16 hours. When the project reached the transplantation stage—after the researchers had developed a methodology for designing and assembling the synthetic genome—they switched to a faster-growing *Mycoplasma* species, *M. mycoides*, with its larger genome (1.1 millibases [mb], compared to 583 kb) but with a doubling time of only 90 minutes.

The investigators began by designing the complete natural sequence (because they knew that it works), without leaving out any nonessential genes, and making sure that the sequence was correct, as a single base error¹⁹ could be lethal. The genome was then divided into 101 cassettes, each on the order of 5 to 6 kb in

¹⁹ Hutchison noted that many sequences in the databases are not exact—in fact, the researchers found about 30 significant base errors.

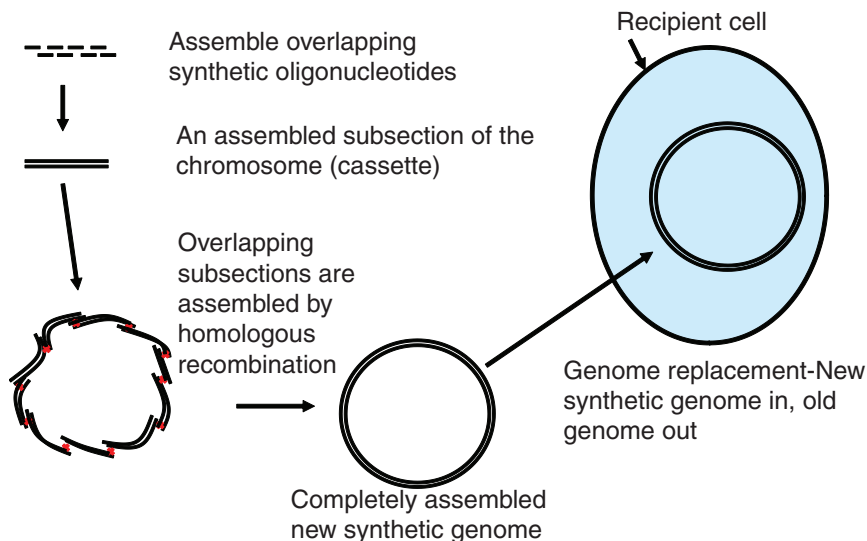


FIGURE WO-26 Overview of steps in making a cell controlled by a synthetic genome. SOURCE: J. Craig Venter Institute (2011). Reproduced with permission.

length, and outsourced production of the cassettes. They assembled the overlapping cassettes in stages to make 144-kb quarter genomes (Gibson et al., 2009, 2010). Inasmuch as the quarter genomes were too large to clone in *E. coli*, the investigators developed a technique for cloning them in *Saccharomyces cerevisiae* (Benders et al., 2010). Finally, the scientists developed a transplantation procedure for replacing the complete genome of a recipient bacterial cell with the complete genome of the donor bacterial cell (Lartigue et al., 2007) and a technique for transplanting a bacterial genome that has been assembled in *S. cerevisiae* back into another bacterial cell (Lartigue et al., 2009).

Hutchison emphasized that the genome transplantation procedure they developed is distinctly different from natural transformation. Transplantation results in a clean replacement of the recipient genome by the donor genome, whereas transformation results in recombination between the donor and recipient genomes. Hutchison went on to state that the “synthetic cell” they produced grew at roughly the same rate as its wild-type counterpart, had the same appearance by electron microscopy, contained nearly identical proteins, and had the same donor cell genome with some minor differences (including two deletions of 4 and 6 kb and 14 missing genes). Currently, the Venter Institute scientists are generalizing the methods described here to other bacterial species, revisiting their original intention to define the minimal *Mycoplasma genitalium* genome, and experimenting with reorganizing the genome during the assembly process. In the future, syn-

thetic biologists may be able to transplant not just naturally occurring genomes but also novel genomes with useful properties that contain mixed-and-matched genes from a variety of sources.

HOW SYSTEMS AND SYNTHETIC BIOLOGY APPROACHES ARE BEING USED TO UNDERSTAND AND MANAGE BIOFILMS

Biofilms are a protective mode of growth that allows bacteria to survive in hostile environments. They are composed of populations or communities of microorganisms that adhere to biotic or abiotic surfaces. These microorganisms are usually encased in an extracellular polysaccharide matrix that they themselves synthesize and may be found on essentially any environmental surface in which sufficient moisture is present.

Biofilms may form:

1. on solid substrates in contact with moisture,
2. on soft tissue surfaces in living organisms, or
3. at liquid–air interfaces.

Typical locations for biofilm production include rock and other substrate surfaces in marine or freshwater environments. Biofilm communities are also commonly associated with living organisms. Plant tissues are commonly associated with microbial populations on their external surfaces. Internal mammalian tissues such as teeth and intestinal mucosa, which are constantly bathed in a rich aqueous medium, rapidly develop complex aggregations of microorganisms enveloped in an extracellular polysaccharide envelope that they themselves produce.

Speaker Peter Greenberg of the University of Washington defined a biofilm as a “structured community of bacterial cells enclosed in a self-produced polymeric matrix.” (Dr. Greenberg’s contribution to the workshop summary report can be found in Appendix A, pages 213-222.) Some experts qualify the definition further and consider only such structured communities that are attached to a surface to be true biofilms. Efforts to identify the composition of biofilms have led to the recognition of multiple polysaccharides, amyloid fibers which the bacterial cells use to adhere to surfaces and, in some cases, extracellular DNA (Whitchurch et al., 2002).

Biofilms are the cause of many persistent, chronic infections for which surgical removal—for example, in cystic fibrosis lung infections, bone infections, and heart valve infections—is a critical component of treatment (Costerton et al., 1999). Their protective nature makes biofilms inherently resistant to many antibiotic compounds regularly used to control non-encapsulated bacterial infections. According to speaker Paul Freemont of the Imperial College London, approximately 60 percent of all hospital-associated infections—more than one million

cases per year—are due to biofilms that have formed on indwelling catheters or implanted medical devices.

Biofilm communities are also ubiquitous in and on environmental surfaces. Biofilms are known to readily form in water pipes and in the ducts of heating, ventilation, and air conditioning (HVAC) systems, impeding heat transfer. Even a 100 micron-thick biofilm, according to speaker Timothy Lu of the Massachusetts Institute of Technology, can block energy transfer efficiency by up to 10 to 15 percent (Schnepf, 2010; Wanner, 2006). (Dr. Lu's contribution to the workshop summary report can be found in Appendix A, pages 278-324.) Biofilms also readily form on foods, serving as an important source of food contamination. Because of their threat to human and environmental health and their resistance to treatment, biofilm detection and dispersal has been the focus of a significant amount of systems and synthetic biology research. This section summarizes some of that work.

Systems Biologists Discover New Ways to Treat Biofilms

Speaker Peter Greenberg described how research on social activities of microbes has led to the development of novel ways to think about infection control. Quorum sensing is bacterial cell-to-cell signaling that allows for coordination of group activities (Parsek and Greenberg, 2005). The signals accumulate only in environments that support a sufficiently dense population (i.e., a quorum) of signal-generation bacteria (Fuqua and Greenberg, 2002; Fuqua et al., 1994). Quorum sensing controls virulence gene expression and has become a target for development of new therapies. Greenberg also discussed biofilms—organized groups of bacteria that can tolerate standard antibiotic treatment. Greenberg and colleagues discovered that iron (Fe) was an important signal for the development of mushroom-like structures that grow on the surface of *Pseudomonas aeruginosa* biofilms attached to glass and that a host iron chelator, lactoferrin, can block biofilm development (Banin et al., 2005; Singh et al., 2002). At low iron concentrations (i.e., sufficient for biofilm growth but still low), the biofilms that form are flat and thin. The researchers further discovered that even in high-iron environments, mutant *P. aeruginosa* cells that are unable to import iron form flat biofilms. They identified several iron receptors, including two receptors that identify iron chelated to ferrioxamine—the desferrioxamine, or DFO, receptors.

Recognizing the critically important role that iron plays in biofilm formation, Greenberg's team collaborated with a research group that had figured out how to replace the iron attached to DFO with gallium (Ga), an inert metal with the same ionic radius as iron, to see if gallium would interfere with the cells' DFO-Fe uptake systems and iron metabolism and thereby block biofilm development (Banin et al., 2008). The researchers demonstrated that, compared to traditional treatment with the antibiotic gentamicin, which kills some of the bacterial cells on the biofilm's surface while leaving cells in the biofilm's interior unscathed, or

Ga alone, treatment with a DFO-Ga complex effectively kills the entire biofilm population (Banin et al., 2008). DFO-Ga has been used in an animal model to potentiate the standard-of-care treatment for corneal infections.

This approach, according to Greenberg, represents a new way to treat bacterial biofilms. It is also testament to insights that are enabled by a systems-level approach to understanding biology. Greenberg went on to observe that, “I think this is a burgeoning field now, coming up with novel ways, based on the biology of the bacteria on biofilms, to attack them.”

How Systems Biology Is Leading to a Better Understanding of Antibiotic Resistance

Speaker Kim Lewis’ work at Northeastern University with persisters serves as another example of how a systems-level approach is leading to a better understanding of biofilm biology and potentially new approaches to managing biofilm aggregation and disaggregation. (Dr. Lewis’ contribution to the workshop summary report can be found in Appendix A, pages 254-278.) Lewis referred to the limited efficacy of existing antibiotics against susceptible cells as “the paradox of chronic infections.” As Lewis explained, *E. coli*, in its free-living state, is antibiotic susceptible but, once it settles on a catheter or another biological implant and forms a biofilm, it becomes incredibly difficult to treat. *E. coli* is not the only microorganism to form these encapsulated communities. Biofilm diseases include pediatric infections of the middle ear by *Haemophilus influenzae*, dental diseases caused by *Streptococcus* and *Actinomyces*, infection of medical devices such as catheters and prosthetic hips and knees by *Staphylococcus aureus* and *S. epidermidis*, and, *Pseudomonas aeruginosa*–associated cystic fibrosis infections. An estimated 65 percent of all infections in developed countries are caused by biofilms (Lewis, 2007).

When a biofilm is treated with low concentrations of an appropriate antibiotic, the vast majority of cells die, but a small fraction persist and repopulate the biofilm, thereby sustaining infection, Lewis explained (Lewis, 2010). These “persister” cells are not mutants, but phenotypic variants that are dormant and, therefore, immune to antibiotic assault (Shah et al., 2006). Unlike resistant cells—which prevent bactericidal antibiotics from binding to their targets—persisters are tolerant of antibiotics because target molecules are inactive as a result of dormancy. “In order to understand tolerance, we need to appreciate that bactericidal antibiotics kill not by stopping functions, but by creating either corrupted products or toxic products that then kill the cell,” Lewis observed. “If the target is inactive, there will be no corrupted or toxic product and no death” (Kohanski et al. 2010a; Lewis, 2007). The small proportion of biofilm cells that are persisters, therefore, function as a pathogen refuge in the presence of antibiotic.

Lewis’ group examined intracellular toxins known to induce dormancy and found that these molecules also rendered cells highly tolerant to antibiotics

(Schumacher et al., 2009). One such toxin, called TisB, is activated by the bacterial SOS response, which also increases mutation rates and, thereby, opportunities for antibiotic resistance to emerge (Dorr et al., 2009, 2010). Lewis observed that when sublethal antibiotic exposures trigger the SOS response, it can lead to the creation of persisters that are multidrug tolerant.

He and coworkers then analyzed pathogen isolates from patients with chronic infections, whose exposure to periodic high doses of antibiotics would be expected to select for comparatively high levels of persistence (LaFleur et al., 2010; Lewis, 2007, 2010; Mulcahy et al., 2010). This is indeed what the researchers found, Lewis said, and these results clearly demonstrate that the ability to make persisters plays a key role in infection, and one distinct from resistance. “In acute infection, it is very important for the pathogen to be able to have resistance, both intrinsic and acquired,” Lewis explained. He went on to observe that chronic infections favor persister cells and tolerance, both of which are reinforced by selective pressure in the form of repeated high doses of antibiotic.

Lewis’ team is exploring the use of broad-spectrum prodrugs, such as metronidazole, a prodrug²⁰ activated by nitrate reductase in bacteria (Lewis, 2007). Initial data have established proof-of-principle that the approach works. However, in terms of developing drugs to target persisters, there is no easy, or realistic, target, as persister formation involves multiple pathways. RelE, HipA, and TisB are just a few of many components. Lewis’ team has been using HTS in order to identify prodrug compounds that might show promise in targeting persister cells.

How a Systems-Level Understanding of Biofilms Is Influencing Antibiotic Drug Discovery

Part of why antibiotic discovery is still in a “Dark Age” is that most environmental microorganisms—more than 99 percent—are unculturable. Lewis and colleagues wanted to see if they could culture these microorganisms by growing them in their natural environment. In order to do this they built a diffusion chamber, embedded marine sediment inside the chamber, and then placed the chamber back in the marine sediment environment. In effect, the bacteria were “tricked” into perceiving the chamber as their natural environment. The recovery rate was about 40 percent, compared to <0.05 percent under standard culture conditions (Kaeberlein et al., 2002). The researchers have been using the same diffusion chamber technique and other similar *in situ* methods to recover a growing number of previously uncultivated microorganisms (Bollmann et al., 2007; Gavrish et al., 2008; Lewis et al., 2010).

Observing that some organisms in the diffusion chamber were able to grow only in the presence of other species from the same environment, the scientists

²⁰ Prodrugs are benign until activated by a bacteria-specific enzyme, so they kill bacterial but not host cells.

hypothesized that cultivability requires the presence of some sort of environmental growth factor. Indeed, in a subsequent study, they found that siderophores²¹ from neighboring species induce growth of uncultured marine bacteria (D'Onofrio et al., 2010). Some bacteria lack the ability to produce their own siderophores and are chemically dependent on other species in their environment. Lewis said, “[unculturable bacteria] live in a precisely defined environment with a precise neighbor. That’s how they know where they are.”

Lewis and his team have found the same phenomenon in the human gut, where about half the bacteria are as yet uncultivated. Pairing different strains together, these investigators have identified “helper strains” that permit growth of previously uncultivated strains. They have also isolated a new class of growth factor—not a siderophore—that they believe may play a role in managing Crohn’s disease. The growth factor is produced by *Faecalibacterium prausnitzii*.

Synthetic Biology Approaches to Managing Biofilms

The easiest way to remove biofilms from surfaces is mechanically by, for example, brushing one’s teeth or surgically removing a biofilm infection. Unfortunately, mechanical removal of biofilms may not be practical in many situations, such as in industrial settings, where the current practice is to use biocides like chlorine bleach and quaternary ammonia. These compounds remove cells on the biofilm’s surface but do not remove cells living deep within the biofilm capsule—a situation similar to what happens when antibiotics, even antibiotics to which the bacterial infection has not evolved resistance, are used to treat biofilms in the human body.

Speaker Tim Lu and colleagues are exploring the potential to use engineered bacteriophages as a novel way to target and disaggregate biofilms once formed (Lu and Collins, 2007). A bacteriophage is any one of a number of viruses that infect bacteria. Bacteriophages are among the most common biological entities on Earth. Discovered as a therapeutic agent in the early 1900s, early efforts to commercialize bacteriophages were challenged by poorly controlled clinical trials. With the introduction of antibiotics over 70 years ago into clinical practice, very few investigators continued with their research in using phages as a therapeutic alternative to antibiotics. With antibiotic resistance having emerged as a major global health problem phage therapy is being seriously considered as a viable option in the treatment and control of antibiotic-resistant “superbugs.” Like antibiotics, phages do not readily penetrate biofilms. In order to get around this physiological barrier scientists are reengineering bacteriophages to express enzymes that digest the polysaccharide matrix capsule so that the phages can gain access to the interior cells of the biofilm. Investigators are also exploring

²¹ Siderophores are low-molecular-weight compounds with a high binding affinity for insoluble iron-III; microorganisms release siderophores to scavenge iron-III and then transport it back into the cell.

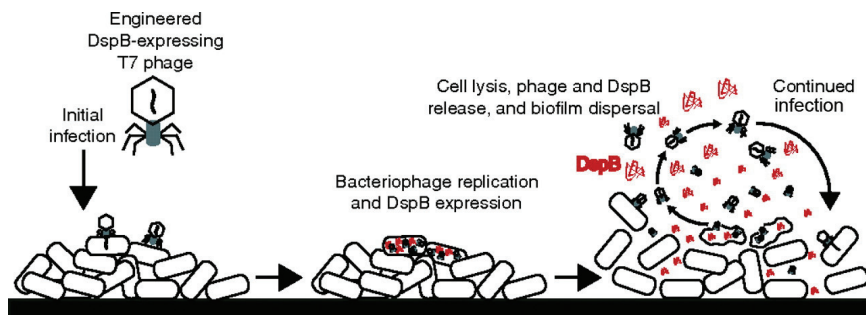


FIGURE WO-27 Two-pronged attack strategy for biofilm removal with enzymatically active DspB-expressing T7DspB phage. Initial infection of *E. coli* biofilm results in rapid multiplication of phage and expression of DspB. Both phage and DspB are released upon lysis, leading to subsequent infection as well as degradation of the crucial biofilm EPS component, β -1,6-N-acetyl-D-glucosamine.

SOURCE: Lu and Collins (2007).

the use of phages—in combination with co-administered antibiotics—as a way to prevent, or minimize, the evolution of resistance to antibiotics.

Lu discussed several efforts to develop enzymes that can break down the extracellular matrix of biofilms and reengineering phages to incorporate these enzymatic modules. Bacteriophages kill bacterial cells and are relatively easy to produce and deliver. By reengineering phages to express enzymatic modules, Lu and his colleagues hope to create a therapeutic product that is easy to produce and deliver and that is able to penetrate the biofilm's interior through expression of the biofilm-degrading enzymatic modules (Lu and Collins, 2007).

As a proof-of-concept that phages can be used not just to kill but also to deliver biofilm matrix-removing enzymatic machinery into bacterial cells, much like a Trojan horse, the researchers re-engineered bacteriophage by incorporating the genetic circuitry for DspB.²² The goal was to create a phage that attacked not only the bacterial cells themselves but also the biofilm matrix. Figure WO-27 illustrates Lu's experimental approach.

Lu and colleagues were able to demonstrate that, compared to non-enzymatic bacteriophages, biofilm removal was significantly enhanced when engineered enzymatic bacteriophages were used to attack and break down the biofilm's protective matrix (Lu and Collins, 2007). After 48 hours, based on cell counts, the untreated biofilms were observed to have about 10^7 colony forming units (CFU)/biofilm. Over the same time course, biofilms treated with natural phages had more than 10^4 CFU/biofilm, and biofilms treated with the engineered phages had less than about 10^3 CFU/biofilm (Lu and Collins, 2007).

²² DispersinB® (DspB) is an antibiofilm enzyme, which has been shown to inhibit and disperse biofilms.

Lu went on to discuss the possibility of engineering bacteriophages that could not only deliver biofilm matrix-degrading enzymes but also the genetic circuitry required to potentiate bactericidal antibiotic pathways that produce reactive oxygen leading to DNA damage. Both Lu and Collins discussed the possibility of shutting off the SOS repair pathway as a way to potentiate the effectiveness of bactericidal antibiotics. As illustrated in Figure WO-28, they found that engineering lysogenic phages to express the *lexA3* repressor of the SOS pathway increased killing by quinolones by several orders of magnitude *in vitro* and increased survival of infected mice *in vivo* (Lu and Collins, 2009).

If left untreated, only 10 percent of mice infected with *E. coli* survived. When treated with antibiotic-potentiating phage, 80 percent of infected mice survived (Lu and Collins, 2009). These investigators also reported that the use of antibiotic-potentiating phage with antibiotics decreased the number of resistant bacterial cells that emerged later on (Lu and Collins, 2009). They tested a range of phage and antibiotic concentrations and, in almost all cases, the combination therapy enhanced bacterial killing. Lu remarked that engineering phage to target non-SOS genetic networks and/or overexpress multiple factors can produce effective antibiotic adjuvants (Lu and Collins, 2009).

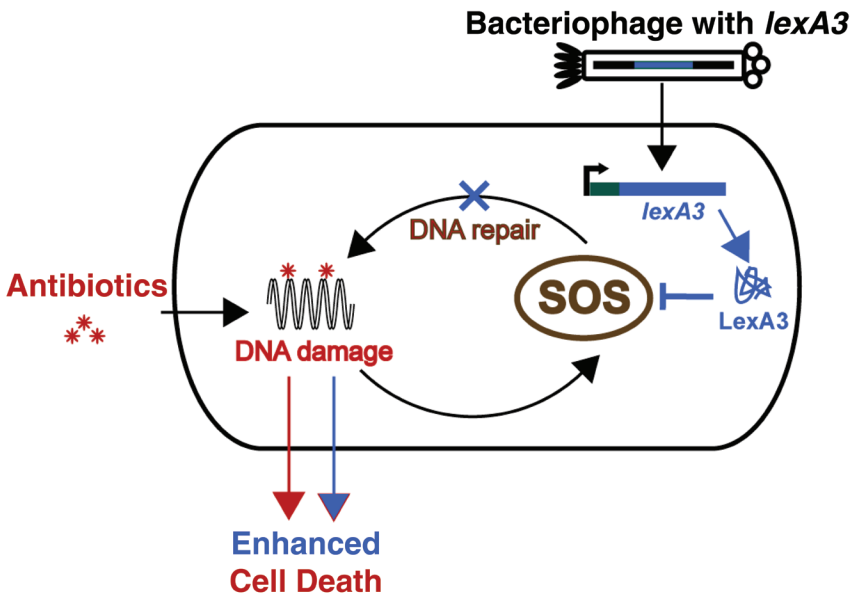


FIGURE WO-28 Targeting bacterial defense networks. Schematic of combination therapy with engineered phage and antibiotics. Bactericidal antibiotics induce DNA damage via hydroxyl radicals, leading to induction of the SOS response. SOS induction results in DNA repair and can lead to survival.

SOURCE: Lu and Collins (2009).

Lu observed that the ability to engineer bacteriophages using synthetic biology methods is becoming easier and faster. The goal, Lu said, is to be able to use phages not just as a means of cell killing but also as a platform for delivering rationally designed antimicrobial agents into the biofilm matrix. After the genetic circuit controlling whatever it is that one wants to express has been identified, one can simply “pop” that circuitry into the bacteriophage and see if it works. Alternatively, one could take a nonrational approach and insert random modules into phages and select for those that work. It is not clear which approach is going to win out, Lu said.

THE PROMISE OF SYNTHETIC BIOLOGY

While there are only a handful of examples of synthetic biology research transitioning from basic to applied research, the growing number of proof-of-concept studies have reinforced both the value and the promise of the synthetic biology approach for product development. The few examples of discoveries that are being actively scaled up for widespread applications have the potential to revolutionize several industries—from drug discovery to materials manufacturing.

Speaker David Berry of Flagship Ventures observed that the biotechnology industry has evolved from a “deconstructive” approach—identifying single factors that make an impact—to a hybridized deconstructive-constructive approach—designing systems-level manufacturing platforms to efficiently produce those single factors. (Dr. Berry’s contribution to the workshop summary report can be found in Appendix A, pages 105-117.) The power of synthetic biology, Berry remarked, is in its potential to streamline and simplify chemical processing and to facilitate faster and less expensive production. Synthetic biology tools and approaches are also being used to design and engineer novel single-factor products, such as protein therapeutics.

Metabolic Engineering as a Platform for the Production of Pharmaceuticals and Other Chemical Products

One can envision a future when a microorganism is tailor-made for production of a specific chemical from a specific starting material, much like chemical engineers build refineries and other chemical factories from unit operations.

—Keasling (2010)

That future may not be too far away. In many parts of the world, *Anopheles* mosquitoes have evolved resistance to quinine-based antimalarial drugs, such as chloroquine. As a result, the World Health Organization (WHO) in 2003 recommended the use of the non-quinine-based artemisinin as the drug of choice to treat malaria. Speaker Jay Keasling of the University of California, Berkeley, observed

that, due to an over delivery of the drug artemisinin following the 2003 WHO recommendation coupled with a subsequent price drop—to a point where it is now more profitable for farmers to grow food crops than *Artemisia annua* plants from which artemisinin is derived—the once plentiful artemisinin stockpile has been essentially depleted, creating what may soon become a huge unmet demand²³ for an essential antimalarial treatment.

One often-cited example of a synthetic biology project that is working its way toward widescale industrial application is the creation of an engineered yeast that produces artemisinic acid, the immediate precursor to the antimalarial drug artemisinin. Artemisinin is highly effective against multidrug-resistant strains of malaria. But, because it is derived from the plant *Artemisia annua* (sweet wormwood), and not easily produced on an industrial scale, it is frequently and chronically in short supply (Specter, 2009). Jay Keasling and colleagues engineered a *Saccharomyces cerevisiae* yeast to produce artemisinic acid by modifying an existing metabolic pathway in the yeast and adding in a gene from *A. annua* to convert the product into the drug precursor (Ro et al., 2006).

Keasling's work aims to engineer a microorganism to become a biofactory that produces artemisinic acid, the precursor to artemisinin. The ultimate goal of Keasling's research is to reduce the cost of artemisinin-based antimalarial drugs by an order of magnitude by engineering a microbe that could produce artemisinin from an inexpensive, renewable resource. Stephanopoulos's research group is also using metabolic engineering to explore and refine a novel microbial-based platform for the production of a range of pharmaceutical and chemical products, including a key intermediate molecule in the biosynthesis of the anticancer drug paclitaxel (Taxol®) (Ajikumar et al., 2010; Liu and Khosla, 2010).

Keasling's work involved overcoming a multitude of significant technical and design challenges (Dietrich et al., 2010; Keasling, 2010). Keasling and his group used synthetic biology techniques to effectively substitute a microbe for the *A. annua* plant, whereby the reengineered microbe becomes a biofactory producing the same artemisinin chemical derived from the *A. annua* plant. They inserted into *E. coli*, a microbe that produces farnesyl pyrophosphate, all of the genes involved in converting farnesyl pyrophosphate into artemisinic acid. The first enzyme in the pathway is amorphaadiene synthase, which converts farnesyl pyrophosphate into amorphaadiene. Because they could not get access to the gene, Keasling's team inserted a similar gene from tobacco that converts farnesyl pyrophosphate into 5-*epi*-artistolochene instead of amorphaadiene. Although the substitution worked, the yield of artemisinic acid was very low—not nearly close enough to the estimated 1 gram per liter that the researchers needed to make it an economically viable option for commercial applications.

²³ According to an artemisinin demand analysis conducted by the Boston Consulting Group, the predicted demand for the next several years is 250 to 300 million treatments annually.

They then synthesized the gene for amorphadiene synthase and optimized it for expression in *E. coli* by changing the codon usage, leading to a two-order-of-magnitude increase in production but still far below what was necessary before commercialization could be considered an option (Martin et al., 2003). Keasling's team used directed evolution and other technologies to further optimize production and balance other parts of the multi-gene pathway (Anthony et al., 2009; Pfeleger et al., 2006; Pitera et al., 2007). Although the final pathway, the conversion of amorphadiene into the desired end product, artemisinic acid, had been proposed in the literature, none of the genes had been identified.

In order to identify the genes involved in the final step in the pathway, Keasling and his team constructed a yeast—*Saccharomyces cerevisiae*—that produces amorphadiene with the goal of using the yeast system as a probe to screen a library of *Artemisia annua* genes and identify those involved in the hydroxylation of amorphadiene into artemisinic acid and then inserting these genes into *E. coli* (Paradise et al., 2008; Ro et al., 2006). Keasling's research group developed a technique for isolating artemisinin-producing cells from the *A. annua* trichome oil sacs in order to develop a cDNA library from those cells' genomes. Keasling's team was able to identify an enzyme that was not only functional but also catalyzed the entire three-step oxidation reaction converting amorphodiene to artemisinic acid (Ro et al., 2006). Remarkably, Keasling explained, because of the toxicity of artemisinic acid, the engineered yeast cells maintained a series of pumps that excreted the toxic end product to the outside of the cell wall. Lowering the pH caused the product to fall off the cell wall. Keasling said, "This gave us the perfect purification process." The same product can readily be transferred back into the *E. coli* chassis (Chang et al., 2007).

Keasling then found a way to link together all of the various reengineered pathways, or partial pathways, especially given that some of the metabolites produced by the reengineered pathways are toxic. Keasling said, "If you think about it, when we engineer metabolic pathways, we are basically expressing the genes that encode the enzymes in that pathway and flooding the cell with protein that catalyzes the transformations in that metabolic pathway. But we don't connect those in any way, so the metabolites are able to drift around the cell. . . . It is as if the plumber threw the plumbing in your house and just expected the water to get from the street to your shower." In order to build a system that would transport metabolites from one reaction to the next, Keasling's team needed to either build what would be the equivalent of pipe threads to link the pathways together or at least find a way to hold the pathways close together in order to increase the chance that the metabolites would naturally diffuse from one reaction to the next. So they built a synthetic protein scaffold with binding domains that recruit the pathway enzymes and, in doing so, increase yield of the final end product even more—to about 500 mg per liter (Dueber et al., 2009).

The yeast developed by Keasling's team has the potential to allow for production of artemisinin at a much larger scale and at a significantly lower cost than

conventionally manufactured artemisinin, which could help save millions of lives annually (Khalil and Collins, 2010; Ro et al., 2006; Royal Academy of Engineering, 2009; Specter, 2009). To accomplish this, Keasling helped start Amyris Biotechnologies, which has already increased the amount of artemisinic acid each cell can produce by a factor of more than one million and, through a partnership with Sanofi Aventis, is hoping to bring artemisinin derived from synthetically-produced artemisinic acid to the market soon. Sanofi Aventis has completed the scale-up process and, at the time of this workshop (March 2011), was outfitting a production facility in Eastern Europe. Keasling expected that the drug will be available by the end of 2011 or early 2012 and remarked that initially it will be available at cost with a long-term goal of reducing the price 10-fold. The cost of their synthetically-produced artemisinin is expected to be less than one dollar per treatment course, approximately one-tenth the current price of naturally produced artemisinin (Specter, 2009).

The Evolution of Resistance

Given that resistance to quinine-based antimalarial drugs is the main reason novel antimalarial compounds—and ways to produce those compounds—are needed, an obvious question is: what is the likelihood of resistance to artemisinin developing? Keasling explained that WHO has recommended that artemisinin be used only as a co-therapy, which is how he and his team are developing the drug and will be marketing it. His intention is to sell the microbially produced drug at a lower cost than conventional commercial drug production. Out-competing monotherapy production, he said, is one way to help with resistance. Developing derivatives of the drug is another. While he and his team have not considered the manufacture of derivatives of artemisinin, they can provide others with the artemisinic acid intermediate which others can chemically “decorate” in any way they choose. “No doubt people will be doing that,” he said.

A Metabolic Engineering Platform for the Discovery and Production of New Therapeutic Molecules

Speaker Greg Stephanopoulos’ work at the Massachusetts Institute of Technology exploits metabolic engineering as a platform for the discovery and production of new therapeutic molecules and revolves around the same isoprenoid pathway that Keasling uses in his research. (Dr. Stephanopoulos’ contribution to the workshop summary report can be found in Appendix A, pages 417-429.) Isoprenoids are chemical precursors to many pharmaceuticals and other chemicals. Stephanopoulos noted that the isoprenoid pathway could be reengineered to generate upwards of 50,000 to 100,000 different compounds.

The isoprenoid pathway can be split into upstream and downstream pathways, both of which can be reengineered and optimized in ways to maximize

production of the desired compound. The upstream pathway yields formation of a key intermediate molecule, isopentenyl pyrophosphate (IPP); the downstream pathway converts IPP into any of tens of thousands of different compounds. In addition to artemisinin and paclitaxel (Taxol®), some of the more commonly known compounds produced by the isoprenoid pathway are menthol, lycopene, and polyisoprene (rubber).

By engineering both the upstream pathway leading to IPP production and the downstream pathway leading to synthesis of taxadiene, a key intermediate in Taxol® production, as well as the biochemical step that immediately follows taxadiene synthesis, Stephanopoulos's team has been able to dramatically increase microbial-based Taxol® production (Ajikumar et al., 2010). Instead of engineering the upstream mevalonate pathway, as Keasling and his team did, Stephanopoulos's group focused on the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway. Stephanopoulos remarked that not only have Keasling's group and others done a substantial amount of work with the mevalonate pathway, leaving less to learn, he wanted to challenge the conventional wisdom that the MEP pathway is not a good pathway for production of isoprenoids. As it turns out, the MEP pathway is about 25 percent more efficient in the synthesis of many downstream molecules.

Specifically, the scientists added extra copies of four genes known to be rate-limiting steps for IPP production to varying degrees (i.e., *DXP*, *CDP-ME*, *ME-cPP*, and *DMAPP*) and transferred two genes—the genes for geranylgeranyl diphosphate (GGPP) synthase and taxadiene synthase—that convert IPP into taxodiene—into *E. coli*. The researchers also reengineered an additional component of the downstream pathway that converts taxadiene into taxadien-5a-ol, improving taxadien-5a-ol production by 2,400-fold. By over-expressing the upstream and downstream pathways to varying degrees, Stephanopoulos's team was able to create an optimized system that produces taxadiene on the order of 1-2 g/liter, representing a 15,000-fold increase over the control (Ajikumar et al., 2010). During their experiments, the researchers discovered that production of taxadiene is inversely correlated with synthesis of another, unrelated metabolite, indole, and that balancing the pathways to optimize taxadiene production also modulates indole levels. To confirm that they had efficiently balanced the upstream and downstream pathways, the researchers used metabolic control analysis, a quantitative method for predicting how changes in various parameters such as enzyme concentration(s) will likely impact metabolite production (Quant, 1993).

Stephanopoulos observed that metabolic engineering can also be used for compound discovery by using metabolic pathway scaffolds to screen libraries of synthetic molecules and identify those compounds that yield the greatest amount of end product. The same metabolic engineering approach can also be used for biofuel production and discovery (Alper and Stephanopoulos, 2009).

Synthetic Biology Approaches to Developing Novel Protein Therapeutics

Speaker George Georgiou of the University of Texas at Austin identified two areas of protein therapeutic discovery and development where synthetic biology is having a significant impact: enzyme therapeutics and monoclonal antibody discovery. (Dr. Georgiou's contribution to the workshop summary report can be found in Appendix A, pages 202-213.) He observed that both areas are immediately relevant to emerging infectious disease management, as both enzymes and monoclonal antibodies have important antimicrobial properties.

Synthetic Biology and Enzyme Therapeutics

While the focus of Georgiou's research is on the use of enzymes for the treatment of tumors, not for antimicrobial applications, he and his colleagues' work nevertheless serves as a proof of principle that this approach may have a variety of applications beyond cancer therapy. Many tumors have metabolic defects that cause them to enter an apoptotic²⁴ process and eventually die if deprived of a particular amino acid. Many central nervous system tumors, such as glioblastoma multiforme, for example, are highly sensitive to methionine depletion; if methionine is removed from their environment the tumor cells die within 3 days. In theory, methionine depletion is a potentially powerful therapeutic approach. Investigators have been attempting to develop a bacterial enzyme for depleting systemic methionine for the past several decades. The challenge, Georgiou said, is that bacterial enzymes are highly immunogenic, causing anaphylactic shock and death in preclinical trials using experimental animals.

Georgiou and his team set out to develop a human enzyme that would have the desired therapeutic effect without the immunogenicity of its heterologous bacterial counterpart. The methionine-degrading bacterial enzyme L-methionine-g-lyase (MGL) is structurally similar to the human enzyme cystathionine g-lyase (CGL). Using computational design and HTS, these investigators engineered a modified CGL with methionine-depleting capacity and reformulated the compound to have a circulation half-life²⁵ of 35 hours. Georgiou's team then developed a process for expressing the engineered enzyme in *E. coli* so that they could generate enough product to use in preclinical animal studies. Georgiou and his group are currently collaborating with an academic good manufacturing practice (GMP) facility in preparation for clinical research. In addition, Georgiou's group has two other enzyme therapeutics in development, one for the systemic depletion of arginase and the other for the systemic depletion of asparagine.

²⁴ Apoptosis is a process of programmed cell death by which cells undergo an ordered sequence of events that lead to death of the cell, as occurs during growth and development of the organism, as a part of normal cell aging, or as a response to cellular injury.

²⁵ Half-life (biological) is the time it takes for a substance to lose half of its pharmacologic, physiologic, or radiologic activity.

Synthetic Biology and Antibody Discovery

Novel antibodies are typically discovered in one of two ways: the disease target molecule is purified and an antibody specific to that target is produced, or, antibodies are isolated from antigenically-responsive patients (see Figures WO-29A and B, respectively).

Georgiou remarked that the first method is not as easy as it sounds, especially with complex targets; investigators often need to make and sort through thousands of antibodies before finding one that is specific enough to have a therapeutic effect. The second method, in addition to being time-consuming, is also limited by the fact that “clonable” antibodies are not the same as antibodies that are actually eliciting the bulk of the immune response. The antibodies responsible for about 80 to 90 percent of humoral immunity, Georgiou said, are “hiding” in the bone marrow.

Georgiou and his team of investigators developed a set of “third wave” antibody discovery technologies for “mining” the hidden antibodies (see lower half of Figure WO-29B). The technologies involve isolating the desired bone marrow plasma cells and sequencing the entire genetic repertoire, mining the repertoire and identifying antigen-specific antibodies, synthesizing the antibody genes, and then expressing those genes in *E. coli* (Reddy et al., 2010). The researchers have applied the methodology to a variety of experimental animal species by immunizing animals with various protein antigens and then identifying antibodies with varying affinities to those proteins. Georgiou and colleagues have also developed a methodology to engineer aglycosylated monoclonal antibodies (Jung et al., 2010).

Georgiou offered his opinion that antibodies make for useful antimicrobials because of their specificity, predictability, and profitability for their commercial manufacturers. A number of antibodies are currently in late-stage development for the treatment of a variety of infectious diseases. In 2009, Merck & Co. signed a licensing agreement for a monoclonal antibody for use in the treatment of *Clostridium difficile* that was co-developed by Mederix Inc. and Massachusetts Biological Laboratories (News and Analysis, 2009). Elusys Therapeutics, Inc. (Pine Brook, New Jersey) is also developing an antibody engineered by Georgiou’s laboratory for use as a post-exposure prophylactic therapy for inhalational anthrax (see Leysath et al., 2009; Maynard et al., 2002; Mohamed et al., 2005). In addition to conventional antibodies, several investigators and commercial firms, such as Symphogen (Lyngby, Denmark), are developing polyclonal recombinant antibodies for use as broad-spectrum anti-infective therapeutics.

What Synthetic Ecosystems Are Teaching Biologists About Antibiotic Resistance and Antibiotic Drug Discovery

The study of how organisms interact with each other—and with their environment—falls under the purview of ecosystems analysis. Collins described two studies demonstrating the power of what he called “synthetic ecosystems”—

A. Monoclonal antibodies to purified disease target molecule

e.g., *B. anthracis* PA (Anthrax), Her2 (Herceptin, breast cancer)

- Hybridomas, B cell immortalization, display technologies

B. Isolation of monoclonal antibodies from responding patients

e.g., neutralizing Ab to HIV1, broadly neutralizing antibodies to influenza

- Human hybridomas, single-cell PCR cloning from blood B cells

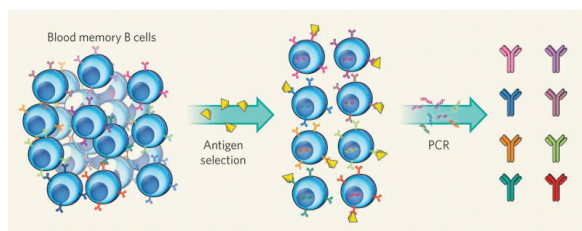


FIGURE WO-29A Antibody discovery strategies.
SOURCE: Georgiou (2011).

B. Isolation of monoclonal antibodies from responding patients

- Very time consuming, difficult
- “Clonable” peripheral B lymphocytes (B memory cells) generally do not represent the long-term protective Abs in circulation which are produced by bone marrow cells
- No information on the molecular composition of protective responses

C. “Third Wave” Antibody Discovery

- Experimental analysis and mining of the polyclonal immune response

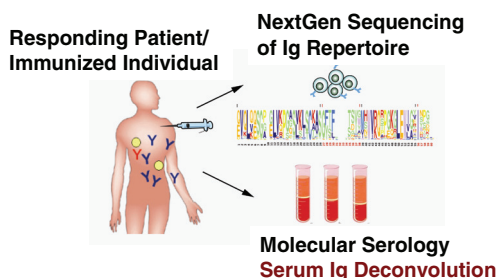


FIGURE WO-29B Isolation of monoclonal antibodies from responding patients.
SOURCE: Georgiou (2011).

engineered systems that provide for the coexistence of multiple microbes (Lee et al., 2010). Collins and his team engineered a bioreactor that allowed them to control, on a day-to-day basis, the level of antibiotic that the resident *E. coli* organisms were exposed to and track the evolution of resistance. They found that the large majority of bacterial cells evolve only very low-level antibiotic resistance and that the small minority of cells that do evolve high-level resistance protect their less-resistant neighbors by producing and secreting indole, a signaling molecule that turns on oxidative stress protective mechanisms. Collins referred to the highly resistant indole-producing cells as “bacterial charity workers.”

In the second synthetic ecosystem study, Collins and colleagues pitted *E. coli* against *Candida albicans* in what they viewed as a large-scale scheme of Andrew Fleming’s original “microbial wars” work. The scientists observed that *E. coli* always won. They investigated further and discovered that *E. coli* produces a small molecule that kills *C. albicans*. Currently, the researchers are trying to identify the molecule, with the ultimate goal of synthesizing it. Collins observed that the same synthetic ecosystem scheme could be used to pit any two microbes against each other and search for organisms that produce bactericidal molecules.

Building Synthetic Biological Diagnostic and Detection Devices

One of synthetic biology’s “early win” situations, in Paul Freemont’s opinion, was with the modification of biological systems to detect biological signals. While most classical biosensors, such as blood sugar level monitors, rely on electrochemistry—converting a concentration of a molecule into a digital display—synthetic biosensors are entirely genetically encoded. Genetically encoded biosensors are very simple devices. They typically use transcription regulators to bind to the analyte of interest, resulting in either the expression or repression of a reporter gene that codes for a particular output signal. Some biosensors utilize fluorescently-tagged proteins that respond directly to the analyte; others make use of aptamers.

Freemont and his research group have been working with genetically coded biosensors to see if they could design a biosensor capable of detecting the acyl-HSL quorum-sensing signals—discussed by Greenberg—that diffuse in and out of bacterial cells as biofilms develop. Freemont agreed with Greenberg that regulation of the gene circuit activated by acyl-HSL quorum sensing is complex, with other factors at play. Biofilm development is one of those factors and there is a clear relationship between quorum sensing and biofilm development (Kirisitis and Parsek, 2006).

Sixty percent of all hospital-associated infections are biofilms on indwelling medical devices. In fact, development of a urinary tract infection is widely considered almost inevitable for patients undergoing long-term indwelling catheterization. Freemont’s team wanted to develop a biosensor to detect *P. aeruginosa* biofilms on medical devices.

The synthetic biology approach taken to design the device included consideration of input (quorum sensing), sensing (what type of genetically encoded sensor would be used), output (fluorescent, luminescent, or enzymatic proteins), and the chassis (the host system). As proof-of-concept, the scientists' initial goal was to engineer a three-stage cell-free amplification device that could detect nanomolar concentration levels of AHL and, within 3 to 6 hours, send a fluorescent signal indicating detection to an output device. The actual device would involve the LasR transcription factor binding to AHL and the LasR-AHL complex dimerizing and inducing transcription of AHL-responsive promoters. A schematic illustration of a simple biofilm biosensor is presented in Figure WO-30.

A cell-free design is essentially a biochemical mixture of the contents of the cell that has the ability to transcribe and translate a genetically encoded design into a device. Through modeling and *in silico* simulations, combined with *in vivo* testing of various parts—such as the various promoters controlled by LasR—Freemont's team has developed two devices ("V" and "L") for testing (MacDonald et al., 2011).

In one set of experiments, when the devices were tested on streaks of *Pseudomonas aeruginosa*, fluorescence production was readily observable at the tip of the devices. In another set of experiments that involved forming biofilms in 96-well plates, the devices responded differently to different strains of *P. aeruginosa*. These results are very exciting, Freemont remarked, because they represent the first time that an apparently effective biosensor device has progressed from the modeling stage all the way through *in vivo* testing. Freemont observed that, "This whole project has told us that if you get away from all the complexity and you look at the performance and the characteristics and the modeling, and you actually think of this as a device—and all you want is for it to work within certain parameters—then you can make quite a lot of progress without knowing absolutely everything about what's going on." The challenge now, he said, is to develop the

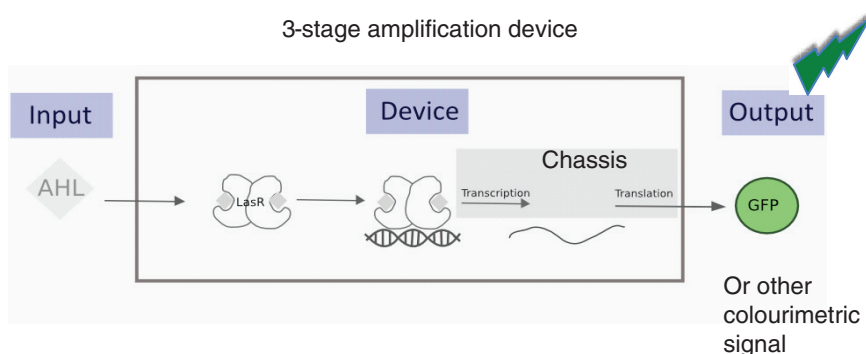


FIGURE WO-30 A simple biofilm biosensor.

SOURCE: Freemont (2011).

device into one that is cell-free and can actually detect biofilm development on catheters and other surfaces in real-world settings. That effort is ongoing.

Use of a Cell-Free Design Versus a Living Chassis

Freemont explained that the choice to use a cell-free system was based partly on a decision to stay away from the use of genetically modified organisms. Additionally, cell-free systems are simple to set up, as they have only three major components: the cell-free extract (the machinery required for transcription and translation), the premix (energy and resources required for the device to operate), and a DNA template. In terms of time, there is a lag as transcription begins, followed by a linear increase in production of the fluorescence, and ending with another lag as all of the components of the biochemical mixture are used up. Moreover, and arguably of greatest importance, cell-free systems can be very clearly defined.

Freemont remarked that “the great beauty of cell-free systems” is that they are easier to define than systems with a living organism’s entire genetic program operating in the background. Without the extra genetic coding of a host organism, cell-free systems can be built up from detailed modeling. Freemont and his research team are currently exploring the relationship between the properties of synthesized parts in cell-free systems and those same parts obtained *in vivo*. Freemont’s team has built a microfluidics platform for testing thousands of proteins and obtaining information on basic characteristics for each protein. It remains an open question whether this approach and the information gathered from it will be useful in the future design and construction of biological devices.

Implications of Synthetic Biology for Energy and the Environment

The potential applications for synthetic biology devices extend far beyond medicine. French’s work with whole-cell arsenic biosensors for drinking water, is just one example of the many ways that synthetic biology is being used in environmental applications (Joshi et al., 2009). Other investigators are developing similar systems for detecting other toxins (van der Meer and Belkin, 2010). In one of the earliest engineered environmental biosensors, Looger et al. (2003) designed a protein that can bind and detect TNT in soil. Future applications include engineering biosensors to detect heavy metals and other common persistent toxins in soil and water (Royal Academy of Engineering, 2009).

Scientists are also engineering microbes that can bioremediate oil spills, organic pollutants, and other environmental contaminants. For example, Hannink et al. (2001) added a modified nitroreductase (*nfsI*) gene from *Enterobacter cloacae* to tobacco plants, allowing the plant to tolerate, take up, and break down 2,4,6-trinitrotoluene (TNT). Bacterial enzymes inserted into tobacco plants demonstrated the ability of the engineered plants to remediate a range of halo-

generated organic pollutants (Mena-Benitez et al., 2008). One of the pollutants is 1,2-dichloroethane (1,2-DCA), which is used as an intermediate in the synthesis of vinyl chloride and other industrial chemicals and is listed by the U.S. Environmental Protection Agency as a priority pollutant and a probable human carcinogen. Researchers at the University of Cambridge added the gene for the enzyme pentaerythritol tetranitrate reductase (PETNR), normally found in *Enterobacter cloacae*, into tobacco plants, allowing the plant to break down TNT (French et al., 1999).

Building Microbial-Based Fuel Security

While there is a great deal of interesting work being done in the biotherapeutic market space, Berry suggested that the energy and fuel market, as well as other chemical product markets—including specialty chemicals, commodity chemicals, petrochemicals, agricultural chemicals, and other chemicals—completely dwarfs that sort of opportunity. Indeed, synthetic biology is providing a novel means to help address energy challenges through the design of organisms that can more efficiently manufacture biofuels and are less wasteful than the current processes used to make ethanol-based fuels (Fortman et al., 2008; French, 2009; Royal Academy of Engineering, 2009). Much of this research and development work is being sponsored and conducted by the U.S. Department of Energy and at private firms.

The market for synthesized microbial-based fuel production is driven not just by rising oil prices but also by increased consumer demand for environmentally friendly products and growing government regulations to curb greenhouse gas emissions. Berry estimated that 95 percent of the global market for petroleum and other products manufactured via chemical transformations have never been addressed biologically. He predicted a \$3 trillion to \$5 trillion-plus market for synthetic biology applications. Berry, a venture capitalist, estimated an additional \$3 trillion-plus market for synthetic biology.

As three examples of how synthetic biology is being applied to the commercial development of nonpharmaceutical chemically-derived products, Berry mentioned Mascoma (Lebanon, New Hampshire), LS9, Inc. (south San Francisco, California), and Joule Unlimited, Inc. (Cambridge, Massachusetts). Mascoma is focused on the production of cellulosic ethanol fuels using engineered microbial organisms. Leveraging 20 years of effort to optimize a process first described in 1991, the company has used metabolic engineering techniques to develop a microbial-based system with near 90 percent efficiency (Lynd et al., 1991, 2005). According to Berry, the firm has ramped up to near-commercial scale at a demonstration plant in Rome, New York, and expects to break ground on a full commercial-scale refinery in Kinross, Michigan, soon.

While investigators have been studying the biofuel potential of ethanol since the 1970s, ethanol is not the only potential biofuel alternative. LS9 scientists have

harnessed the fatty acid biosynthesis pathway in *E. coli* to produce a fat molecule, with an efficiency of about 94 percent, which looks very much like diesel fuel. That molecule is then converted into diesel through subsequent processing. Additionally, LS9 is using the same system as a platform for producing other multi-billion-dollar market chemicals. As Berry observed, this company has put itself in a position of basically being a biorefinery of sorts, where you can systematically make a single organism to go after different productions that leverage a common process. LS9 has ramped up production to demonstration scale at a facility in Okeechobee, Florida, and expects to break ground on a commercial plant in 2012.

A major challenge for all heterotrophic processes is the dependency of sugar as a core feedstock ingredient. The reality of this was made clear through the company's development of LS9. LS9's feedstock is any sugar source, which includes sugar cane, corn, cellulosics, and others. When the company was founded in 2005, the price of sugar was much lower than it is today. As a way to hedge against this sort of price fluctuation, Berry and colleagues began exploring the possibility of using CO₂ as an input instead of sugar cane syrup, eliminating the need for dependency on not just a commodity but also arable land. Founded in 2007 by Flagship VentureLabs, Joule scientists have metabolically engineered a photosynthetic microbe to convert solar energy and waste CO₂ into usable fuel (Robertson et al., 2011). The firm is also using the platform to produce other fuels and chemicals as well.

Joule opened a pilot plant in Leander, Texas, in 2010. According to Berry, the company expects to break ground on a commercial plant by the end of 2011. One advantage that Joule has over companies like Mascoma and LS9 is modularity²⁶ and scalability—there is no risk involved with the scale-up of a solar panel, or reactor, as all solar panels function in the same way (as opposed to the scale-up of industrial fermenters, whose scale-up requires overcoming new chemical engineering challenges).

Synthetic Biology Meets Materials Engineering

As discussed earlier in this chapter, Chris Voigt's work with spider silk protein-secreting *Salmonella* bacteria could have widescale material engineering applications. Spider silk has a similar tensile strength as steel, yet it is much lighter. By modifying the type III secretion system of *Salmonella* using synthetically designed genes, these investigators have essentially turned the bacterial cell into a small spider silk production factory (Widmaier et al., 2009). Large-scale production of spider silk by engineered *Salmonella* could lead to novel super-strong and lightweight weavable materials, which could have major implications

²⁶ As Berry went on to state: "If you know one reactor works, you know two, or ten, or a hundred work because they are just repeats—much like the scaling of solar power. It's not risk free, but very heavily risk mitigated. Fermenters scale in three dimensions, which do have the fundamental chemical engineering challenges."

for the aeronautic, automotive, and other industries (Royal Academy of Engineering, 2009).

Synthetically engineered organisms could also one day produce the materials from which the next generation of aircraft, automobiles, and buildings are constructed. The 2010 iGEM team from Newcastle University developed a product called BacillaFilla (see Figure WO-31) in which an engineered *B. subtilis* uses quorum sensing to grow into and fill cracks in concrete and produce a mixture of calcium carbonate, levan glue, and filamentous cells that hardens and repairs the crack (Cathcart, 2011; Newcastle University 2010 iGEM Team, 2010). With further development, the product could help extend the life of concrete structures or even pave the way toward “self-healing” concrete.

While most systems and synthetic biologists who work with biofilms are seeking novel ways to treat biofilms, Lu noted that there are also some potential applications of biofilms. One of those applications is in materials engineering. Biofilm cells have evolved ways to produce very complex three-dimensional structures with unique properties (Epstein et al., 2011). Lu and his research team are exploring the possibility of capitalizing on the self-assembly mechanisms that enable biofilm cells to do what they do and leveraging the same mechanisms to produce new types of materials.

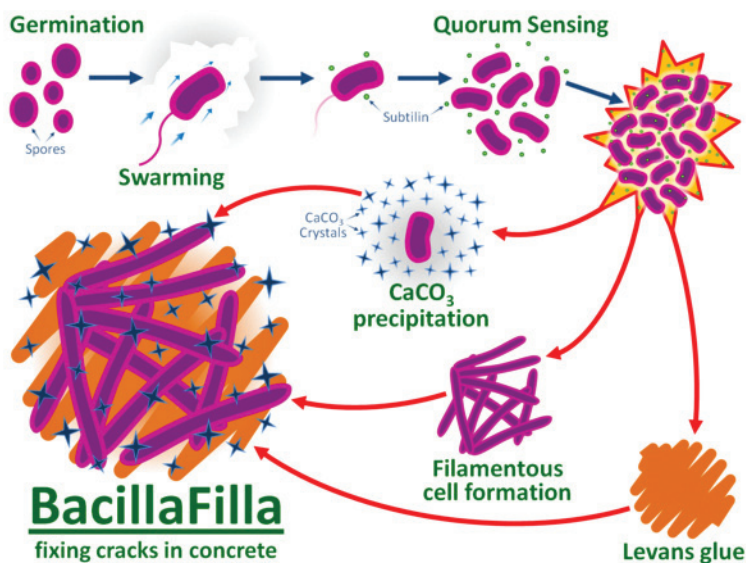


FIGURE WO-31 Cartoon describing Newcastle University 2010 iGEM team’s BacillaFilla. SOURCE: <http://2010.igem.org/Team:Newcastle>.

Living Computers

Finally, the foundation is being laid for researchers to begin to develop computers made out of living cells (e.g., Baumgardner et al., 2009). While it is unlikely that biological computers would ever replace conventional computers in the near term they would certainly outperform conventional computers in certain situations, such as inside engineered cells. Cells that have been engineered to deliver a drug could be programmed to deliver doses directly to the target sites. The basic biological switches and oscillators developed over the past decade and previously discussed represent the first steps in this direction (Khalil and Collins, 2010; Royal Academy of Engineering, 2009).

CURRENT CHALLENGES IN SYNTHETIC BIOLOGY

Despite the field's rapid development over the past decade, many of the speakers and the Forum's members discussed what they perceived to be the many significant challenges that would need to be overcome for many of the promises of synthetic biology to become realities. Some speakers observed that significant technical hurdles such as the lack of standardization and incompatibility of bioparts must still be addressed before true "plug-and-play" engineering of biological systems can be achieved. It was also felt that a far better understanding of complex biological systems will be required before novel organisms could be designed from the bottom up. Coordination and ownership challenges might also need to be overcome in order to ensure that experiments are conducted safely and that discoveries can be built upon while still addressing intellectual property concerns. Finally, many participants discussed the legitimate ethical and regulatory concerns regarding the proprietary and potential safety issues of "designing life" that must continue to be transparently discussed and addressed by the scientific community as the field grows and evolves.

Technical and Scientific Challenges

We are still like the Wright brothers, putting pieces of wood and paper together.

—Luis Serrano (Kwok, 2010)

The field has had its hype phase. Now it needs to deliver.

—Martin Fussenegger (Kwok, 2010)

At its core, synthetic biology represents remarkable progress in the technical capacity to not just mix and match genetic material from different species but also to design and build genetic systems that do not exist in nature. Yet, arguably, it is the technical challenges more than anything else that are keeping synthetic biolo-

gists from achieving what some consider the ultimate prize: the ability to design an organism and effectively create new life. These technical challenges include:

- a lack of complete knowledge about how biological systems—from genetic circuits to whole cells to entire organisms—function,
- the inability to standardize parts and mix and match parts in different host organisms, and
- the problem of evolution (even synthetic biological systems change over time in unpredictable ways).

Bottom-up approaches in particular are far from reaching a point at which synthetic biologists will be able to build, from scratch, systems capable of doing novel things, let alone systems capable of self-replication and otherwise functioning as living organisms. Currently, the most interesting and potentially applicable synthetic biology approaches are all top down, reengineering existing organisms to perform functions that they normally would not do, such as produce a chemical precursor to an antimalarial drug, spider silk protein, or biofuels.

Does Systems Biology Trump Synthetic Biology?

Our understanding of physical laws and knowledge of material properties allow us to engineer bridges that do not collapse and car engines that convert energy into mechanical energy. Engineering biology, however, is different. Even the simplest bacterium comprises a system whose complexity is humbling.

—Fritz et al. (2010)

When asked whether systems biology trumps synthetic biology, many speakers said, “Yes.” Arguably the greatest challenge confronting the synthetic biologist is that scientists still do not understand many of the fundamental principles about how life “works.” Sauro remarked that there is a great deal of basic biological knowledge that needs to be generated before synthetic biology can create the “wow” results that are expected of it. The complexity of even basic cellular physiology is far beyond that of a circuit board or a transistor and there are few such physiological processes that biologists fully comprehend. It is not enough to understand the actual molecular parts that they are manipulating; synthetic biologists must also appreciate how those parts operate within the context of the host cell. In effect, synthetic biologists are trying to engineer life without knowing all the ground rules. As a result, despite efforts to develop the best possible predictive models, there is still a high degree of trial and error required to get synthesized biological parts to work together to form functioning circuits. As systems biology advances and scientists gain a better understanding of how the parts of a cell or other biological system interact, synthetic biology will become more predictive—and easier.

The Challenge of Standardization

“The beauty of synthetic biology,” Freemont observed, “is that it’s modular.” Others agreed. Voigt remarked that the vision for the future is to advance the design of large genetic systems to a point where it might be possible to simply “pop in” modules for various functions. Freemont discussed his research team’s ongoing efforts to construct a biosensor device that can detect the formation of *Pseudomonas aeruginosa* biofilms on indwelling catheters before the infections develop into full-blown urinary tract infections. The device that he and his colleagues have built—that his students refer to as the “infector detector”—can potentially be modularized with different inputs creating different outputs.

Another challenge facing synthetic biologists is that there is still a lack of standardized, well-characterized, and interoperable biological parts. The BioBricks® Foundation and the Registry of Standard Biological Parts are making progress in developing a common toolbox of biological components for synthetic biologists to use. Yet, the functions and properties of most parts remain poorly defined (Kelly et al., 2009). Sauro observed that biological parts in general (not necessarily BioBricks®) are poorly characterized, as the tradition of characterizing biological components has been losing ground to the use of high-throughput methods for collecting large quantities of data.

Part of the problem, Sauro observed, is that synthetic biologists rarely reported exactly what they did in their experiments, making the experimental results difficult for others to replicate. In particular, exact DNA sequences are often omitted from published papers (Peccoud et al., 2011). Moreover, synthetic biologists’ ability to make quantitative measurements is still relatively crude. This is especially true of high-resolution, system-wide, measurements.

Without a robust toolbox of standardized parts that function in a predictable and reliable manner, synthetic biologists are forced to design biological circuits *ad hoc*, limiting how complex their designed circuits and systems can be (Kwok, 2010). As Sauro observed, poor standardization also limits what can be done with new knowledge gained through synthetic biology experimentation. Improved standardization would allow researchers to electronically exchange designs and replicate experiments, send designs to “bio-fabrication centers” for assembly, and store designs in repositories and for publication purposes. Sauro pointed to the Synthetic Biology Open Language (www.sbolstandard.org), a platform for exchanging data among different software applications and connecting different types of synthetic biology information (e.g., modeling results, experimental results, biological parts data), as an example of an initiative aimed at providing standardization.

Despite the challenge of standardization, many researchers nonetheless are using BioBricks® in their research (Shetty et al., 2008). Acknowledging “serious quality control issues” with the Registry of Standard Biological Parts, French stated that he was a “fan” of BioBricks®. Without knowing how a biological

system works, one can use parallel combinatorial methods to assemble multiple combinations of parts and see which parts and combinations work(s), eliminating much of the unpredictability associated with the design process. BioBricks® also serve as the raw material for iGEM, an annual synthetic biology competition for students (see Box WO-2).

BOX WO-2
The International Genetically Engineered Machine Competition



The iGEM competition resembles a giant science fair for budding synthetic biologists. iGEM is a global synthetic biology competition involving mostly undergraduate students, although participants range from high school students to world experts in the field. At the heart of the competition is the BioBricks® standard, a format for interchangeable, composable DNA parts. Several months before the actual competition, competing teams receive a kit of DNA parts. Working at their own schools over a summer, teams design and build synthetic systems that operate in living cells. Examples of recent projects include an arsenic biosensor, wintergreen-scented bacteria, and color-coded microbes. Chris French remarked that, because students are unfettered by considerations of practicality or feasibility, they tend to come up with very interesting ideas. For example, in 2005, competitors developed a bacterial photography system (Levskaya et al., 2005); and in 2006, one group engineered a strain of *E. coli* that generated the aroma of bananas during the exponential growth phase and oil of wintergreen during the stationary phase. All of the parts used in the student projects are components that have been deposited in the Registry of Standard Biological Parts (<http://partsregistry.org>). The whole-cell biosensor for the detection of arsenic in groundwater that French described (see text) was a 2006 iGEM project. Beyond building biological systems, the broader goals of iGEM include growing and supporting a community of science guided by social norms.

SOURCE: Image from The International Genetically Engineered Machine Competition (http://ung.igem.org/wiki/images/d/de/IGEM_basic_Logo_stylized.png); text from Presidential Commission for the Study of Bioethical Issues (2010).

The Challenge of the Robust Chassis

As with the parts, there is no standard chassis. In the context of synthetic biology, the chassis refers to the cell or organism onto which an engineered DNA construct or part is embedded in order to produce the desired device or system (Royal Academy of Engineering, 2009). Synthetic biologists typically insert their engineered circuits into *Escherichia coli*, but they also use *Bacillus subtilis*, *Saccharomyces spp.*, and other microbial species. The lack of a standard chassis has presented a problem for those investigators seeking to develop a standardized set of biological parts since parts that work in one cellular environment may not work in another (Kwok, 2010).

The current effort among top-down synthetic biologists to create a minimal cell by stripping a simple bacterium down to only those parts required for basic survival represents an attempt to develop a standard model organism (Glass et al., 2006). It is critical that any engineered DNA or other component that is inserted into an organism not interfere with the normal metabolic processes of the organism (Royal Academy of Engineering, 2009). A major challenge with engineering a synthetic pathway inside a living cell is managing depletion of the metabolite serving as the source of the engineered reaction(s). Westerhoff explained that depletion of a metabolite jeopardizes cell function and, eventually, the cell will die. Another major challenge is the likelihood that a change in metabolite concentration will elicit a homeostatic response in the host cells that shuts down the engineered system. Building a robust chassis means building a chassis that is buffered against otherwise noticeable fluctuations in metabolite concentration.

When testing new parts in new chassis, Sauro emphasized the importance of allowing the engineered system enough time to reach a steady state. He described an experiment that he and his colleagues conducted with what he described as a “very, very simple experiment,” that is, a GFP attached to a *lac*-inducible promoter which will synthesize GFP when lactose is present and is being metabolized. When the rate of GFP synthesis was plotted over time, it demonstrated an exponential increase in production followed by a sharp plateau and then a downward plunge over the course of several hours. The investigators are still not sure why GFP synthesis collapsed so quickly. However, when they extended the experiment out to 16 hours, the system actually reached a steady state of production, remaining in the exponential phase. Sauro remarked that most synthetic biology experiments are conducted over a very short time period and emphasized the need to extend experiments over longer time periods. Sauro also emphasized the importance of considering the media in which host cells are grown. For example, the growth curve of *E. coli* is very different in different nutrient media. He surmised that cells undergo morphological changes in the presence of difference amino acids, which can affect the results of synthetic biology experiments.

What Does the Future Hold: Coordination, Growth, and Ownership Challenges

Given that an estimated \$7 trillion is spent every year on biomedical research worldwide, Hans Westerhoff emphasized the urgency of developing worldwide platforms—and standards—for combining and coordinating efforts not just within synthetic and systems biology but across all areas of biomedical research. He described a European Union initiative to link all of the various and currently disjointed *in silico* human research programs, most of which currently are focused on single organ systems (e.g., Noble, 2008; Thomas, 2009), and construct an *in silico* human (the “virtual physiological human”; see www.vph-noe.ed) similar in concept to the *in silico* trypanosome that he and his colleagues are constructing (Bakker et al., 2010; Kohl and Noble, 2009). Both projects seek to create web-interfaced, experiment-based mathematical models of the physiologies of their respective organisms, providing a means for collaborative investigation and quantitative prediction. Bakker et al. (2010) predict that the modeling enabled by a “silicon trypanosome” will provide investigators with a way to identify the most suitable targets for developing novel antiparasitic drugs.

As a nascent and cutting-edge field, there is significant interest among both professional and amateur scientists alike in the developments of synthetic biology. As information about how to work with DNA has proliferated on the Internet and the cost of obtaining a basic DNA synthesis machine has dropped from hundreds of thousands of dollars to less than one thousand dollars on eBay®, a growing movement of “backyard,” amateur scientists has emerged who are conducting synthetic biology experiments. These so-called do-it-yourself (DIY) biologists operate outside of the infrastructure and constraints of traditional research institutions and without the support of large-scale grants. They are often compared to the early computer hackers who assembled the first home computers in their backyard garages.

However, because these “biohackers” are working with living organisms that could escape into the environment, there has been a significant debate within the traditional scientific community about whether to embrace or constrain the DIY biology movement. Some see it as a positive force that is helping to attract new scientists who could push the envelope of innovation. Others fear the risk of accidental release or the intentional creation of agents of bioterrorism should the DIY biology movement—practiced by individuals who lack formal scientific training and work in makeshift facilities—become further enabled to conduct synthetic biology experiments and more knowledgeable (Feuer, 2010). What is clear is that this movement, and others involving nontraditional life scientists, is not going away and the regulatory and organizational challenges they present must be addressed.

The iGEM competition, described earlier, is one way that the scientific community has responded to this challenge. By providing a curated community in which budding synthetic biologists from a variety of backgrounds can work to-

gether to develop innovative projects, iGEM encourages innovation and engagement while promoting laboratory standards and best practices. iGEM even helps link members up with online courses about synthetic biology to encourage ongoing study and learning (iGEM, 2011). Indeed, iGEM, the Registry of Standard Biological Parts and the BioBricks® Foundation are at the forefront of the kind of open-source science that many synthetic biologists argue is the best way to encourage innovation and progress in the field. The open source model of science promoted and embraced by many working in synthetic biology does, however, present some challenges, particularly in terms of intellectual property rights.

Given the wide array of sectors that synthetic biology could influence, and the potentially significant wealth that its research findings could generate, many of this workshop's participants felt that it was important for the community to address the issue of intellectual property more rigorously. The challenge before the scientific and regulatory communities is to strike the appropriate balance between allowing researchers to benefit from their discoveries while simultaneously encouraging an environment of information sharing so that accomplishments can be built upon.

Regulatory Challenges

The novel technologies and innovative approaches being developed and used by synthetic biologists represent new ground for the regulatory agencies. The concern is exacerbated by the reality that the United States is entering a more austere budgetary climate, with doubts about whether expected increases in the Food and Drug Administration budget, including increased investments in regulatory science, will actually happen. The concern is especially great for large-scale multiplex profiling devices, or systems, given the difficulties regulators have had in the past with other (non-synthetic) multiplex assays (e.g., companion diagnostics to guide therapeutic decisions, and cancer diagnostics). Forum member George Poste observed that, "I have great concerns as to whether there is going to be both the resource availability as well as the intellectual agility needed to deal with some of these issues."

From the beginning—just a mere decade ago—scientists have openly discussed how the synthetic biology community should regulate itself and whether there should be limits placed on the type of research conducted (Cho and Relman, 2010; Church, 2005). Some organizations have proposed recommendations for self-governance in an effort to proactively address the issues raised by these activities (Garfinkel et al., 2007). Indeed, the scientific literature, as well as the National Academies and other research institutions, has considered the potential risks of the "dark side" of synthetic biology and the technologies that it employs (Choffnes et al., 2006; Fraser and Dando, 2001; IOM/NRC, 2006; Parliamentary Office of Science and Technology, 2008; Relman et al., 2006; Royal Academy of Engineering, 2009). In order to improve the regulatory climate, Poste suggested

that the scientific community develop its own robust and rigorous standardization protocols. Others agreed and emphasized the need for early dialogue between scientists and regulatory authorities in order to get a better sense of what type of information regulators will need to interpret synthetic (and systems) biology data. The National Academy of Engineering hosted a workshop on how engineering ethics might contribute to the development of synthetic biology (Hollander, 2010).

The Presidential Commission for the Study of Bioethical Issues did not recommend that any immediate regulations or oversight be placed on synthetic biology research, noting that the field was still in its infancy and the potential benefits of the research outweighed the potential risks. Rather, it recommended that the government adopt a strategy of “prudent vigilance” by encouraging innovative research while proactively monitoring risks and updating regulations as necessary (Kaiser, 2010; Presidential Commission for the Study of Bioethical Issues, 2010).

Just as gene splicing sparked a global ethical and regulatory debate in the 1970s, the ability to manipulate living systems and the potential to create new life forms pose significant ethical and regulatory challenges as well. In the case of synthetic biology, the call for collaboration is not just a matter of combining and coordinating efforts among scientists in order to accelerate the generation of knowledge; it is also a matter of safety.

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Appendix A

Contributed Manuscripts

A1

COMMERCIAL APPLICATIONS OF SYNTHETIC BIOLOGY

David A. Berry^{1,2}

An Overview of Venture Capital

Venture capital is financial capital invested into high-potential companies. The role of venture capital is to support the entrepreneurial talent that takes basic science and breakthrough ideas to market by building companies. This risk capital ultimately supports some of the most innovative and promising companies—those that have gone on to change existing industries or create new ones altogether (Thompson Reuters, 2011).

Venture capital is a distinct asset class. Venture capital firms, which are professional, institutional capital managers, make investments by purchasing equity in a company. The stock acquired is an illiquid investment that requires the growth of the company for the investors to ultimately reap any potential return. It is this inability of venture capitalists to rapidly enter and exit investments, or “flip” them, that aligns their goals with those of the entrepreneurs. Venture capital is intrinsically a long-term investment (Thompson Reuters, 2011).

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² Key words: venture capital, biological engineering, synthetic biology, microbiome, diesel, photosynthesis, genome engineering.

Venture capitalists invest out of a fund, a vehicle that deploys capital on behalf of third-party investors. The investors in these funds, called limited partners, are often pension funds, foundations, corporations, endowments, and wealthy individuals, among others. Given the low liquidity associated with their investment into venture capital funds, limited partners expect large returns—better than those in the stock market—from the funds in which they invest. The funds represent a commitment of capital with a fixed life, typically 10 years. The general partner, a group of partners with fiduciary responsibility for the firm with the legal form of a partnership, manages the capital in the fund. The committed capital is called by the general partner from the limited partners to make a portfolio of investments. Ultimately, when investments mature and become liquid, the profits are shared, with the majority going back to the limited partners and the rest shared by the general partner.

Funding provided by venture capitalists typically takes the form of “rounds,” where a given amount of money is invested into a company at a valuation agreed upon between the management and the investors. Prior to an investment, the equity ownership is divided among the founders, management, and others. The valuation sets a share price against which the venture capital firm buys shares. At each round, the earlier investors and management team strive to increase the valuation for the subsequent round(s) of investment. The higher the valuation of a round, the less dilution (reduction in ownership) the existing shareholders take. While each round contemplates a share price that defines a paper value for an investor’s or an employee’s shares, little actual value is created. Only at a sale event or initial public offering do investors and the management team see a tangible financial return, which can take 5-8 years, if not longer.

Venture capital firms statistically see 100 business plans, take a deep look at 10 of these proposals, and invest in one. This process involves an assessment of the management team, the proposed business, its potential to exclude competition, the market being pursued, and how well the opportunity fits with the firm’s goals.

With an investment, a partner will typically get involved with a company by taking a seat on the board of directors, where he or she works closely with the management team on company strategy and growth. The venture capital industry plays an important role in the economy. Companies supported by early venture capital account for 21 percent of the U.S. gross domestic product by revenue, and 11 percent of private-sector jobs despite the fact that fewer than 1,000 new businesses get venture capital funding any given year (National Venture Capital Association, 2009).

A Brief History of the Venture Capital Industry

Venture capital is said to have originated in 1946 with the founding of the first two firms: American Research and Development Corporation (ARDC) and

J. H. Whitney & Company (Wilson, 1985). Georges Doriot, referred to as the “father of venture capitalism,” the former dean of Harvard Business School and founder of INSEAD, founded ARDC along with Karl Compton, the former president of MIT, as well as Ralph Flanders (Ante, 2008). ARDC sought to invest in businesses run by soldiers returning from World War II. The firm is most famous for investing \$70,000 in Digital Equipment Corporation in 1957—a company that when it had its initial public offering in 1968 was valued at \$355 million for a return to ARDC of over 1,200-fold. Employees of ARDC went on to found leading venture funds including Greylock Partners and Flagship Ventures, among others (Kirsner, 2008).

Two major government changes allowed venture capital to emerge as a fully fledged industry. First, the Small Business Investment Act of 1958 enabled the Small Business Administration to license Small Business Investment Companies to help finance and manage small entrepreneurial businesses. Second, in 1978, the Employee Retirement Income Security Act was altered to allow corporate pension funds to invest in venture capital. These two acts together supported the framework for venture capital and facilitated substantial investment in it.

Successes of the venture capital industry in the 1970s and 1980s, with companies including Digital Equipment Corporation, Apple, and Genentech resulting, led to rapid growth of the industry. With rapid growth came diminished returns. In the early 1990s the numbers of firms and managers shrank in response to declining investment performance. At the same time, the more successful firms retrenched, starting a wave of increased returns that began in 1995 and continued through the Internet bubble in 2000 (Metrick, 2007). Once again, with grossly increased returns, the investment into the sector and the number of funds skyrocketed. Beginning in March 2000, the NASDAQ crashed, and many funds suffered from a second contraction.

After the Internet bubble, the funds raised by venture firms shrank substantially. Amounts of committed capital increased through 2005 to a level much less than in 2000, and they remained flat until the economic meltdown in 2008. During the decade from 2000 to 2010, venture capital returns also fell dramatically to the point that the median 10-year return of all U.S. funds was less than the stock market (Thomson Reuters, 2011). These events resulted in another substantial contraction in the industry. The industry is currently responding to this most recent contraction. The number of funds has decreased as the average fund size has risen. This dynamic has caused venture funds to focus on either earlier-stage investments, later-stage investments (similar to private equity), or a combination. Other funds have started focusing on flipping assets by investing before or to induce specific value-creation events. This has created a new environment where only a small number of funds are focused on the earliest stage—that which venture capital is most associated with and most successful at—with several others focused on a more transactional business. This evolution is still in process, but it has been changing the nature of companies that receive investment as well.

At the earliest stage of investments, venture capitalists have returned to investing in outstanding teams and under the assumption that they can create great companies. A number of approaches have been taken to inspire innovation and support a new era of breakthrough companies. Various firms have taken different approaches. CMEA, for example, invests in proven entrepreneurs “pre-napkin” (before the idea), on the belief that they will come up with ideas. Polaris’ Dogpatch Labs has created an environment where multiple entrepreneurs share a common environment and with light money attempt to prove out their concepts. Y Combinator gives entrepreneurs an education and a small amount of money to try out their ideas. Andreessen Horowitz has similarly created an infrastructure to support the earliest stages of companies and to allow them to focus their capital on the company. “Super Angels” such as Peter Thiel have also emerged to provide important early stage funding. Several companies produced from some of these efforts have emerged as important venture-backed companies. Flagship VentureLabs has created an internal infrastructure of serial entrepreneurs to co-iterate its own innovations and use that as a basis to build companies.

Flagship VentureLabs

Flagship VentureLabs was built with a focus on increasing the efficiencies of innovation and entrepreneurship. In the broadest context, both traditional entrepreneurship and venture capital have intrinsic benefits and inefficiencies. Entrepreneurs, for example, typically perform well when capital constrained, but, by the same token, avoid asking critical questions because if an undesirable answer results, they are unemployed. Venture capital has the advantage of large sample sizes and substantial funding, but it is limited in its investments to only those which it can see, and all of its investments must fundamentally go through a common set of efforts (i.e., financial infrastructure, legal, etc.). Fundamentally, Flagship VentureLabs removes the constraints from the typical elements of traditional ecosystems; that is, by harnessing the key constituents and requirements all under one roof, with the common goal of the betterment of humankind through innovation and entrepreneurship.

The focus of Flagship VentureLabs is to develop breakthrough technologies to match large unmet needs in life sciences and sustainability through the vehicle of startup companies. New companies come from a breakthrough innovation without a set utility or from work within Flagship VentureLabs identifying the intersection between the potential for technology solution and market pull. In the former case, a team is nucleated around the technology, including the inventor, to heavily iterate the concept and pressure test it against markets, intellectual property opportunities, team-building potential, and other features with the attempt at nonrationally identifying the “sweet spot.” In certain cases, this process results in the pseudolinear formation of a company focused on commercializing the technology. In others, however, through a progressive set of explorations,

the company may end up far from its origins, potentially not including the base technology. In the latter case, the defined intersection creates a hypothesis. If the hypothesis has already been manifest by others in a company or in academia (either singularly or through a combination of efforts), a simple investment may be warranted. In the absence of such a proof point, the concept then goes through heavy conceptual iteration with the attempt to prove the hypothesis wrong, and in the combination of not being able to make it fail and the generation of significant key stakeholder support (including industry and key opinion leaders), a company will be launched. Ultimately, this approach results in taking new ideas and forming companies several years before such an opportunity is likely to be compelling. The following discusses efforts in three such technology-based companies originating from Flagship VentureLabs covering both life sciences and sustainability.

Seres Therapeutics: Rethinking Drug Development

In an effort to reduce side effects, drug development has focused its efforts on target specificity, particularly on features including affinity, low off-target effects, pharmacokinetics, pharmacodynamics, and others. The Human Genome Project and systematic understandings of the functions of kinases have helped to drive this increasing target specificity. Nonetheless, the biology of diseases is complex and multi-factorial. Focusing drugs to single actors may reduce side effects but it also limits the spectrum of efficacy. The growing recognition of the nature of disease is driving the understanding of more complex biology and the development of drugs focused on the multitude of key factors.

One particular example is with the human microbiome. Microorganisms have long been thought of as independently functioning pathogens. Recently, however, the commensal and mutualistic natures of various microorganisms that inhabit the body have started to be characterized (Dethlefsen, 2011). The interactions between the multitude of organisms, as well as between the organisms and the host, play an important role in normal physiology broadly (Reid et al., 2011). Accordingly, disruptions in the microbiome, whether by antibiotics, diet, infection, or other means, can alter the microbiome and induce or simply increase the likelihood of a wide range of diseases, ranging from *Clostridium difficile* infection and inflammatory bowel disease to obesity and diabetes (Kau et al., 2011).

The complexity of the microbiome, including not only the interrelation between a number of species and the host but also the physical formation of the communities in specific niches (Rickard et al., 2003), is important to take into account when developing therapeutics aimed at diseases where the microbiome plays an important role. Seres Therapeutics was founded specifically to develop drugs based on the complexity of the microbiome. Probiotics and single biologics affect a limited scope of disease and, thus, have limited efficacy in complex diseases such as those involving the microbiome (Shen et al., 2009). By creat-

ing synthetic microbiomes aimed at disrupting pathogenic communities, Seres provides a therapeutics means by which a normal microbiome can be restored. Understanding biology and synthetically recapitulating conditions that can recover from a disease-associated insult enables a new class of therapeutics to be designed and developed that are focused on the etiology of underlying disease.

Sustainability

Persistently high fossil fuel prices, increasing dependency on foreign fuel supplies, and insecurity relating to the sources of petroleum have created substantial market pull for alternative solutions in the \$6 trillion petrochemical industry. Outside of government-mandated markets, such as ethanol of late, fuels and chemicals are fungible products driven by price and purity, as well as supply and demand. Markets therefore require products with a known utility that meet certain industrial specifications while doing so at a competitive cost point. Consumers have not shown a willingness to pay for benefits such as greenhouse gas mitigation or domestic sourcing, so products made as alternatives must do so while competing head-to-head with the incumbents using the same metrics.

Fuels have traditionally originated from biology in some form or another. Fossil fuels are thought to ultimately derive from processing of ancient biomass through a process that takes millions to hundreds of millions of years. Historically, humans have also found faster cycle time sources of energy, namely the burning of trees for heat and energy, as well as the removal of spermaceti from whales as a source of wax. All of these resources have limited renewal potential and substantial environmental impact (Tertzakian, 2009). Given the central role biology has had in fossil fuels historically, it stands to reason that biology would be well positioned to be at the forefront of the future of sustainable fuels.

Biological engineering has evolved rapidly over the last 50-plus years. Breakthroughs in genomics research, increased genetic manipulation potential, and more complete knowledge of the inner workings of cells have set a stage for cells to be engineered to achieve desired functionalities. Moreover, the time from conception to proof of concept, and that from proof of concept to commercial viability, has reduced substantially. Historically, these periods have decreased threefold every 10 years. Given the technological potential enabled, the market needs can now drive the technological direction, thus leading toward an intersection between market pull and the potential for technology solution.

LS9: Ultraclean Renewable Diesel

In 2005, the U.S. government had built a robust market demand for ethanol by outlining a replacement timeline for methyl tert-butyl ether (MTBE), a fuel oxygenate that had been associated with groundwater contamination and potential increased cancer risks, with ethanol (U.S. Environmental Protection Agency,

2011a). Twelve billion gallons were mandated by 2012, effectively defining a market growth. This mandate was soon supplemented with the renewable fuel standard (RFS), and subsequently RFS2, ultimately requiring 36 billion gallons produced per year by 2022 (U.S. Environmental Protection Agency, 2011b). Corn ethanol was thus given ample runway to launch, and blenders were incorporating biologically based products into fuel nationwide. The intent of the MTBE replacement with ethanol, however, was replacing an oxygenate, not deeming ethanol a fuel. Nonetheless, outspoken investors were enthusiastically supportive of building the future of American renewable fuel on ethanol, asserting that it could be cheaper than and as efficient as petrochemically derived fuels (Khosla, 2006) despite the disadvantaged domestic cost structure and intrinsic lower energy density. The market was becoming well positioned for a viable alternative.

LS9 originated by asking the question, “If you could make any fuel from biology, what would you make?” The ideal fuel to be produced from biology would be diesel, given its high energy density and its use throughout the world as a primary transportation fuel. A market-acceptable biologically produced diesel must compete in a low-cost commodity market without subsidies, requiring an efficient biological pathway and process. Translating these needs into specific technological tasks required that the cell be engineerable to be feedstock agnostic (i.e., able to use any form of sugar), that the most efficient pathway of producing the product was available, that the product was to be made directly and secreted, and that it entailed both straightforward separations (a feature of the product) and no downstream processing (the final product is made by the cell).

Using the defined market constraints, various pathways to produce a straight-chain hydrocarbon were defined and evaluated. The fatty acid biosynthesis pathway not only has exceptionally high energy efficiency at over 90 percent but also produces a molecule that is chemically identical to diesel, requiring potentially fewer biological steps. Fatty acids are activated with coenzyme A (CoA) or acyl carrier protein (ACP) to make fatty acyl-CoA or fatty acyl-ACP (Zhang and Rock, 2008), which serve as the biological precursor products for fuel synthesis. These products are then modified to make a desired end product. The same pathways can be leveraged to make a series of other petrochemicals including fatty acid methyl esters, olefins, fatty alcohols, and others, in addition to alkanes (diesel) (Rude et al., 2011).

The product itself is insufficient for a commercial host and process. The identified market constraints require that the cell chassis has flexibility in feedstock, be optimized to maximize carbon flux to end product, and secrete the end product. Feedstock costs, driven by sugar prices, have risen dramatically over the past 6 years. Alternatives require the liberation of sugar from cellulosic biomass, which is done through exogenous enzymes at present. The expression of hemicellulases into the host already engineered to produce alkanes or other derivatives can enable consolidated bioprocessing, thereby reducing process costs (Magnuson et al., 1993). This is achievable, for example, with the endogenous production

of glycosyl hydrolases such as xylanase (Xsa) from *Bacteroides ovatus* and an endoxylanase catalytic domain (Xyn10B) from *Clostridium stercorarium*, which together hydrolyze hemicelluloses to xylose, which is usable in *E. coli* central metabolism (Adelsberger et al., 2004; Steen et al., 2010; Whitehead and Hespell, 1990). Optimizing the host requires focusing the flux of the sugar input through central metabolism to the product. Specifically, *fadD* and *fadE* knockouts block the first two steps of the β -oxidation pathway, increasing end-product production three- to fourfold (Steen et al., 2010). Secreting the end product eliminates end-product inhibition and streamlines bioprocessing, thus increasing flux and reducing operating costs by supporting continuous operations (Berry, 2010). Expressing a leaderless version of TesA eliminates end-product inhibition, drives secretion, and notably also positively affects chain length with a natural preference for C14 fatty acids (Cho and Cronan, 1995; Jiang and Cronan, 1994; Steen et al., 2010).

Through this approach, an industrial chassis has been rationally developed to systematically meet commercial needs. By specifically including features necessary to ensure diverse feedstock utility, drop-in product synthesis, and lowest cost processing, LS9's technology has been designed specifically to drive market pull.

Joule Unlimited: Renewable Solar Fuels

An intrinsic challenge of using a sugar-based feedstock is the price volatility associated with the commodity. Joule Unlimited was founded to develop a platform that could eliminate dependence on sugar feedstocks while still producing fuels in a way that meets market needs. A systematic exploration of sources of carbon that can be routed into central metabolism rapidly identifies photosynthesis, nature's solution to carbon dioxide assimilation driven by solar energy, as a compelling, though insufficient, pathway. The Department of Energy's Aquatic Species program, based on explorations of algal biofuels between 1976 and 1996, concluded that photosynthesis could support viable fuel processes, but it requires a set of key innovations to do so (Sheehan et al., 1998; Weyer et al., 2010; Zhu et al., 2008). At the outset of Joule Unlimited, the fundamental limitations of algal fuels were examined and coupled with market needs to design an entirely new and distinct approach, whose only similarity to algae was the use of photosynthesis.

A thorough exploration of market needs identified that an ideal solution would directly produce secreted fungible fuel directly from sunlight and carbon dioxide without a dependency on arable land, freshwater, or other costly reagents, while having a cost that could meet or beat fossil fuel equivalents in the absence of subsidies and at the same time scale modularly such that smaller-scale plants could be used to validate large-scale deployments. The simultaneous technological solution to meet all of these needs demands a genetically tractable cyanobacteria engineered to not need exogenous factors and to produce secreted fuel grown in a

modular bioreactor leveraging the two-dimensional scaling of light and incorporating fundamental process needs including proper mixing. A schematic of the Joule Unlimited approach, Helioculture™, is provided in Figure A1-1.

Cyanobacteria had previously been engineered to express recombinant proteins, but it had not been systematically engineered on a genome scale owing primarily to a lack of engineering tools (Alvey et al., 2011). A concerted effort using *E. coli* engineering over 50 years serves as a roadmap of the needs for genome engineering in cyanobacteria. Using these tools coupled with a systemic genome engineering effort allows one to overcome a theoretical maximum light use and net productivity for algal biofuels of ~2,000 gallons per acre per year by enabling photoautotrophs, for the first time, to function like industrialized heterotrophs, whose phases of growth and production are separated (Berry, 2010; Robertson et al., 2011). Systematic re-regulation of central metabolism directs 95 percent of photosynthetic activity to specific product synthesis versus up to 50 percent in un-engineered organisms, allowing for productivity ~95 percent of the light-exposed time through continuous operations versus substantial down time with batch processes, minimizing maintenance energy requirements, and limiting the energy lost to photorespiration.

At the same time, the engineered cells are grown in a reactor system designed to be low cost and linearly scalable. Low-cost product separation, cell mixing, and proper gas transfer to the cells are all incorporated into the reactor design. Coupled together, this systems approach allows for ~12 percent theoretical maximal photonic energy conversion versus 1.5 percent for traditional algal processes (Figure A1-2), which translates to unprecedented areal productivities of 25,000 gallons of ethanol per acre per year or 15,000 gallons of diesel per acre per year.

The high productivities achieved through the Joule Unlimited approach allow for cost points as low as \$20/barrel for fungible diesel. Maximizing solar energy capture, carbon dioxide fixation as a replacement for sugar use, and organism productivity creates a system that can be market competitive while providing for the environmental benefits that have been sought in fossil fuel replacements. Specifically, Joule Unlimited's technology eliminates the need for arable land, requires no freshwater, and reduces life-cycle greenhouse gases by over 90 percent by using carbon dioxide as a feedstock. By coupling the technical needs for a total solution with a market need, Joule Unlimited has uniquely developed a process that can produce a sugar-independent diesel in a highly scalable manner, overcoming the challenges of alternative approaches.

Conclusions

Systematic changes in venture capital have altered the entrepreneurial ecosystem. Flagship VentureLabs is pioneering a new approach of technology development through companies by building technologies that specifically address the intersection between the potential for technology solution with market pull

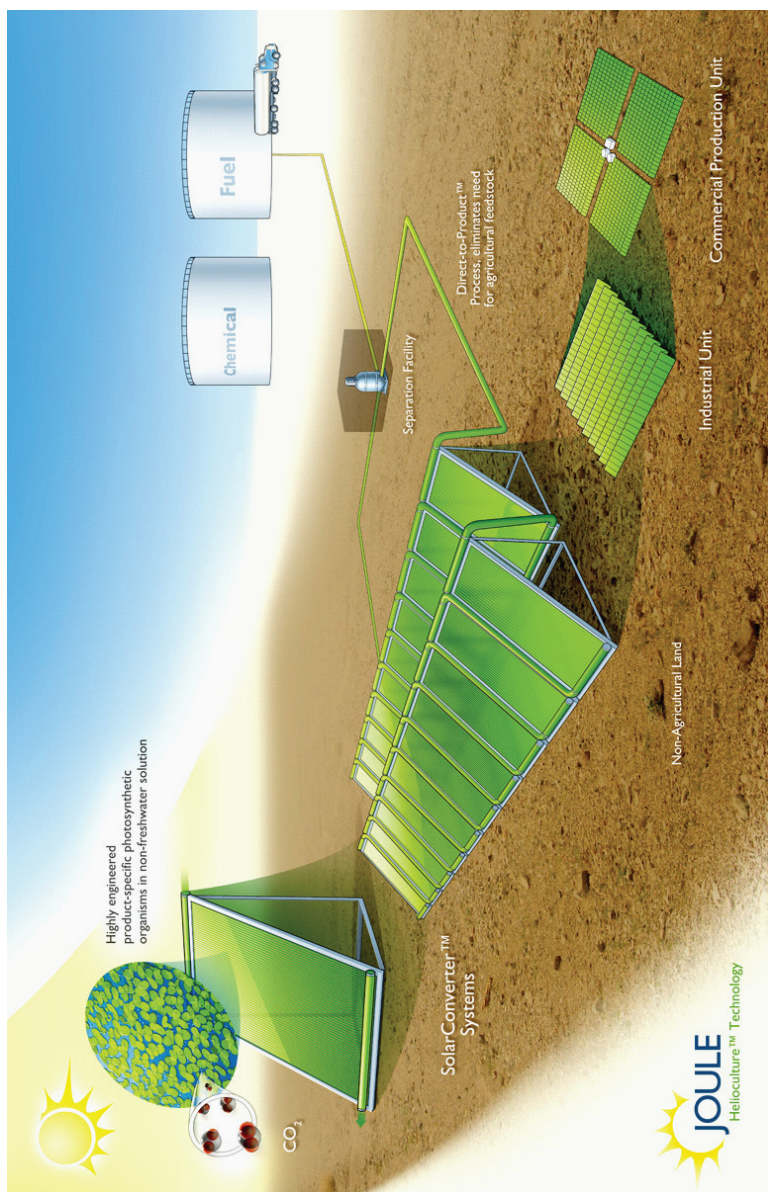


FIGURE AI-1 Schematic of the Joule Unlimited Heliculture systems approach. Specific cyanobacteria are engineered to convert sunlight, CO₂, and nonfreshwater to diesel (or other fuels and chemicals). The engineered organisms are housed within a Solar Converter, a single reactor unit designed to interconnect with others and therefore scale linearly.

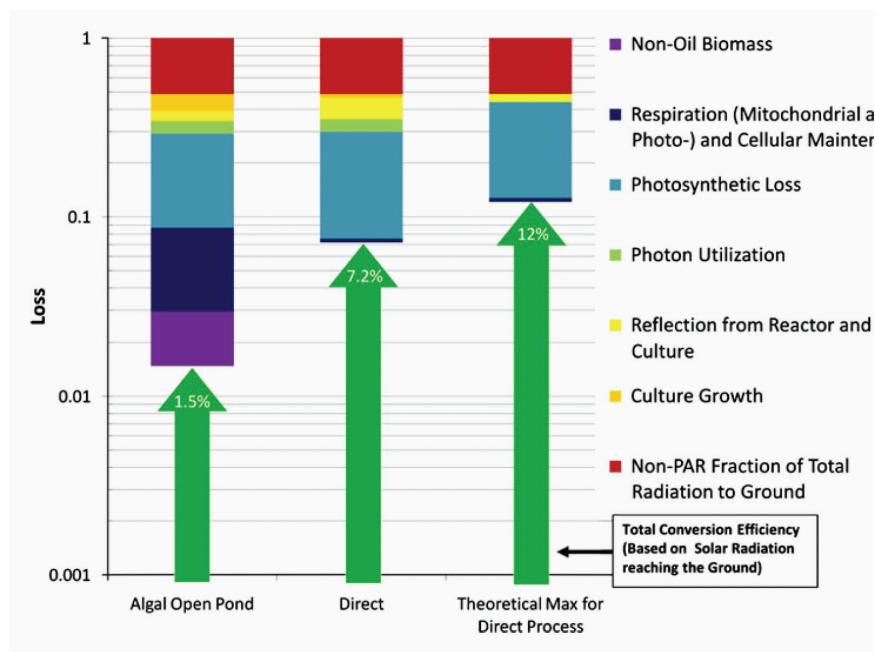


FIGURE A1-2 A summation of the accumulated photon losses for algal and direct fuel processes, as well as a theoretical maximum photonic energy conversion. The losses are shown through individual contributions accumulated serially and illustrated on a logarithmic scale, beginning with the percent of photosynthetically active radiation empirically measured at the ground. Total energy conversion efficiency as a function of the losses is indicated by the green arrows.

SOURCE: Adapted from Robertson et al. (2011).

driving invention and innovation toward market needs. The resultant companies are designed by exploring cutting-edge capabilities and iterating against market needs—not just from an evolutionary standpoint, but additionally identifying the true needs of an industry across multiple facets. Synthetic biology is a new and rapidly developing tool that has particular utility in meeting broad-based and distinct market needs, particularly through its ability to create functional modules in a cell-based system. By leveraging the potential of synthetic biology with market-driven needs, Flagship VentureLabs has been able to spearhead a set of breakthrough innovations in both life sciences and sustainability. This approach now takes market potential before traditional research approaches have made for a compelling and investable technology-driven opportunity and, through heavy iteration, can bring it to bear ahead of time through broad-based collaborations with industry and academia. This approach can be broadly leveraged to develop

a series of future breakthrough technologies in a variety of important market sectors.

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A2

SYNTHETIC BIOLOGY: APPLICATIONS COME OF AGE³

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Abstract

Synthetic biology is bringing together engineers and biologists to design and build novel biomolecular components, networks and pathways, and to use these constructs to rewire and reprogram organisms. These re-engineered organisms will change our lives over the coming years, leading to cheaper drugs, ‘green’ means to fuel our cars and targeted therapies for attacking ‘superbugs’ and diseases, such as cancer. The *de novo* engineering of genetic circuits, biological modules and synthetic pathways is beginning to address these crucial problems and is being used in related practical applications.

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The circuit-like connectivity of biological parts and their ability to collectively process logical operations was first appreciated nearly 50 years ago (Monod and Jacob, 1961). This inspired attempts to describe biological regulation schemes with mathematical models (Glass and Kauffman, 1973; Savageau, 1974; Kauffman, 1974; Glass, 1975) and to apply electrical circuit analogies to biological pathways (McAdams and Arkin, 2000; McAdams and Shapiro, 1995). Meanwhile, breakthroughs in genomic research and genetic engineering (for example, recombinant DNA technology) were supplying the inventory and methods necessary to physically construct and assemble biomolecular parts. As a result, synthetic biology was born with the broad goal of engineering or ‘wiring’ biological circuitry — be it genetic, protein, viral, pathway or genomic — for manifesting logical forms of cellular control. Synthetic biology, equipped with the engineering-driven approaches of modularization, rationalization and modeling, has progressed rapidly and generated an ever-increasing suite of genetic devices and biological modules.

The successful design and construction of the first synthetic gene networks — the genetic toggle switch (Gardner et al., 2000) and the repressilator (Elowitz and Leibler, 2000) (Box A2-1) — showed that engineering-based methodology could indeed be used to build sophisticated, computing-like behaviour into biological systems. In these two cases, basic transcriptional regulatory elements were designed and assembled to realize the biological equivalents of electronic memory storage and timekeeping (Box A2-1). Within the framework provided by these two synthetic systems, biological circuits can be built from smaller, well-defined parts according to model blueprints. They can then be studied and tested in isolation, and their behaviour can be evaluated against model predictions of the system dynamics. This methodology has been applied to the synthetic construction of additional genetic switches (Gardner et al., 2000; Atkinson et al., 2003; Bayer and Smolke, 2005; Deans et al., 2007; Dueber et al., 2003; Friedland et al., 2009; Ham et al., 2006, 2008; Kramer and Fussenegger, 2005; Kramer et al., 2004), memory elements⁶ (Gardner et al., 2000; Friedland et al., 2009; Ham et al., 2006; Ajo-Franklin et al., 2005) and oscillators (Elowitz and Leibler, 2000; Atkinson et al., 2003; Fung et al., 2005; Stricker et al., 2008; Tigges et al., 2009; Danino et al., 2010), as well as to other electronics-inspired genetic devices, including pulse generators⁷ (Basu et al., 2004), digital logic gates⁸ (Anderson et al., 2007; Guet et al., 2002; Rackham and Chin, 2005; Rinaldo et al., 2007;

⁶ Memory elements – Devices used to store information about the current state of a system.

⁷ Pulse generators – Circuits or devices used to generate pulses. A biological pulse generator has been implemented in a multicellular bacterial system, in which receiver cells respond to a chemical signal with a transient burst of gene expression, the amplitude and duration of which depend on the distance from the sender cells.

⁸ Digital logic gates – A digital logic gate implements Boolean logic (such as AND, OR or NOT) on one or more logic inputs to produce a single logic output. Electronic logic gates are implemented using diodes and transistors and operate on input voltages or currents, whereas biological logic gates operate on cellular molecules (chemical or biological).

Stojanovic and Stefanovic, 2003; Win and Smolke, 2008), filters⁹ (Basu et al., 2005; Hooshangi et al., 2005; Sohka et al., 2009) and communication modules (Danino et al., 2010; Basu et al., 2005; Kobayashi et al., 2004; You et al., 2004).

Now, 10 years after the demonstration of synthetic biology's inaugural devices (Gardner et al., 2000; Elowitz and Leibler, 2000), engineered biomolecular networks are beginning to move into the application stage and yield solutions to many complex societal problems. Although work remains to be done on elucidating biological design principles (Mukherji and van Oudenaarden, 2009), this foray into practical applications signals an exciting coming-of-age time for the field.

Here, we review the practical applications of synthetic biology in biosensing, therapeutics and the production of biofuels, pharmaceuticals and novel biomaterials. Many of the examples herein do not fit exclusively or neatly into only one of these three application categories; however, it is precisely this multivalent applicability that makes synthetic biology platforms so powerful and promising.

Biosensing

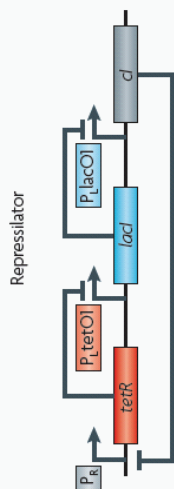
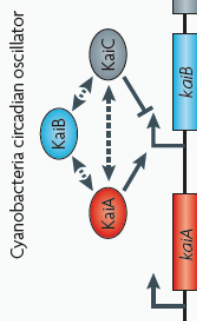
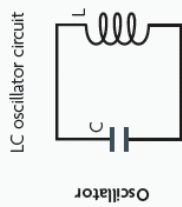
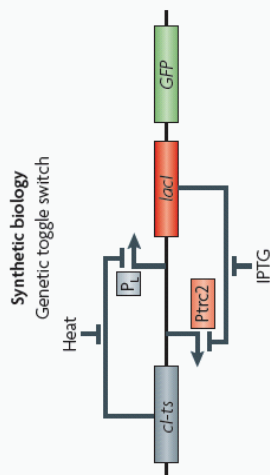
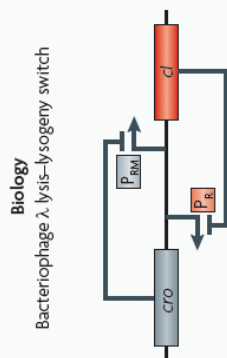
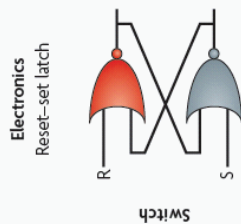
Cells have evolved a myriad of regulatory circuits — from transcriptional to post-translational — for sensing and responding to diverse and transient environmental signals. These circuits consist of exquisitely tailored sensitive elements that bind analytes and set signal-detection thresholds, and transducer modules that filter the signals and mobilize a cellular response (Box A2-2). The two basic sensing modules must be delicately balanced: this is achieved by programming modularity and specificity into biosensing circuits at the transcriptional, translational and post-translational levels, as described below.

Transcriptional biosensing. As the first dedicated phase of gene expression, transcription serves as one method by which cells mobilize a cellular response to an environmental perturbation. As such, the genes to be expressed, their promoters, rNA polymerase, transcription factors and other parts of the transcription machinery all serve as potential engineering components for transcriptional biosensors. Most synthetic designs have focused on the promoters and their associated transcription factors, given the abundance of known and characterized bacterial, archaeal and eukaryotic environment-responsive promoters, which include the well-known promoters of the *Escherichia coli lac*, *tet* and *ara* operons.

Both the sensory and transducer behaviours of a biosensor can be placed under synthetic control by directly engineering environment-responsive promoter sequences. In fact, this was the early design strategy adopted for establishing inducible expression systems (Brown et al., 1987; Deuschle et al., 1989; Hu and

⁹ Filters – Algorithms or devices for removing or enhancing parts or frequency components from a signal.

BOX A2-1 Early Synthetic Biology Designs: Switches and Oscillators



Switches and oscillators that occur in electronic systems are also seen in biology and have been engineered into synthetic biological systems.

Switches

In electronics, one of the most basic elements for storing memory is the reset-set (RS) latch based on logical NOR gates*. This device is bistable in that it possesses two stable states that can be toggled with the delivery of specified inputs. Upon removal of the input, the

circuit retains memory of its current state indefinitely. These forms of memory and state switching have important functions in biology, such as in the differentiation of cells from an initially undifferentiated state. One means by which cellular systems can achieve bistability is through genetic mutual repression. The natural $P_{R-}P_{RM}$ genetic switch from bacteriophage λ , which uses this network architecture to govern the lysis-lysogeny decision, consists of two promoters that are each repressed by the gene product of the other (that is, by the Cro and CI repressor proteins). The genetic toggle switch (Gardner et al., 2000) constructed by our research group is a synthetically engineered version of this co-repressed gene regulation scheme. In one version of the genetic toggle, the P_L promoter from λ phage was used to drive transcription of *lacI*, the product of which represses a second promoter, *P_{trc2}* (a *lac* promoter variant). Conversely, *P_{trc2}* drives expression of a gene (*cl-ts*) encoding the temperature-sensitive (*ts*) λ CI repressor protein, which inhibits the P_L promoter. The activity of the circuit is monitored through the expression of a GFP promoter.

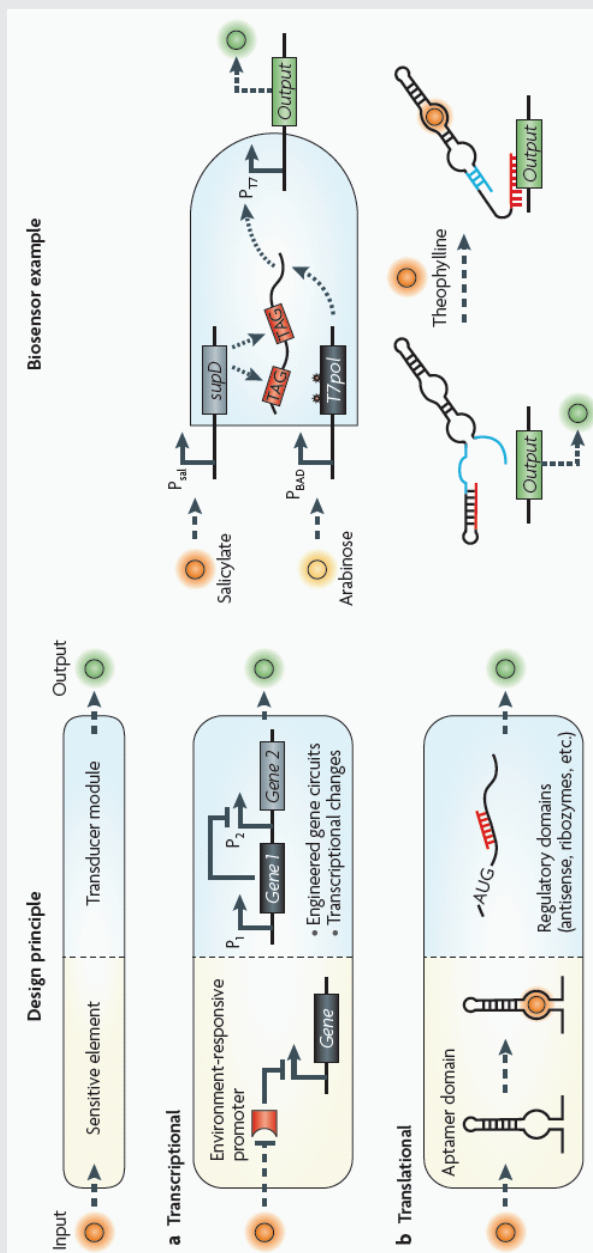
The system can be toggled in one direction with the exogenous addition of the chemical inducer isopropyl- β -D-thiogalactopyranoside (IPTG) or in the other direction with a transient increase in temperature. Importantly, upon removal of these exogenous signals, the system retains its current state, creating a cellular form of memory.

Oscillators

Timing mechanisms, much like memory, are fundamental to many electronic and biological systems. Electronic timekeeping can be achieved with basic oscillator circuits — such as the LC circuit (inductor L and capacitor C) — which act as resonators for producing periodic electronic signals. Biological timekeeping, which is widespread among living organisms (Dunlap, 1999), is achieved with circadian clocks and similar oscillator circuits, such as the one responsible for synchronizing the crucial processes of photosynthesis and nitrogen fixation in cyanobacteria. The circadian clock of cyanobacteria is based on, among other regulatory mechanisms, intertwined positive and negative feedback loops on the clock genes *kaiA*, *kaiB* and *kaiC*. Elowitz and Leibler constructed a synthetic genetic oscillator based not on clock genes but on standard transcriptional repressors (the repressilator) (Elowitz and Leibler, 2000). Here, a cyclic negative feedback loop composed of three promoter-gene pairs, in which the 'first' promoter in the cascade drives expression of the 'second' promoter's repressor, and so on, was used to drive oscillatory output in gene expression.

* NOR gate – A digital logic gate that implements logical NOR, or the negation of the OR operator. It produces a HIGH output (1) only if both inputs to the gate are LOW (0).

BOX A2-2 Synthetic Biosensors: Transcriptional and Translational Architectures and Examples



Biosensors consist of two basic modules (see the figure): sensitive elements for recognizing and binding analytes, and transducer modules for transmitting and reporting signals.

Transcriptional

Transcriptional biosensors (part **a**) are built by linking environment-responsive promoters to engineered gene circuits for programmed transcriptional changes. In the example shown, a transcriptional AND gate was designed to sense and report only the simultaneous presence of two environmental signals (for example, salicylate and arabinose) (Anderson et al., 2007). At one gate input, the researchers encoded an environment-responsive promoter (for example, P_{BAD}) that activates transcription of a T7 RNA polymerase gene in response to a single environmental signal (for example, arabinose). The gene, however, carries internally encoded amber stop codons (red spiked circles) that function to block translation of its transcript. Activation of the second gate input is the key to unlocking translation; specifically, translation can be induced when a second promoter (for example, P_{saI}) activates transcription of the *supD* amber suppressor tRNA in response to a second unique signal (for example, salicylate). In other words, only when the two environmental signals are simultaneously present can the T7 RNA polymerase be faithfully expressed and used to activate an output T7 promoter. This is an example of how sophisticated specificity can be programmed into a transducer module by creatively linking the sensory information of multiple sensitive elements. Furthermore, the design is transcriptionally modular in that different sets of environment-responsive promoters can be interfaced to the AND gate.

Translational

Translational biosensors (part **b**) are typically built by linking RNA aptamer domains to RNA regulatory domains. The example shown is an OFF 'antiswitch'. Here, the small molecule theophylline is recognized and bound by the aptamer stem of the RNA biosensor. This causes a conformational change in the molecule that liberates the antisense domain from its sequestering stem loop and allows it to inhibit translation of an output reporter (Bayer and Smolke, 2005).

Davidson, 1987; Lutz and Bujard, 1997). By introducing, removing or modifying activator and repressor sites, a promoter's sensitivity to a molecule can be tuned. Synthetic mammalian transactivation systems are generic versions of this strategy in which an environmentally sensitive transcription factor is fused to a mammalian transactivation domain to cause inducer-dependent changes in gene expression. Synthetic mammalian biosensors based on this scheme have been created for sensing signals such as antibiotics (Fussenegger et al., 2000; Gossen and Bujard, 1992; Weber et al., 2002), quorum-sensing molecules (Neddermann et al., 2003; Weber et al., 2003b), gases and metabolites (Malphettes et al., 2005; Mullick et al., 2006; Weber et al., 2006; Weber et al., 2004), and temperature changes (Boorsma et al., 2000; Weber et al., 2003a). Fussenegger and colleagues have even incorporated this transgene design into mammalian circuits, creating synthetic networks that are responsive to electrical signals (Weber et al., 2009).

Although the engineering of environment-responsive promoters has been valuable, additional control over modularity¹⁰ and specificity can be achieved by embedding environment-responsive promoters¹¹ in engineered gene networks. Achieving true modularity with genetic parts is inherently difficult because of unintended interference among native and synthetic parts and therefore requires careful decoupling of functional modules. One such modular design strategy was used by Kobayashi *et al.* (2004) to develop whole-cell *E. coli* biosensors that respond to signals in a programmable fashion. In this design, a sensory module (that is, an environment-responsive promoter and associated transcription factor) was coupled to an engineered gene circuit that functions like a central processing unit. *E. coli* cells were programmed to respond to a deleterious endogenous input — specifically, DNA-damaging stimuli, such as ultraviolet radiation or mitomycin C. The gene circuit, which was chosen to be the toggle switch (Box A2-1), processes the incoming sensory information and flips from an 'OFF' to an 'ON' state when a signal threshold is exceeded. Because the biosensor has a decoupled, modular nature, it can be wired to any desired output, from the expression of a standard fluorescent reporter to the activation of natural phenotypes, such as biofilm formation (for example, through expression of *traA*) or cell suicide (for example, through expression of *ccdB*).

Sometimes a single signal may be too general to characterize or define an environment. For such situations, Anderson *et al.* (2007) devised a transcriptional AND gate that could be used to integrate multiple environmental signals into a single genetic circuit (Box A2-2), therefore programming the desired level of biosensing specificity. Genetic biosensors of this sort could be useful for communicating the state of a specific microenvironment (for example, in an industrial

¹⁰ Modularity – The capacity of a system or component to function independently of context.

¹¹ Environment-responsive promoters – Promoters that directly transduce environmental signals (for example, heavy metal ions, hormones, chemicals or temperature) that are captured by their associated sensory transcription factors.

bioreactor) within a 'sea' of environmental conditions, such as temperature, metabolite levels or cell density.

Translational biosensing. RNA molecules have a diverse and important set of cellular functions (Eddy, 2001). Non-coding RNAs can splice and edit RNA, modify ribosomal RNA, catalyse biochemical reactions and regulate gene expression at the level of transcription or translation (Eddy, 2001; Doudna and Cech, 2002; Guerrier-Takada et al., 1983; Kruger et al., 1982). The regulatory subset of non-coding RNAs (Lee et al., 1993; Stougaard and Nordstrom, 1981; Wagner and Simons, 1994) is well-suited for rational design (Isaacs et al., 2006) and, in particular, for biosensing applications. Many regulatory RNA molecules are natural environmental sensors (Gelfand et al., 1999; Johansson et al., 2002; Lease and Belfort, 2000; Majdalani et al., 2002; Mandal et al., 2003; Mironov et al., 2002; Morita et al., 1999; Winkler et al., 2002; Winkler et al., 2004), and because of their ability to take on complex structures defined by their sequence, these molecules can mediate diverse modular functions across distinct sequence domains. Riboswitches (Winkler and Breaker, 2005), for instance, bind specific small-molecule ligands through aptamer¹² domains and induce conformational changes in the 5' UTR of their own mRNA, thereby regulating gene expression. Aptamer domains that are modelled after riboswitches are versatile and widely used sensitive elements for RNA-based biosensing. The choice and number of aptamer domains can provide control over specificity. Building an entire RNA-based biosensor typically requires coupling an aptamer domain (the sensitive element) with a post-transcriptional regulatory domain (the transducer module) on a modular RNA molecule scaffold.

Antisense RNAs¹³ (Wagner and Simons, 1994; Good, 2003) are one such class of natural regulatory RNAs that can control gene expression through post-transcriptional mechanisms. By linking a riboswitch aptamer to an antisense repressor on a single RNA molecule, Bayer and Smolke (2005) engineered *trans*-acting, ligand-responsive riboregulators¹⁴ of gene expression in *Saccharomyces cerevisiae* (Box A2-2). Binding of the aptamer to its ligand (for example, the small molecule theophylline) induces a conformational change in the RNA sensor that either sequesters the antisense domain in a stable stem loop (ON switch) or liberates it to inhibit translation of an output gene reporter (OFF switch). As a result of the cooperative dependence on both ligand and target mRNA, this biosensor shows binary-like switching at a threshold ligand concentration, similar

¹² Aptamer – Oligonucleic acids that bind to a specific target molecule, such as a small molecule, protein or nucleic acid. Nucleic acid aptamers are typically developed through *in vitro* selection schemes but are also found naturally (for example, RNA aptamers in riboswitches).

¹³ Antisense RNAs – RNAs that bind segments of mRNA *in trans* to inhibit translation.

¹⁴ Riboregulators – Small regulatory RNAs that can activate or repress gene expression by binding segments of mRNA *in trans*. They are typically expressed in response to an environmental signalling event.

to the genetic toggle design. Importantly, this detection threshold can be adjusted by altering the RNA sequence and therefore the thermodynamic properties of the structure. In principle, the ‘antiswitch’ framework is modular; in other words, aptamers for different ligands and antisense stems targeting different downstream genes could be incorporated into the scaffold to create new sensors. In practice, developing new sensors by aptamer and antisense replacement often involves re-screening compatible secondary structures to create functioning switches. In the future, this platform could be combined with rapid, *in vitro* aptameric selection techniques (Cox et al., 2002; Ellington and Szostak, 1990; Hermann and Patel, 2000; Tuerk and Gold, 1990) for generating a suite of RNA biosensors that report on the levels of various mRNA species and metabolites in a cell. However, here it should also be noted that aptamers show specificity for a biased ligand space, and as a result aptamers for a target ligand cannot always be found.

Another method for transducing the sensory information captured by aptamer domains is to regulate translation through RNA self-cleavage (Winkler et al., 2004; Winkler and Breaker, 2005). RNA cleavage is catalysed by ribozymes, some of which naturally possess aptameric domains and are responsive to metabolites (Winkler et al., 2004). Yen *et al.* (2004) took advantage of this natural framework and encoded ligand-sensitive ribozymes in the mRNA sequences of reporter genes. In the absence of its cognate ligand, constitutive autocleavage of the reporter mRNA resulted in little or no signal. The RNA biosensor is flipped when the cognate ligand is present to inhibit the ribozyme’s activity. Similar to the ‘antiswitch’ framework (and with the same technical challenges), these engineered RNAs could potentially be used as endogenous sensors for reporting on a variety of intracellular species and metabolites.

Post-translational biosensing. Signal transduction pathways show vast diversity and complexity. Factors such as the nature of the molecular interactions, the number of interconnected proteins in a cascade and the use of spatial mechanisms dictate which signals are transmitted, whether a signal is amplified or attenuated and the dynamics of the response. Despite the multitude of factors and interacting components, signal transduction pathways are essentially hierarchical schemes based on sensitive elements and downstream transducer modules, and as such can be rationalized for engineering protein-based biosensors.

The primary sensitive element for most signal transduction pathways is the protein receptor. Whereas environment-responsive promoters and RNA aptamers are typically identified from nature or selected with high-throughput combinatorial methods, protein receptors can be designed *de novo* at the level of molecular interactions. For instance, Looger *et al.* (2003) devised a computational method for redesigning natural protein receptors to bind new target ligands. Starting with a ‘basis’ of five proteins from the *E. coli* periplasmic binding protein (PBP) superfamily, the researchers replaced each of the wild-type ligands with a new, non-native target ligand and then used an algorithm to combinatorially explore all

binding-pocket-residue mutations and ligand-docking configurations. This procedure was used to predict novel receptors for trinitrotoluene (TNT; a carcinogen and explosive), L-lactate (a medically-important metabolite) and serotonin (a chemical associated with psychiatric conditions). The predicted receptor designs were experimentally confirmed to be strong and specific *in vitro* sensors, as well as *in vivo* cell-based biosensors.

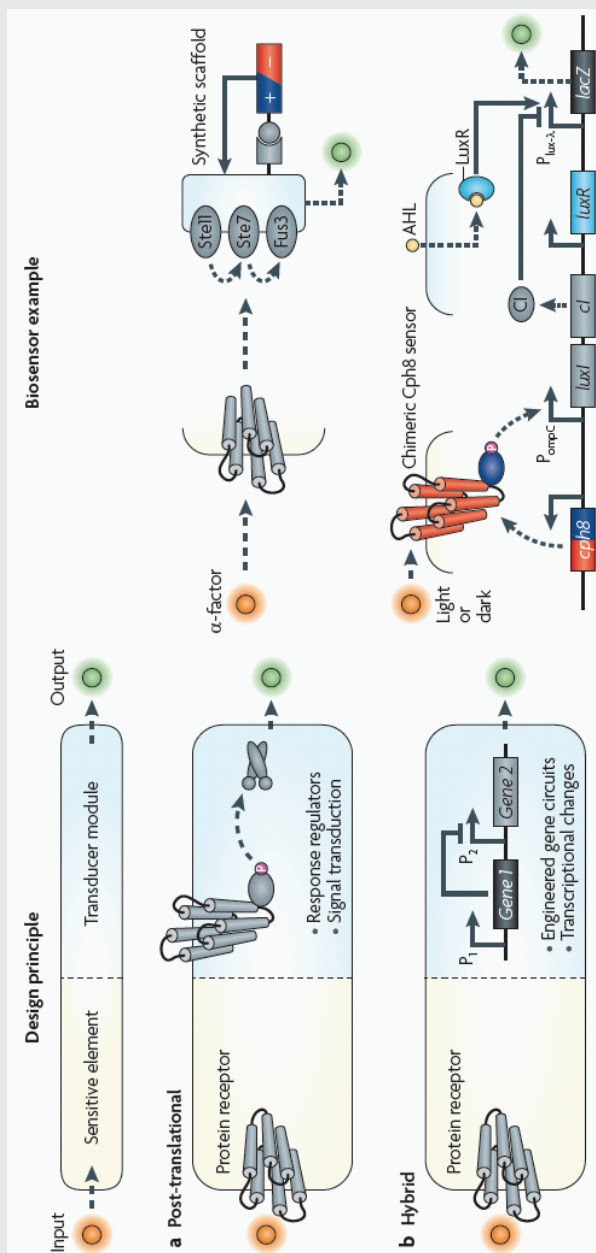
Protein receptors, such as the ones discussed above, are typically membrane-bound; they trigger protein signalling cascades that ultimately result in a cellular response. However, several synthetic methods can be used to transmit captured sensory information in a tunable and desirable manner. Skerker *et al.* (2008) rationally rewired the transmission of information through two-component systems¹⁵ by identifying rules governing the specificity of a histidine kinase to its cognate response regulator. Alternatively, engineered protein scaffolds can be designed to physically recruit pathway modulators and synthetically reshape the dynamical response behaviour of a system (Bashor *et al.*, 2008) (Box A2-3). This constitutes a modular method for programming protein-based biosensors to have any desired response, including accelerated, delayed or ultrasensitive responses, to upstream signals.

Hybrid approaches. Combining synthetic transcriptional, translational and post-translational circuits into hybrid solutions and harnessing desired characteristics from each could lead to the creation of cell-based biosensors that are as robust as those of natural organisms. Using a synthetic hybrid approach, Voigt and colleagues (Levskaya *et al.*, 2005; Levskaya *et al.*, 2009; Tabor *et al.*, 2009) developed *E. coli*-based optical sensors. A synthetic sensor kinase was engineered to allow cells to identify and report the presence of red light. As a result, a bacterial lawn of the engineered cells could faithfully ‘print’ a projected image in the biological equivalent of photographic film. Specifically, a membrane-bound photoreceptor from cyanobacteria was fused to an *E. coli* intracellular histidine kinase to induce light-dependent changes in gene expression (Levskaya *et al.*, 2005) (Box A2-3). In a clever example of its use, the bacterial optical sensor was applied in image edge detection (Tabor *et al.*, 2009). In this case, by wiring the optical sensor to transcriptional circuits that perform cell–cell communication (the quorum-sensing¹⁶ system from *Vibrio fischeri*) and logical functions (Box A2-3), the researchers programmed only the cells that receive light and directly neighbour cells that do not receive light to produce a pigment, allowing

¹⁵ Two-component systems – Among the simplest types of signal transduction pathways. In bacteria, they consist of two domains: a membrane-bound histidine kinase (sensitive element) that senses a specific environmental stimulus, and a cognate response regulator (transducer domain) that triggers a cellular response.

¹⁶ Quorum sensing – A cell-to-cell communication mechanism in many species of bacteria, whereby cells measure their local density (by the accumulation of a signalling molecule) and subsequently coordinate gene expression.

BOX A2-3
Synthetic Biosensors: Post-translational and Hybrid Architectures and Examples



A basic biosensor has two modules (see the figure): the sensitive element recognizes and binds analytes, whereas the transducer module transmits and reports signals.

Post-translational

Post-translational biosensors (part **a**) consist of membrane-bound protein receptors that trigger signal transduction cascades through signalling proteins, such as response regulators of two-component systems. In the example shown, a synthetic protein scaffold was engineered to physically localize the pathway components of the yeast mitogen-activated protein kinase (MAPK) pathway, which here is being triggered by the mating α -factor (Bashor et al., 2008). By recruiting pathway positive and negative modulators (\pm) to the scaffold, the system can be tuned to enable desired responses to upstream signals (for example, accelerated, delayed or ultrasensitive responses).

Hybrid

The hybrid example (part **b**) shows a synthetic genetic edge detection circuit (Tabor et al., 2009). The sensitive element is a light-dark sensor, Cph8, made as a chimera of the photoreceptor domain of the cyanobacteria phytochrome Cph1 and the kinase domain of *Escherichia coli EnvZ*. This synthetic sensor activates an engineered gene circuit that combines cell-cell communication (genes and promoters of the Lux operon) with a logical AND gate ($P_{lux\>}$) to trace the edges of an image. Specifically, the absence of light triggers Cph8 kinase activity, which correspondingly activates the *ompC* promoter. Cells not receiving light will therefore produce the cell-cell communication molecule 3-oxohexanoyl-homoserine lactone (AHL; yellow circle) through expression of its biosynthetic enzyme LuxI. In addition, these cells will produce the transcriptional repressor Ci (grey oval). AHL binds to the constitutively expressed transcription factor LuxR (light blue oval) to activate expression from the $P_{lux\>}$ promoter, which is simultaneously and dominantly repressed by Ci. The result is that only cells that receive light (and therefore do not express the transcriptional repressor Ci) and are nearby to AHL-producing dark cells will activate the final gate and produce pigment through β -galactosidase activity (encoded by *lacZ*).

the edges of a projected image to be traced. This work demonstrates that complex behaviour can emerge from properly wiring together smaller genetic programs, and that these programs can lead to unique real-world applications.

Therapeutics

Human health is afflicted by new and old foes, including emergent drug-resistant microbes, cancer and obesity. Meanwhile, progress in medicine is faced with challenges at each stage of the therapeutic spectrum, ranging from the drying up of pharmaceutical pipelines to limited global access to viable medicines. In a relatively short amount of time, synthetic biology has made promising strides in reshaping and streamlining this spectrum (Box A2-4). Indeed, the rational and model-guided construction of biological parts is enabling new therapeutic platforms, from the identification of disease mechanisms and drug targets to the production and delivery of small molecules.

Disease mechanism. An electrical engineer is likely to prototype portions of a circuit on a ‘breadboard’ before printing it as an entire integrated circuit. This allows for the rigorous testing of submodules in an isolated, well-characterized environment. Similarly, synthetic biology provides a framework for synthetically reconstructing natural biological systems to explore how pathological behaviours may emerge. This strategy was used to give mechanistic insights into a primary immunodeficiency, agammaglobulinaemia, in which patients cannot generate mature B cells and as a result are unable to properly fight infections (Ferrari et al., 2007). The researchers developed a synthetic testbed by systematically reconstructing the various components of the human B cell antigen receptor (BCr) signalling pathway in an orthogonal environment.¹⁷ This allowed them to identify network topology features that trigger BCR signalling and assembly. A rare mutation in the immunoglobulin- β -encoding gene was identified in one patient and introduced into the synthetic system, in which it was shown to abolish assembly of the BCR on the cell surface, thereby linking this faulty pathway component with disease onset. Pathogenic viral genomes can similarly be reconstructed for studying the molecular underpinnings of infectious disease pandemics. For instance, synthetic reconstruction of the severe acquired respiratory syndrome (SARS) coronavirus (Becker et al., 2008) and the 1918 Spanish influenza virus (Tumpey et al., 2005) helped to identify genetic mutations that may have conferred human tropism and increased virulence.

Drug-target identification. Building up synthetic pathways and systems from individual parts is one way of identifying disease mechanisms and therapeutic

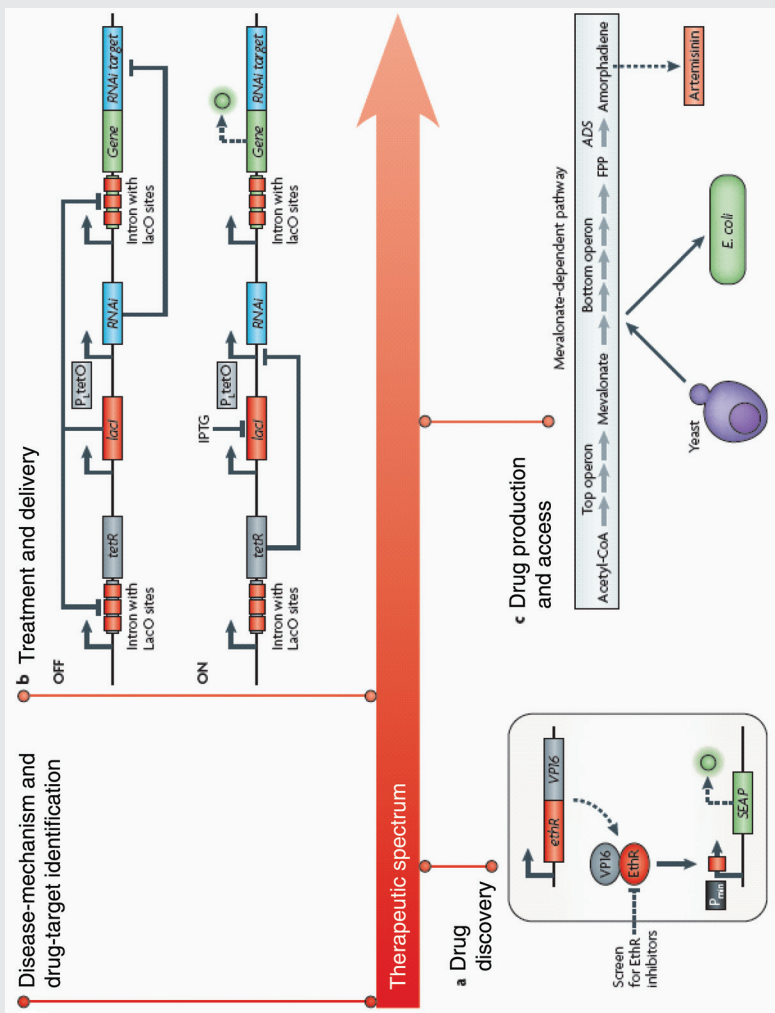
¹⁷Orthogonal environment – A cellular environment or host into which genetic material is transplanted to avoid undesired native host interference or regulation. Orthogonal hosts are often organisms with sufficient evolutionary distance from the native host.

targets. Another is to deploy synthetic biology devices to systematically probe the function of individual components of a natural pathway. Our group, for instance, has engineered modular riboregulators that can be used to tune the expression of a toxic protein or any gene in a biological network (Isaacs et al., 2004). To achieve post-transcriptional control over a target gene, the mRNA sequence of the riboregulator 5' UTR is designed to form a hairpin structure that sequesters the ribosomal binding site (RBS) and prevents ribosome access to it. Translational repression of this *cis*-repressed mRNA can be alleviated by an independently regulated transactivating RNA that targets the stem-loop for unfolding. Engineered riboregulators have been used to tightly regulate the expression of CcdB, a toxic bacterial protein that inhibits DNA gyrase,¹⁸ to gain a better understanding of the sequence of events leading to induced bacterial cell death (Dwyer et al., 2007). These synthetic biology studies, in conjunction with systems biology studies of quinolones (antibiotics that inhibit gyrase) (Dwyer et al., 2007), led to the discovery that all major classes of bactericidal antibiotics induce a common cellular death pathway by stimulating oxidative damage (Kohanski et al., 2007, 2008). This work provided new insights into how bacteria respond to lethal stimuli and paved the way for the development of more effective antibacterial therapies.

Drug discovery. After a faulty pathway component or target is identified, whole-cell screening assays can be designed using synthetic biology strategies for drug discovery. As a demonstration of this approach, Fussenegger and colleagues (Weber et al., 2008) developed a synthetic platform for screening small molecules that could potentiate a *Mycobacterium tuberculosis* antibiotic (Box A2-4). ethionamide, currently the last line of defence in the treatment of multidrug-resistant tuberculosis, depends on activation by the *M. tuberculosis* enzyme EthA for efficacy. However, due to transcriptional repression of *ethA* by the protein EthR, ethionamide-based therapy is often rendered ineffective. To address this problem, the researchers designed a synthetic mammalian gene circuit that featured an EthR-based transactivator of a reporter gene and used it to screen for and identify EthR inhibitors that could abrogate resistance to ethionamide. Importantly, because the system is a cell-based assay, it intrinsically enriches for inhibitors that are non-toxic and membrane-permeable to mammalian cells, which are key drug criteria as *M. tuberculosis* is an intracellular pathogen. This framework, in which drug discovery is applied to whole cells that have been engineered with circuits that highlight a pathogenic mechanism, could be extended to other diseases and phenotypes.

¹⁸DNA gyrase – A type II DNA topoisomerase that catalyses the ATP-dependent supercoiling of closed-circular dsDNA by strand breakage and rejoining reactions. Control of chromosomal topological transitions is essential for DNA replication and transcription in bacteria, making gyrase an effective target for antimicrobial agents (for example, the quinolone class of antibiotics).

BOX A2-4 The Impact of Synthetic Biology on the Therapeutic Spectrum



Drug discovery

Part **a** of the figure shows a synthetic mammalian gene circuit that enabled drug discovery for antituberculosis compounds (Weber et al., 2008). The antibiotic ethionamide is rendered cytotoxic to *Mycobacterium tuberculosis* by the enzyme EthA in infected cells. Because EthA is natively repressed by EthR, resistance to ethionamide treatment is common. In the gene circuit, a fusion of EthR and the mammalian transactivator VP-16 binds a minimal promoter (P_{min}) with a synthetic EthR operator site and activates expression of the reporter gene *SEAP* (human placental secreted alkaline phosphatase). This platform allows for the rapid screening of EthR inhibitors in mammalian cells.

Treatment and delivery

Part **b** shows a synthetic mammalian genetic switch for tight, tunable and reversible control of a desired gene for therapeutic or gene-delivery applications. In the OFF configuration (upper panel), expression of the gene of interest (green) is repressed at the levels of both transcription and translation. Constitutively expressed LacI repressor (red) binds to the *lac* operator sites in the transgene module of the gene of interest, therefore repressing its transcription. Any transcriptional leakage is repressed at the level of translation by an interfering RNA (blue), which targets the gene's 3' UTR. The system is switched ON (lower panel) by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG), which binds LacI repressor proteins and consequently relieves both forms of repression.

Drug production and access

The discovery of drugs does not always translate to the people who need them the most because drug production processes can be difficult and costly. Antibiotics are industrially produced from microbes and fungi, and are therefore widespread and cheap. Conversely, many other drugs are isolated from hosts that are not as amenable to large-scale production and are therefore costly and in short supply. Such drugs include the antimalaria drug artemisinin and the anticancer drug taxol. Fortunately, global access to drugs is being enabled by hybrid synthetic biology and metabolic engineering strategies for the microbial production of rare natural products. In the case of artemisinin (part **c**), there exist two biosynthetic pathways for the synthesis of the universal precursors to all isoprenoids, the large and diverse family of natural products of which artemisinin is a member. The native isoprenoid pathway found in *Escherichia coli* (the deoxyxylulose 5-phosphate (DXP) pathway) has been difficult to optimize, so instead researchers have synthetically constructed and tested the entire *Saccharomyces cerevisiae* mevalonate-dependent (MEV) pathway in *E. coli* in a piece-wise fashion (for example, by separating the 'top' and 'bottom' operons). The researchers initially used *E. coli* as a simple, orthogonal host platform to construct, debug and optimize the large metabolic pathway (Martin et al., 2003). They then linked the optimized heterologous pathway to a codon-optimized form of the plant terpene synthase *ADS* to funnel metabolic production to the specific terpene precursor to artemisinin. This work allowed them to build a full, optimized solution that could be ultimately and seamlessly deployed back into *S. cerevisiae* for cost-effective synthesis and purification of industrial quantities of the immediate drug precursor of artemisinin (Fo et al., 2006). FPP, farnesyl pyrophosphate.

Therapeutic treatment. Synthetic biology devices have additionally been developed to serve as therapies themselves. Entire engineered viruses and organisms can be programmed to target specific pathogenic agents and pathological mechanisms. For instance, in two separate studies (Lu and Collins, 2007, 2009) researchers used engineered bacteriophages to combat antibiotic-resistant bacteria by endowing them with genetic mechanisms that target and thwart bacterial mechanisms for evading antibiotic action. The first study was prompted by the observation that biofilms,¹⁹ in which bacteria are encapsulated in an extracellular matrix, have inherent resistance to antimicrobial therapies and are sources of persistent infections. To more effectively penetrate this protective environment, T7 phage was engineered to express the biofilm matrix-degrading enzyme dispersin B (DspB) upon infection (Lu and Collins, 2007). The two-pronged attack of T7 expressing DspB and phage-induced lysis fuelling the creation and spread of DspB resulted in the removal of 99.997% of the biofilm bacterial cells. In the second study (Lu and Collins, 2009), it was suggested that inhibition of certain bacterial genetic programs could improve the effectiveness of current antibiotic therapies. In this case, bacteriophages were deliberately designed to be non-lethal so as not to elicit resistance mechanisms; instead, a non-lytic M13 phage was used to suppress the bacterial SOS DNA-damage response by overexpression of its repressor, *lexA3*. The engineered bacteriophage significantly enhanced killing by three major classes of antibiotics in traditional cell culture and in *E. coli*-infected mice, potentiated killing of antibiotic-resistant bacteria and, importantly, reduced the incidence of cells with antibiotic-induced resistance.

Synthetically engineered viruses and organisms that are able to sense and link their therapeutic activity to pathological cues may be useful in the treatment of cancer, in which current therapies often indiscriminately attack tumours and normal tissues. For instance, adenoviruses were programmed to couple their replication to the state of the p53 pathway in human cells (Ramachandra et al., 2001). Normal p53 production would result in inhibition of a crucial viral replication component, whereas a defunct p53 pathway, which is characteristic of tumour cells, would allow viral replication and cell killing. In another demonstration of translational synthetic biology applied to cancer therapy, Voigt and colleagues (Anderson et al., 2006) developed cancer-targeting bacteria and linked their ability to invade the cancer cells to specific environmental signals. Constitutive expression of the heterologous invasin (*inv*) gene (from *Yersinia pseudotuberculosis*) can induce *E. coli* cells to invade both normal human cell lines and cancer cell lines. So, to preferentially invade cancer cells, the researchers placed *inv* under the control of transcriptional operons that are activated by environmental signals specific to the tumour microenvironment. These engineered bacteria could be made to carry or synthesize cancer therapies for the treatment of tumours.

¹⁹ Biofilms – Surface-associated communities of bacterial cells encapsulated in an extracellular polymeric substances (EPS) matrix. Biofilms are an antibiotic-resistant mode of microbial life found in natural and industrial settings.

Therapeutic delivery. In addition to engineered therapeutic organisms, synthetic circuits and pathways can be used for the controlled delivery of drugs as well as for gene and metabolic therapy. In some cases, sophisticated kinetic control over drug release in the body may yield therapeutic advantages and reduce undesired side effects. Most hormones in the body are released in time-dependent pulses. Glucocorticoid secretion, for instance, has a circadian and ultradian²⁰ pattern of release, with important transcriptional consequences for glucocorticoid-responsive cells (Stavreva et al., 2009). Faithfully mimicking these patterns in the administration of synthetic hormones to patients with glucocorticoid-responsive diseases, such as rheumatoid arthritis, may decrease known side effects and improve therapeutic response (Stavreva et al., 2009). Periodic synthesis and release of biologic drugs can be autonomously achieved with synthetic oscillator circuits (Elowitz and Leibler, 2000; Atkinson et al., 2003; Fung et al., 2005; Stricker et al., 2008; Tiggles et al., 2009) or programmed time-delay circuits (Weber et al., 2007). In other cases, one may wish to place a limit on the amount of drug released by programming the synthetic system to self-destruct after a defined number of cell cycles or drug release pulses. Our group has recently developed two variants of a synthetic gene counter (Friedland et al., 2009) that could be adapted for such an application.

Gene therapy is beginning to make some promising advances in clinical areas in which traditional drug therapy is ineffective, such as in the treatment of many hereditary and metabolic diseases. Synthetic circuits offer a more controlled approach to gene therapy, such as the ability to dynamically silence, activate and tune the expression of desired genes. In one such example (Deans et al., 2007), a genetic switch was developed in mammalian cells that couples transcriptional repressor proteins and an RNAi module for tight, tunable and reversible control over the expression of desired genes (Box A2-4). This system would be particularly useful in gene-silencing applications, as it was shown to yield >99% repression of a target gene.

Additionally, the construction of non-native pathways offers a unique and versatile approach to gene therapy, such as for the treatment of metabolic disorders. Operating at the interface of synthetic biology and metabolic engineering, Liao and colleagues (Dean et al., 2009) recently introduced the glyoxylate shunt pathway²¹ into mammalian liver cells and mice to explore its effects on fatty acid metabolism and, more broadly, on whole-body metabolism. Remarkably, the researchers found that when transplanted into mammals, the shunt actually increased fatty acid oxidation, evidently by creating an alternative cycle. Furthermore, mice expressing the shunt showed resistance to diet-induced obesity when

²⁰ Ultradian – Periods of cycles that are repeated throughout a 24-hour circadian day.

²¹ Glyoxylate shunt pathway – A two-enzyme metabolic pathway unique to bacteria and plants that is activated when sugars are not readily available. This pathway diverts the tricarboxylic acid (TCA) cycle so that fatty acids are not completely oxidized and are instead converted into carbon energy sources.

placed on a high-fat diet, with corresponding decreases in total fat mass, plasma triglycerides and cholesterol levels. This work offers a new synthetic biology model for studying metabolic networks and disorders, and for developing treatments for the increasing problem of obesity.

Finally, the discovery of drugs and effective treatments may not quickly — or ever — translate to the people who need them the most because drug production processes can be difficult and costly. As discussed below, synthetic biology is allowing rare and costly drugs to be manufactured more cost-effectively (Box A2-4).

Biofuels, pharmaceuticals and biomaterials

Recent excitement surrounding the production of biofuels, pharmaceuticals and biomaterials from engineered microorganisms is matched by the challenges that loom in bringing these technologies to production scale and quality. The most widely used biofuel is ethanol produced from corn or sugar cane (Fortman et al., 2008); however, the heavy agricultural burden combined with the suboptimal fuel properties of ethanol make this approach to biofuels problematic and limited. Microorganisms engineered with optimized biosynthetic pathways to efficiently convert biomass into biofuels are an alternative and promising source of renewable energy. These strategies will succeed only if their production costs can be made to compete with, or even outcompete, current fuel production costs. Similarly, there are many drugs for which expensive production processes preclude their capacity for a wider therapeutic reach. New synthetic biology tools would also greatly advance the microbial production of biomaterials and the development of novel materials.

Constructing biosynthetic pathways. When engineering for biofuels, drugs or biomaterials, two of the first design decisions are choosing which biosynthetic pathway or pathways to focus on and which host organism to use. Typically, these decisions begin with the search for organisms that are innately capable of achieving some desired biosynthetic activity or phenotype (Alper and Stephanopoulos, 2009). For biofuel production, for instance, certain microorganisms have evolved to be proficient in converting lignocellulosic material to ethanol, biobutanol and other biofuels. These native isolates possess unique catabolic activity, heightened tolerances for toxic materials and a host of enzymes designed to break down the lignocellulosic components. Unfortunately, these highly desired properties exist in pathways that are tightly regulated according to the host's evolved needs and therefore may not be suitable in their native state for production scale. A long-standing challenge in metabolic and genetic engineering is determining whether to improve the isolate host's production capacity or whether to transplant the desired genes or pathways into an industrial model host, such as *E. coli* or *S.*

cerevisiae; these important considerations and trade-offs are reviewed elsewhere (Alper and Stephanopoulos, 2009).

The example of the microbial production of biobutanol, a higher energy density alternative to ethanol, provides a useful glimpse into these design trade-offs. Butanol is converted naturally from acetyl-CoA by *Clostridium acetobutylicum* (Jones and Woods, 1986). However, it is produced in low yields and as a mixture with acetone and ethanol, so substantial cellular engineering of a microorganism for which standard molecular biology techniques do not apply is needed to produce usable amounts of butanol (Tummala et al., 2003; Shao et al., 2007). Furthermore, importing the biosynthetic genes into an industrial microbial host can lead to metabolic imbalances (Inui et al., 2008). In an altogether different approach, Liao and colleagues (Atsumi et al., 2008) bypassed standard fermentation pathways and recognized that a broad set of the 2-keto acid intermediates of *E. coli* amino acid biosynthesis could be synthetically shunted to achieve high-yield production of butanol and other higher alcohols in two enzymatic steps.

Indeed, complementary to efforts in traditional metabolic and genetic engineering is the use of engineering principles for constructing functional, predictable and non-native biological pathways *de novo* to control and improve microbial production. In an exemplary illustration of this, Keasling and colleagues engineered the microbial production of precursors to the antimalarial drug artemisinin to industrial levels (Martin et al., 2003; Ro et al., 2006) (Box A2-4). There are now many such examples of the successful application of synthetic approaches to biosynthetic pathway construction — such approaches have been used in the microbial production of fatty-acid-derived fuels and chemicals (such as fatty esters, fatty alcohols and waxes) (Steen et al., 2010), methyl halide-derived fuels and chemicals (Bayer et al., 2009), polyketide synthases that make cholesterol-lowering drugs (Ma et al., 2009), and polyketides made from megaenzymes that are encoded by very large synthetic gene clusters (Kodumal et al., 2004).

Optimizing pathway flux. After biosynthetic pathways have been constructed, the expression levels of all of the components need to be orchestrated to optimize metabolic flux²² and achieve high product titres. A standard approach is to drive the expression of pathway components with strong and exogenously tunable promoters, such as the P_{Ltet} , P_{Llac} , and P_{BAD} promoters from the *tet*, *lac* and *ara* operons of *E. coli*, respectively. To this end, there are ongoing synthetic biology efforts to create and characterize more reusable, biological control elements based on promoters for predictably tuning expression levels (Alpert et al., 2005; Ellis et al., 2009). Further to this, synthetic biologists have devised a number of alternative methods for obtaining biological pathway balance, ranging from re-configuring network connectivity to fine-tuning individual components. A richer

²² Metabolic flux – The rate of flow of metabolites through a metabolic pathway. The rate is regulated by the enzymes in the pathway.

discussion of these topics, including the fine-tuning of parts, the application of model-guided approaches and the development of next-generation interoperable parts, is presented elsewhere (Lu et al., 2009). In Box A2-5 and Box A2-6 we detail several synthetic biology strategies that specifically pertain to the optimization of metabolic pathway flux. These strategies range from those driven by evolutionary techniques, to those driven by rational design and *in silico* models, to those that combine both approaches.

Programming novel functionality and materials. Beyond facilitating metabolic tasks, synthetic systems can infuse novel functionality into engineered organisms for production purposes or for building new materials. Early work in the field laid the groundwork for constructing basic circuits that could sense and process signals, perform logic operations and actuate biological responses (Voigt, 2006). Wiring these modules together to bring about reliable, higher-order functionality is one of the next major goals of synthetic biology (Lu et al., 2009), and an important application of this objective is the layering of ‘smart’ control mechanisms over metabolic engineering. For instance, circuits designed to sense the bioreactor environment and shift metabolic phases accordingly would further improve biofuel production. Alternatively, autonomous timing circuits could be used to shut down metabolic processes after a prescribed duration of time. Biological timers of this sort have been developed using genetic toggle switches that were deliberately rendered imbalanced through model-guided promoter engineering (Ellis et al., 2009). These genetic timers were used to program the time-dependent flocculation²³ of yeast cells to facilitate the separation of cells from, for instance, the alcohol produced in industrial fermentation processes.

Synthetic control systems can also be used to extract and purify the synthesized product. This is particularly important in the production of recombinant proteins, bioplastics and other large biomaterials, which can accumulate inside cells, cause the formation of inclusion bodies and become toxic to cells if they are present at high titres. To export recombinant spider-silk monomers, Widmaier *et al.* (2009) searched for a secretion system that would enable efficient and indiscriminate secretion of proteins through both bacterial membranes. The *Salmonella* type III secretion system (T3SS) not only fulfils these criteria but also possesses a natural regulatory scheme that ties expression of the protein to be secreted to the secretion capacity of the cell; as a result, the desired protein is only expressed when sufficient secretion complexes have been built. To obtain superior secretion rates of recombinant silk protein, the researchers needed only to engineer a control circuit that hitches the heterologous silk-protein-producing genes to the innate genetic machinery for environmental sensing and secretion commitment.

²³ Flocculation – A specific form of cell aggregation in yeast triggered by certain environmental conditions, such as the absence of sugars. For example, flocculation occurs once the sugar in a beer brew has been fermented into ethanol.

Finally, there is an emerging branch of synthetic biology that seeks to program coordinated behaviour in populations of cells, which could lead to the fabrication of novel biomaterials for various applications. The engineering of synthetic multicellular systems is typically achieved with cell–cell communication and associated intracellular signal processing modules, as was elegantly used by Hasty and colleagues (Danino et al., 2010) to bring about synchronized oscillations in a population of bacterial cells. Weiss and colleagues (Basu et al., 2004, 2005) have similarly done pioneering work in building biomolecular signal-processing architectures that can filter communication signals originating from ‘sender’ cells. These systems, which can be programmed to form intricate multicellular patterns from a solid-phase cellular lawn, would aid the development of fabrication-free scaffolds for tissue engineering.

Future challenges and conclusions

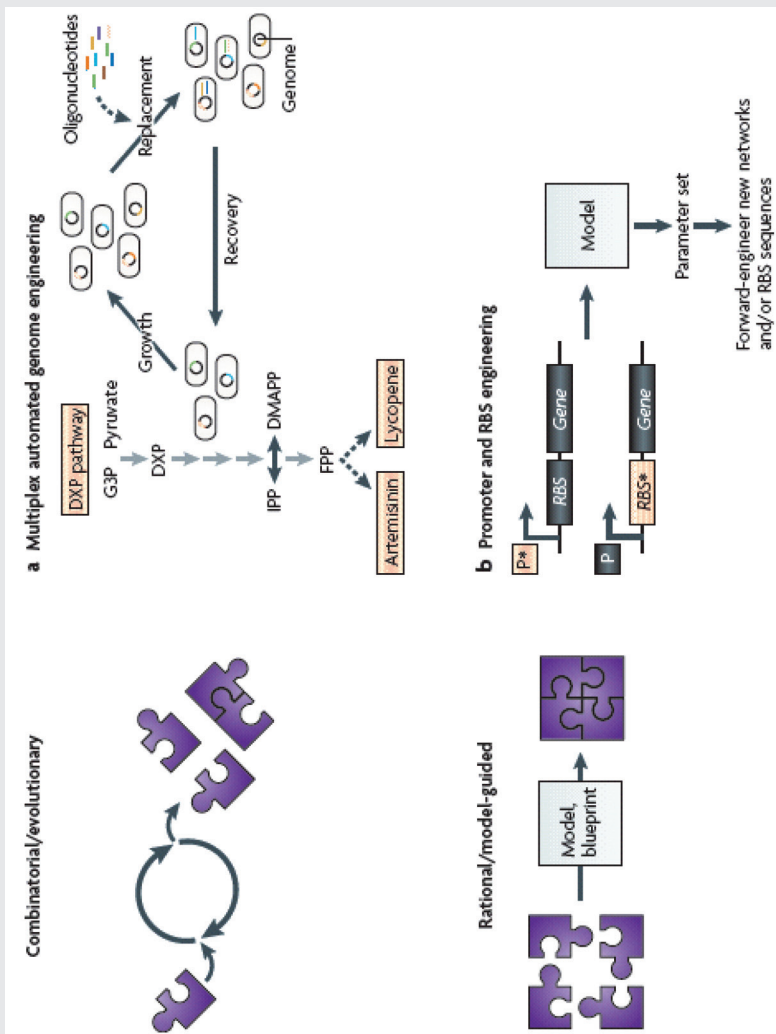
The future of translational synthetic biology hinges on the development of reliable means for connecting smaller functional circuits to realize higher-order networks with predictable behaviours. In a previous article (Lu et al., 2009), we outlined four research efforts aimed at improving and accelerating the overall design cycle and allowing more seamless integration of biological circuitry (Box A2-7).

Beyond the challenge of improving the design cycle, applied synthetic biology would benefit from once again summoning the original inspiration of bio-computing. The ability to program higher-level decision-making into synthetic networks would yield more robust and dynamic organisms, including ones that can accomplish many tasks simultaneously. Furthermore, as adaptive and predictive behaviours are naturally present in all organisms (including microbes) (Mitchell et al., 2009; Tagkopoulos et al., 2008), synthetic learning networks built from genetic and biological parts (Fernando et al., 2009; Fritz et al., 2007) would infuse engineered organisms with more sophisticated automation for biosensing and related applications.

Finally, the majority of synthetic biology is currently practiced in microbes. However, many of the most pressing problems, and in particular those of human health, are inherently problems with mammalian systems. Therefore, a more concerted effort towards advancing mammalian synthetic biology will be crucial for next-generation therapeutic solutions, including the engineering of synthetic gene networks for stem-cell generation and differentiation.

By addressing such challenges, we will be limited not by the technicalities of construction or the robustness of synthetic gene networks but only by the imagination of researchers and the number of societal problems and applications that synthetic biology can resolve.

BOX A2-5
Controlling Metabolic Flux: Evolutionary Strategies and Rational Design



Evolutionary strategies

In the production of artemisinin precursors, the native *Escherichia coli* isoprenoid pathway (the deoxyxylulose 5-phosphate (DXP) pathway) was eschewed in favour of a heterologous pathway so as to circumvent the complex regulatory control imposed by the host (Box A2-4). In an alternative method of relieving regulatory control over the large number of DXP pathway components, Wang *et al.* (2009) diversified and, as a result, optimized the native DXP biosynthetic pathway in *E. coli* (see the figure, part **a**). The researchers developed a rapid, automated method for the *in vivo* directed evolution of pathways, which they termed multiplex automated genome engineering (MAGE). They then applied it to evolve the translational efficiencies of DXP pathway components to achieve maximal lycopene production. Specifically, cells were subjected to cycles of genetic modifications (through oligo-mediated allelic replacement) in an automated fashion to explore sufficient genomic diversity for optimizing biosynthetic pathways at laboratory timescales.

Rational design

At the other end of the spectrum are strategies that rely on quantitative models and blueprints for the rational design of optimized networks and pathways (part **b**). Typically, a component of interest (for example, an engineered promoter (P^*) or ribosomal binding site (RBS *) sequence) will be built into a simple test network. The network and its input-output data will then be fed into a model, which attempts to determine a parameter set that optimally describes the component's dynamics within the framework of the model. Finally, the optimized parameter set will be used to forward-engineer new networks and components. For example, stochastic biochemical models have been developed to capture the expression dynamics of synthetically engineered promoters; these models were subsequently used to predict the correct *in vivo* behaviour of different and more complex gene networks built from the modelled components (Blake *et al.*, 2003; Guido *et al.*, 2006). Similarly, at the level of translation, thermodynamic models that predict the relative translation initiation rates of proteins can be used to rationally forward-engineer synthetic RBS sequences to give desired expression levels (Salis *et al.*, 2009). Such techniques harness modelled genetic parameters (transcriptional or translational) to predict the level of expression of proteins and enzymes in a network. DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; G3P, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate.

In a hybrid rational–combinatorial approach, Dueber *et al.* (2009) suggested that metabolic flux could be controlled by spatially recruiting the enzymes of a desired biosynthetic pathway using synthetic protein scaffolds. To construct the enzyme scaffolding, the researchers tethered protein–protein interaction domains (for example, GBD, SH3 and PDZ domains) from metazoan signalling proteins. These domains recognize and bind cognate peptides that were fused to the enzymes to be recruited (acetoacetyl-CoA thiolase (AtoB) from *Escherichia coli* and HMG-CoA (HMBS) synthase and HMG-CoA reductase (HMGR) from *Saccharomyces cerevisiae*). By varying the number of repeats of an interaction domain, the researchers could additionally control the stoichiometry of the enzymes recruited to the complex. Using the heterologous mevalonate-dependent (MEV) pathway in *E. coli* as a model, they combinatorially (albeit, at a substantially smaller scale) optimized the stoichiometry of the three enzymes responsible for producing mevalonate from acetyl-CoA. Finally, they showed that the optimized synthetic scaffold could substantially increase product titre while reducing the metabolic load on the host; in other words, their high product titres did not require the overexpression of biosynthetic enzymes in the cell.

BOX A2-7
Recommendations for Improving the
Synthetic Biology Design Cycle

- Scaling up to larger and more complex biological systems while simultaneously minimizing interference among parts will require an expanded synthetic biology toolkit and, in particular, libraries of interoperable parts. Eukaryotic systems are fertile grounds for discovering such parts, as many synthetic biology devices are based on a small repertoire of bacterial and archaeal regulatory elements.
- Modelling and fine-tuning of synthetic networks should be emphasized, particularly as the network size and complexity increases. This will facilitate proper matching of input–output behaviours (that is, transfer functions) when distinct modules are connected.
- There is a need to develop new probes and high-throughput methods for the *in vivo* measurement of circuit dynamics to rapidly characterize parts and debug networks.
- Cellular testing platforms need to be developed to quicken the pace of identifying problematic network nodes and ease the failure-prone jumps associated with either building a more complex network or deploying a network in a more complex organism. These testing platforms could be cells engineered to have minimal genomes (Gibson et al., 2008; Glass et al., 2006; Lartigue et al., 2007; Lartigue et al., 2009) or lower model organisms that have been equipped with specific machinery from higher organisms.

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Competing Interests Statement

The authors declare competing financial interests: see Web version for details.

Databases

Entrez Gene: <http://www.ncbi.nlm.nih.gov/gene> *ccdB* | *lexA3* | *traA*

OMIM: <http://www.ncbi.nlm.nih.gov/omim> agammaglobulinaemia

UniProtKB: <http://www.uniprot.org> CI | Cro | DspB | EnvZ | EthA | EthR

Further Information

James J. Collins' homepage: <http://www.bu.edu/abl>

All links are active on the online PDF.

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A3

THE GENOME AS THE UNIT OF ENGINEERING

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Introduction

Recent years have marked a dramatic increase of our capabilities to sequence and synthesize nucleic acids. Ten years ago the first human genome was sequenced, and at the time it was a monumental undertaking requiring billions of dollars in funding and legions of dedicated researchers. Today, the same genome sequence would cost a fraction of the price and soon personal genome sequencing will be commonplace. However, while our understanding of genomes has not yet

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caught up with our ability to generate data, there is no question that the technology revolution has transformed and will continue to drive the biological sciences, in particular the new integrative science known as systems biology.

The same revolution is occurring in DNA synthesis, as prices for DNA drop and technologies are developed for large-scale (and ever longer) syntheses. As with sequencing, the technology revolution will lead understanding, and it can be argued that, although we can synthesize a microbial genome (Gibson et al., 2010), we don't necessarily understand how to design one. That's fine; we can learn by doing, and as synthetic genomes become easier to synthesize, we will undoubtedly generate customized genomes that will test the simple hypothesis of whether they function as intended.

Together, these emerging issues are the essence of what can be seen as the holy grail of both systems and synthetic biology, the genome as the unit of engineering. Synthetic biology can, in many circumstances, be defined as the abstraction of biology to the point where it can be easily engineered. This is first and foremost an operational definition, not an intellectual one. Indeed, there hides in the background of synthetic biology the usually unstated hypothesis that biology was made to be engineered by engineers (a hypothesis we take issue with below). Parallels are often drawn between electrical engineering and synthetic biology, where genetic information like promoters and proteins can be compared to components of a circuit board such as transistors or capacitors. In the view of the new breed of synthetic biologists, standardized components can almost always be used to rationally design genetic elements to perform desired tasks. This view has much merit, since it has already yielded interesting products such as biofuels, pharmaceuticals, and biomaterials. For example, Bayer and Voigt coaxed yeast into making valuable methyl halides from biomass (Bayer et al., 2009), while the Keasling lab reengineered the common lab microbe, *Escherichia coli*, to produce large quantities of amorpha-4,14-diene, a precursor to antimalarial and anticancer drugs (Martin et al., 2003). It is not unreasonable to suspect that synthetic circuits will be introduced in human hosts at some point; for example, Fussenegger has created a synthetic circuit composed of a bacterial uric acid sensor and a fungal urate oxidase (which converts uric acid into a more tolerable compound) that can be used to control uric acid levels in mammalian hosts and thus ameliorate chronic disease states such as gout (Kemmer et al., 2010).

However, it is nonetheless still the case that many of these systems must be optimized in order for them to function as intended, in large part because the constituent parts are neither truly modular nor is their function fully predictable in new contexts. The question thus becomes whether biology is really meant to be engineered the same way a circuit board is, whether engineers will learn to make biology a circuit board, or whether some composite view is more akin to reality. Or, stated another way, the question is whether genetic tinkering (which existed well before synthetic biology) has somehow entered a new, more robust phase or if it has just been relabeled.

Systems biology has taken a different approach to how to tinker with systems. This approach proceeds from an understanding of the system as a whole instead of as an amalgam of component parts. It is top down rather than bottom up. By integrating literally millions of points of data from genomes, transcriptomes, and proteomes across the phylogenetic spectra, systems biologists can draw remarkable conclusions, up to and including the identification of nonobvious and evolutionarily repurposed subsystems. It is this sort of understanding that allows us to realize that the same interactions that govern resistance to antifungals in yeast also govern blood vessel formation in higher organisms (McGary et al., 2010). While a comparable synthetic biology approach might be to repurpose a tractable signaling pathway for blood vessel formation, this approach would require extensive empirical testing. As technology continues to develop for high-throughput analysis of DNA, RNA, and proteins, systems biologists will have the benefit of several billion years of empirical testing and, thus, will hold the intellectual high ground for understanding how organisms truly work. Quantitative modeling of the connectedness of extant systems will, in the end, be more likely to allow us to build a functional genome from scratch than the untested engineering hypothesis that organisms should work like we want them to. In greater detail, this should inevitably lead to the following discussion.

Systems Biology Eats Synthetic Biology

The fiction of synthetic biology is that it is possible to engineer biological systems in modular, composable, scalable, and programmable ways using “parts” to build circuits and eventually systems (and ecosystems) (Bromley et al., 2008; Win et al., 2009). Both the field and the fiction have emerged largely not from scientific research but from the International Genetically Engineered Machines competition, iGEM, which is constrained to rely on “BioBricks” for the construction of student projects (Smolke, 2009). In fact, long before iGEM there were numerous biological engineers, and these biological engineers would, with different degrees of success, use genes (not then known as biological parts) to construct pathways (not then known as circuits) that had particular functions.

What has changed between the long period during which biological engineering has been maturing (indeed, one could argue that biological engineering in the form of selective breeding greatly predated man’s understanding of biology itself) and today to make biological engineering into synthetic biology, besides nomenclature? It could be argued that there are two important milestones: First, many researchers in other engineering disciplines were somehow shut out of the biology because it lacked an engineering flair and was more dissective than synthetic. In this view, the influx of engineers into biology is assisted by recasting biology in terms more familiar to engineers, and this is why we often see biological circuits represented (incorrectly, as I will argue later) as electronic circuits (Khalil and Collins, 2010).

Second, there is not just a quantitative but rather a qualitative or foundational difference in being able to make large amounts of DNA. The ability to remake a whole genome (Gibson et al., 2010) is so great relative to the ability to make a mutation in that genome (or in an episome sharing a cell with that genome) that there must now be a new engineering discipline that approaches genome construction in a wholly different way than traditional biological engineering would have been capable of doing. I think there is some merit to this explanation, although it is a bit like saying that genomics is different than genetics. While we would never argue that we do not have much greater understanding of molecular detail today than we did in Mendel's day, our fantastic increase in knowledge does not invalidate the views of Mendel; rather, this knowledge merely adds depth to the fundamental concept of inheritance. Genomics further explains genetics; it does not remake it (although individual concepts in genetics, such as the role of environment in inheritance, have certainly been remade radically). Nonetheless, it is true that techniques for the rational manipulation of whole genomes will prove to be of enormous value into the future. And while many of us who wish to reconstruct living systems will continue to have "Venter envy," a new generation of techniques such as multiplex automated genome engineering (Wang et al., 2009) are beginning to emerge that will make such manipulations possible even for individual investigators.

In the absence of the synthetic biology "revolution" (redefinition), how might biological engineers who were attempting to manipulate larger and larger amalgams of DNA have managed? Where would they have turned for knowledge and inspiration? I contend that it would not have been electrical engineering—the frequent muse to whom many who call themselves synthetic biologists appeal—but rather to systems biology. Systems biology attempts to understand the interrelationships between all of the molecular and cellular parts of system in a quantitative way, and it has as one of its ultimate goals the modeling of biological organisms down to the molecular level. If we had the same knowledge of organisms that we do of the hardware and software accompanying a computer, the entire organism would not just be a "chassis" for the installation of a synthetic circuit; it would be the unit of engineering itself. Fortunately for both traditional biological engineers and synthetic biologists, our understanding of systems has increased dramatically in recent years, and thus there will come a point where systems biology will not only completely inform biological engineering, but it will overtake the utility of orthogonal, add-on circuits that were largely meant to operate independently of the systems in which they were embedded. Systems biology will eat synthetic biology.

This change in perspective and approach will be absolutely essential as biological engineering moves forward. The utility of orthogonal synthetic circuits is limited for a variety of reasons. First, by failing to take into account the unity of the system, the predictability of circuits must be limited. This can best be seen by thinking about a very simple synthetic circuit that has been around for

a long time: a plasmid that is used for protein overproduction. You can in fact embed a plasmid in a strain and induce protein production. From this vantage, the synthetic circuit has worked. However, it is difficult if not impossible to predict protein yield at the outset with any degree of certainty. Different proteins will express to different extents, will form aggregates to different extents, and will hamper the growth of the organism to different extents, to mention just a few variables that ultimately affect yield. This variability has nothing to do with whether a given “part” has been well characterized or not, and everything to do with the interaction of the part with the system as a whole. Protein overexpression interfaces with the cell’s machinery for transcription, translation, and degradation in myriad ways, and in the absence of a complete understanding of how the circuit interfaces with the system it will be difficult to ultimately predict how the circuit will work. Outcomes will range from making huge amounts of a protein to killing the cell outright. Of course the circuit can always be tinkered with to make more protein, but that was true before it was called a circuit and was merely a plasmid. What has synthetic biology brought to the modular, composable, scalable, and programmable overexpression of proteins that was not previously known? Nothing, because the tinkering that is now possible following the invention of the discipline is the same tinkering that was possible prior to its invention. Collins has pointed out that really tinkering is all that is necessary for engineering to prosper in biology, and he is of course correct. However, understanding and progress in this area will come from an increasingly detailed understanding of systems biology and integrated models of metabolism.

In discussions with Erik Winfree, a more charitable interpretation of the coexplosion of both systems and synthetic biology emerges, which is that these are different means to the same end—a predictable biology. The more complex system, the organism, is at present less predictable, and thus nominally orthogonal subsystems as built by synthetic biologists may allow us to initially develop better models. As those models become more sophisticated, they will of necessity take into account the discoveries and models developed through the study of systems biology.

A second, more important objection is that, by treating systems as though they were mere amalgams of components, synthetic biology ignores what I would argue is the central tenet of biology: organisms evolve. Given that an orthogonal circuit is really just an abstraction, as such circuits draw upon the metabolic, transcription, translation, and other resources of a cell, the circuit must of necessity change the fitness of a cell. To the extent that any synthetic circuit is used in the context of an evolutionary machine—a replicating cell—the possibility exists that mutations will arise that change both the cell and its added circuitry, for better or worse. While one alternative is just to not have cells replicate and to let circuits execute in preset bags of enzymes, this denies one of the great possibilities inherent in biology: the ability to generate larger amounts of complex matter from simpler substrates. An understanding of how to engineer biological systems will therefore proceed in large measure from a better understanding of

the costs and benefits of circuits to the system as a whole, again a province of systems (and evolutionary) biology.

Remaking Organismal Operating Systems

It is with this somewhat different perspective that we can move forward from thinking about how to make orthogonal circuits to thinking about how to manipulate the operating systems of organisms. Ultimately, synthetic biology is not modular, composable, scalable, or programmable because the operating system of biology does not support these features. The operating system of biology is a kludge, the result of billions of years of happenstance and compromise, and it cannot at this juncture be remade by the simple expedient of saying it's not so.

But this does not mean that the operating system cannot be remade.

In looking at the operating system of biology, there is really only one component that is remotely akin to the synthetic biology dream of being modular, composable, scalable, and programmable, and that is DNA (or, more generally, nucleic acids). The simplicity of Watson-Crick base pairing and its implementation in a regular biopolymer meets all of these requirements. Unfortunately, this simplicity is destroyed by translation, which turns a regular biopolymer into many irregular ones. Obviously, from the point of view of organismal fitness, this is a good thing.

Thus, the question becomes, how can we remake the operating system of an organism so that the features of DNA are the features of not just one component but of all the components? My answer to this question is a limited one, but I think it suggests new directions. It seemed to me that since it is possible to have the base-pairing properties of DNA, but with different backbones, such as those seen in RNA, locked nucleic acids (LNAs), and especially peptide nucleic acids (PNAs), that it should be possible to extend base pairing to other components in biological operating systems. In particular, it should be possible to allow proteins to base pair in much the same way that PNAs would.

To this end, my lab has embarked on a journey to expand the genetic code to include four monomers that have nucleobase amino acids with side chains that have pairing properties equivalent to G, A, T, and C. Given the exploits and insights of the Schultz lab and others, this is an engineering feat that should be possible. The punctuation of the genetic code, stop codons, can be reprogrammed to code for amino acids (although ultimately with a systems-level cost), and four base codons (and now even a four base codon-reading ribosome) allow a (still uncertain) expansion of the code and the production of genetically augmented proteins.

Imposing Rationality on Biology

Whereas computers were built from the bottom up, with all of the rationality that engineers could bring to bear on both the hardware and software, biology

and its operating systems were presented to us as a *fait accompli*, and thus have to be engineered from the top down, in a way that can best be described as irrational, despite the fiction of synthetic biology. Biological systems evolved as kludges, and are engineered as kludges. While the underpinnings of synthetic biology seem to be to ignore these kludges, the hope of systems biology is to understand these kludges well enough that engineering short circuits and patches seem seamless.

As a small step, I envision that the addition of nucleobase amino acids to the genetic codes will allow us to begin to engineer biology in a truly rational manner. In my vision, there will be several substantive benefits to this fundamental remaking of a biological operating system. First, engineering protein–protein interactions will become more predictable, as it may be possible to uniquely code for such interactions via strings of nucleobase amino acids. Second, the same benefit will obviously accrue for protein–nucleic acid interactions. Transcription factors and RNA-binding proteins will no longer have semiprogrammable interaction domains, such as zinc fingers, but will instead be fully programmable. Third, it should be possible to rationally design cellular interactions and architectures via membrane proteins that again are tailed with nucleobase amino acids. And finally, this fundamental change in the operating system of a biological system should greatly speed the course of evolution. This conclusion comes from thinking about how transcription factors evolve to acquire new specificities. In general, there are multiple amino acid changes that must occur in order to bind a new DNA sequence, and these changes are not one-to-one, since the “code” for protein–nucleic acid interactions is three-dimensional rather than linear. By switching out the three-dimensional code for a nominally linear one, the number of sequence changes that must be acquired to bind adjacent to a promoter (or to form a new protein–protein interaction, or to make a connection to a new cell) will be proportionately reduced, and thus the acceptance of these changes (evolution) can potentially occur much more quickly.

For the longer term, I think there is the possibility that the operating systems of organisms can be changed in an even more fundamental manner. Merely adding nucleobase amino acids to the repertoire of the genetic code simplifies interactions from three-dimensional and irrational to linear and rational, but it does nothing about the way in which the associated machinery actually performs computations. Signal-transduction pathways will still pass information from the outside world to the inside of cells via a series of just-so scaffolds and complex conformational changes that will remain subject to relatively irrational engineering (and, given how I have tried to define rationality at a global level, this statement takes nothing away from the extraordinary successes of Lim and others in engineering signal transduction) (Bashor et al., 2008).

Where, then, can we find a more rational operating system for biology? In parallel with the “revolution” of synthetic biology, a true revolution in nucleic acid programming has been brewing at Caltech, led by Erik Winfree, Niles

Pierce, Peng Yin, and others (Seelig et al., 2006; Yin et al., 2008; Zhang et al., 2007). These pioneers have taken the seminal work by Adleman (1994), showing that nucleic acids can be used for computation, and further transformed it, showing that nucleic acids can be computers. To my mind, the distinction is subtle but important. An algorithm can be embedded in nucleic acids, as Adleman showed, but nucleic acids may not be the best platform for executing that algorithm (electronics beats biology, at least in terms of speed, on most computations). When instead we attempt to identify how nucleic acids can best compute—how their intrinsic properties can be exploited to do computation—then we begin to make nucleic acid computers of the sort that have emerged from the Caltech researchers. These computers rely on a very simple reaction, strand exchange, in which one nucleic acid binds to and forms a duplex with another. Duplex formation can in turn lead to the displacement of a previous duplex or the alteration of a programmed secondary structure. The displacement can be transient, with new binding sites (so-called toeholds) being revealed and paving the way to the formation of additional and/or different duplexes. Overall, extraordinarily complex executable circuits can be built in a fully rational manner, and these circuits have now been adapted to a variety of interesting computations, from amplification to determining square roots. The comparison with synthetic biology is instructive: while Weiss has pointed out that it has proven difficult to make synthetic circuits with more than six or so layers (Purnick and Weiss, 2009), Winfree has now readily generated a DNA circuit with this many layers and the obvious capability to scale much farther (Qian and Winfree, 2011).

I therefore fantasize about a biological system that is based on a rational biological computer, akin to the strand exchange circuits. As tempting as it is to suggest we should tear down biology and start afresh, this is well beyond my capabilities, and thus even my fantasies must be instantiated incrementally. It is possible to envision how strand-exchange RNA circuits could be embedded in organisms. The outcome of such circuitry could be the production of particular miRNAs that would feed back on the system, in much the same way that Benenson and Weiss have nicely shown that logical miRNAs can be embedded in the current biological operating system (Rinaudo et al., 2007). In couple with a fully realized suite of genetically augmented proteins containing nucleobase amino acids, the possibilities for superimposing a rational operating system on the current irrational one is manifest (albeit still incremental).

In moving from current biological operating systems to a fanciful new operating system built on genetically augmented proteins and strand exchange reactions, I have always been sensitive to the nature of the operating system that we already have (even while attempting to remake it). This “feeling for the organism” (to steal a phrase from McClintock) is not stealth vitalism but rather a sense of how engineering must proceed from the materials at hand. In this regard, while engineers can certainly become biologists and vice versa, each brings their pre-conceptions to the table in a manner that should be explicitly recognized. From

the point of view of a biologist, biological circuits are utterly unlike electronic circuits, irrespective of the analogies that can be drawn (Simpson et al., 2009). Electronic circuits operate at light speed, with spatially defined interconnectivities. Biological circuits operate via chemical diffusion, with molecular recognition and reactivity defining interconnectivity. While software is independent of hardware, and while both types of circuits can make, say, an oscillator, that's no reason to suspect that both types of circuits will be able to operate in the same time regimes or with the same fidelity, since these features will stem in part from capabilities of the materials from which they are constructed. Thus, it may not be unreasonable to suggest that in engineering biology we must build from the capabilities of the materials involved, rather than trying to superimpose a foreign mindset—the current and likely transient *Zeitgeist* of synthetic biology—on those materials.

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A4

SYNTHETIC BIOLOGY—A NEW GENERATION OF BIOFILM BIOSENSORS

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Biofilms: A Survival Strategy

The notion that bacteria live an autonomous, independent, and planktonic lifestyle has been radically challenged with the realization of the abundance of bacterial communities known as biofilms (Hall-Stoodley et al., 2004). Biofilms are structured communities of adherent microorganisms encased in a self-produced complex extracellular polymeric substance (EPS) matrix as shown in Figure A4-1 (Costerton et al., 1999). The advent of microscopy techniques has revealed the complex nature of these structured communities, containing networks of channels for nutrient supply and organized three-dimensional structures. Moreover, bacteria within these biofilms are profoundly physiologically distinct from their planktonic counterparts, and they function in a coordinated manor as a cooperative consortium, more similar to that of multicellular organisms than a unicellular organism (Hall-Stoodley et al., 2004; Lindsay and von Holy, 2006; Parsek and Singh, 2003). The ability to form biofilms is almost ubiquitously found within the bacteria domain of life and indeed within many other classes of microorganisms, including fungi, yeasts, algae, and protoza (Van Houdt and Michiels, 2010). Evidence suggests that biofilm formation is an ancient ability,

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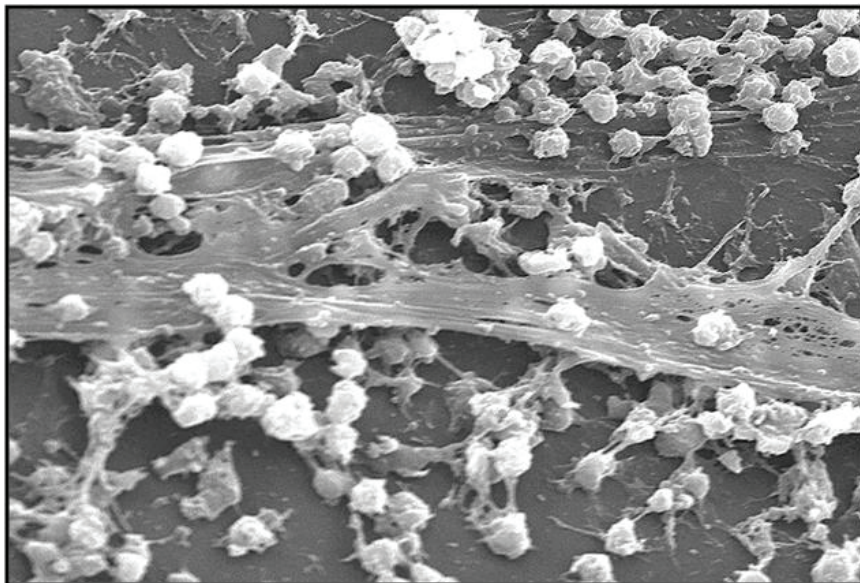


FIGURE A4-1 An electron micrograph of *Staphylococcus aureus* bacteria biofilms on the luminal surface of an indwelling catheter.

SOURCE: Image obtained from Public Health Image Library. Provided courtesy of R. Donlan, and J. Carr, Centers for Disease Control and Prevention. Photo by J. Carr (2005).

with biofilm microcolonies being identified in fossils from 3.3 to 3.4 billion years ago (Hall-Stoodley et al., 2004; Westall et al., 2001). Taken altogether, biofilm formation is an evolutionary conserved and widespread phenomenon.

The advantages of bacteria to abandon their independent lifestyle for that of a community is simply that it provides a more protective mode of growth. Biofilms allow bacteria to colonize and sustain favorable niches independent of larger environmental changes. For example, the EPS, a key component of biofilms, can serve as storage for nutrients and water, helping to stabilize local resources (Costerton et al., 1987; Donlan, 2002; Lindsay and von Holy, 2006). In addition, biofilms provide a defensive role, acting as a physical barrier from environmental dangers such as ultraviolet light, metal toxicity, acid exposure, dehydration, and even immune defenses such as phagocytosis (Donlan, 2002; Lindsay and von Holy, 2006; Parsek and Singh, 2003). These selective advantages of biofilm formation are obvious within the context of hostile and dynamic environments such as that of ancient Earth (Hall-Stoodley et al., 2004). Yet even in today's relatively temperate environment, the formation of biofilms is still selectively advantageous and constitutes a major component of bacterial biomass in natural environments (Costerton et al., 1978, 1999; Lindsay and von Holy, 2006). Bacteria's attempt

to survive by the formation of biofilms extends beyond just natural environments and provides bacteria protection in many man-made environments (Hall-Stoodley et al., 2004). However, the focus of this review is the presence of pathogenic bacterial biofilms within a clinical context.

Biofilms in a Clinical Context

It is only in recent years that the importance of biofilms in clinical settings as a source of pathogenic bacteria has been realised (Lindsay and von Holy, 2006). The persistence of biofilms in hospitals is undoubtedly a significant contribution to hospital-acquired infections or nosocomial infections (Costerton et al., 1999; Donlan and Costerton, 2002; Hall-Stoodley and Stoodley, 2009; Hall-Stoodley et al., 2004; Lindsay and von Holy, 2006). Indeed it has been estimated that biofilms contribute to 65 percent of nosocomial infections (Potera, 1999; Smith and Hunter, 2008). Table A4-1 shows the most frequent pathogenic strains associated with nosocomial infections, the most frequent and problematic being *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *S. epidermidis* (Costerton et al., 1999; Hall-Stoodley et al., 2004). Recent studies estimate that at any one time 9 percent of all in-patients in England and Wales (United Kingdom) suffer from hospital-acquired infections, resulting in approximately 5,000 deaths per year. The costs associated with these hospital-related infections for the U.K. National Health Service are estimated at £1 billion per year (Smith and Hunter, 2008).

TABLE A4-1 The Most Common Causes of Nosocomial Infections

Nosocomial Infections	
ICU pneumonia	Gram-negative rods
Sutures	<i>S. epidermidis</i> and <i>S. aureus</i>
Exit sites	<i>S. epidermidis</i> and <i>S. aureus</i>
Arteriovenous shunts	<i>S. epidermidis</i> and <i>S. aureus</i>
Scleral buckles	Gram-positive cocci
Contact lens	<i>P. aeruginosa</i> and Gram-positive cocci
Urinary catheter cystitis	<i>E. coli</i> and other Gram-negative rods
Peritoneal dialysis (CAPD) peritonitis	A variety of bacteria and fungi
IUDs	<i>Actinomyces israelii</i> and many others
Endotracheal tubes	A variety of bacteria and fungi
Hickman catheters	<i>S. epidermidis</i> and <i>Candida albicans</i>
Central venous catheters	<i>S. epidermidis</i> and others
Mechanical heart valves	<i>S. epidermidis</i> and <i>S. aureus</i>
Vascular grafts	Gram-positive cocci
Biliary stent blockage	A variety of enteric bacteria and fungi
Orthopedic devices	<i>S. epidermidis</i> and <i>S. aureus</i>
Penile prostheses	<i>S. epidermidis</i> and <i>S. aureus</i>

SOURCE: Adapted from Costerton et al. (1999).

Although many nonpathogenic bacteria are able to form innocuous biofilms, it is the formation of pathogenic biofilms that is particularly important for contributing to the high level of hospital-acquired infections. It has been suggested that biofilm formation is analogous to a virulence factor, where biofilm formation increases the likelihood of a pathogen causing an infection.

Three general mechanisms have been proposed to explain the increase of virulence seen for pathogenic bacteria in a biofilm microenvironment (Hall-Stoodley and Stoodley, 2005). These are the survival and transmission of planktonic patho-

BOX A4-1

The Mechanisms of Biofilm-Associated Virulence

Survival and Transmission of Pathogenic Biofilms

Clinical environments such as hospitals are constantly exposed to a consortium of pathogenic bacteria. Many pathogenic bacteria can be easily eradicated when in planktonic mode of growth by biocidal agents. However, if these bacteria form biofilms, they will become significantly more resistant to biocidal agents. Established biofilms can tolerate antimicrobial agents at concentrations of 10-1,000 times that needed to kill genetically equivalent planktonic bacteria (Gander, 1996; Gilbert et al., 2002; O'Toole, 2002; Smith and Hunter, 2008). This high level of resistance makes eradication of pathogenic biofilms in hospitals largely unsuccessful. Indeed, studies have shown pathogenic biofilms to be present on a number of medical surfaces, including indwelling medical devices such as catheters and prostheses (Stickler, 2002), hospital water systems, stethoscopes (Guinto et al., 2002), soap dispensors (Brooks et al., 2002), and other hospital equipment.

The presence of these pathogenic biofilms is problematic because they act as nodes of infection, seeding the regular release of large numbers of pathogenic cells, potentially exposing patients to infectious doses of these organisms (Hall-Stoodley and Stoodley, 2005; Hall-Stoodley et al., 2004). Biofilms have been shown to regularly release cells either by exposure to external mechanical forces, such as by shear forces, or by internal biofilm processes including enzymatic degradation of matrix, release of EPS, and surface binding proteins (Hall-Stoodley et al., 2004; Stoodley et al., 2002). Taken together, the high level of resistance and regular release of pathogenic cells and the presence of pathogenic biofilms increase the potential of a patient being exposed to an infectious dose of pathogenic cells.

Heterogeneity and Selection of Virulent Phenotype

The complex nature of biofilms is that they provide heterogeneous microenvironments within the biofilms. These distinct microenvironments provide different selective pressures and are occupied by genetically distinct populations. Genetic screening of microenvironments has suggested that the biofilm mode of growth accelerates genetic variation in the biofilm populations (Boles et al., 2004; Kirisits et al., 2005; Stewart and Franklin, 2008). It has been shown that *P. aeruginosa* undergoes accelerated genetic

gens from biofilms, the phenotypic heterogeneity of biofilm populations and the potential evolution of infectious phenotypes, and the role of biofilm formation in the regulation of virulence mechanisms. Each of these mechanisms is discussed further in Box A4-1.

The seriousness of this problem has provided strong motivations for developing new antibiofilm therapies and detection methods. The development of new antibiofilm therapies is beyond the scope of this paper, although there are a number of extensive reviews (Lynch and Abbanat, 2010). Here we focus on cur-

diversification in the biofilm mode of growth. This mechanism was dependent on the *recA* gene, a key protein involved in DNA recombination, suggesting that recombination contributes to this variation (Boles et al., 2004). These genetic variations lead to more pathogenic variants; for example, hyperbiofilm formation and antibiotic-resistant phenotypes have been shown to develop in *P. aeruginosa* biofilm models (Drenkard and Ausubel, 2002; Parsek and Singh, 2003).

Virulence Products

Genes contributing to the virulence and pathogenicity of bacterial strains are commonly regulated by the cell-to-cell signaling mechanism known as quorum sensing. Quorum sensing is a mechanism whereby bacteria release and sense small signaling molecules, termed autoinducers. These autoinducers accumulate as a population increases, and, once a sufficient "quorum" (population) is reached, gene expression is induced in a coordinated manner across a population (Fuqua et al., 1994). The advantage of this cooperation is that it allows bacteria to act in concert, but only once a sufficient population has been reached. In terms of pathogenic bacteria, it allows bacterial densities to increase before expression of virulence factors and other pathogenic factors that might trigger immune responses. In *P. aeruginosa*, quorum sensing has been shown to regulate the expression of genes encoding virulence factors, antibiotic resistances, motility, siderophore production, adhesions, cytotoxins, exotoxins, and type III secretion systems (Asad and Opal, 2008; Davies et al., 1998). Furthermore, the importance of quorum sensing for virulence has been confirmed in a number of animal models including mice, nematodes, and insects (Lesic et al., 2007; Mahajan-Miklos et al., 2000; Popat et al., 2008; Rumbaugh et al., 1999).

The relationship between quorum sensing and biofilms is one of positive feedback. It has also been shown that quorum sensing is required for biofilm formation. In *P. aeruginosa* it has been shown that mutants of quorum sensing transcription factors, LasR, produce flat, continuous, and sparse biofilms that had neither the density nor the complex channel structure seen in wild-type biofilms. In addition these mutant strain biofilms lose resistance to the biocide agent sodium dodecyl sulfate (SDS) (Davies et al., 1998). This evidence suggests a key role of quorum sensing in the formation of biofilms and regulation of virulence. Biofilms provide a niche that allows the cell population to increase and sufficient autoinducers to accumulate to thus trigger the expression of genes involved in virulence and pathogenicity (Asad and Opal, 2008; Hall-Stoodley and Stoodley, 2005).

rent methods for biofilm detection, as well as their limitations, and the prospect of synthetic biology to develop completely novel methods for the detection of pathogenic biofilms.

Current Methods of Biofilm Detection

The ability of biofilms to form on a variety of abiotic and biotic surfaces has resulted in colonization of many clinical environments. Detection of these biofilms has obvious advantages: to direct suitable sanitation protocols in biofilm niches, to indicate replacement of any contaminated medical devices such as indwelling catheters, and to direct suitable medication if patient infection ensues. It has now become common practice in hospitals to sample both environmental surfaces and patients, typically urine, for the presence of pathogenic biofilms. There are numerous methods used for detection, including conventional plate counting, phenotypic screens, microscopy methods, and genotypic methods. Two of the most established methods for biofilm detection used in laboratories and clinical settings are the genotypic and phenotypic methods that are described in more detail below.

Phenotypic methods rely on culturing bacterial samples and screening these cultures for the phenotype of biofilm producers. These methods rely on the hypothesis that biofilm formation is a marker of virulence. Three variants of phenotypic detection have been commonly used: the tissue culture plate, the tube method, and the Congo red agar (CRA) method. These methods rely on indirectly assessing biofilm producers by accumulation of biofilm components such as EPS, a polysaccharide involved in cell-cell adhesion and essential for biofilm formation.

Genotypic methods are based upon PCR amplification of specific genetic markers associated with pathogenic biofilms. The most notable example is the use of the *ica* cluster to identify biofilm-producing strains of *Staphylococcus aureus* and *S. epidermidis*. The *ica* cluster contains genes that are essential for these strains to produce EPS. In addition, PCR is commonly used to assess antibiotic resistance; for instance, the presence of the *mecA* gene encoding methicillin resistance can be used to identify strains of methicillin-resistant *S. aureus*.

Each of these methods has its own advantages and limitations. The major limitations of these are described below:

- **Expertise and cost.** Although techniques such as PCR and microscopy techniques have become commonplace in molecular biology laboratories, in hospitals, particularly in developing countries, access to the equipment and expertise required for analysis can be limited (Bose, 2009).
- **In situ and point-of-care detection.** Sampling of biofilms on hospital surfaces can be abrasive to attached cells and may result in damage to

cells, rendering them nonculturable (Lindsay and von Holy, 2006). In addition, sampling of indwelling medical devices such as catheters requires the removal of catheters, whether a biofilm is present or not. This can result in unnecessary stress for patients and wastefulness of such devices (Donlan and Costerton, 2002). Finally, the recovery efficiency of sampling methods commonly used is largely unknown, leaving the possibility of not detecting a representative population.

- **Reliability and reproducibility.** The genotypic methods are generally considered more reliable and sensitive. However, they can be subject to false positives. This is due to the *ica* cluster not being absolutely required for biofilm formation. It has been shown that polysaccharide intercellular adhesin (PIA) synthesis from the *ica* operon alone is not sufficient to produce biofilms and that biofilms can form without producing PIA in staphylococci (Chokr et al., 2006).

For the phenotypic detection methods there is limited sensitivity, reliability, and reproducibility compared to genetic detection. There have been numerous independent studies comparing the different phenotypic methods, which have shown varying success rates using the different phenotypic methods. For example, studies assessing the sensitivities of the CRA method have shown a detection success ranging from 5.26 to 89 percent (Arciola et al., 2006; Bose, 2009; Knobloch et al., 2002; Mathur et al., 2006; Oliveira and Cunha, 2010). In addition, the phenotypic methods are commonly prone to false positives and nondetection of “weak” biofilm-forming strains.

- **Time of response.** The phenotypic detection methods rely upon the culturing of microorganisms collected from samples. These incubation periods are typically 24 to 48 hours and cultures are often analyzed offsite, increasing time for biofilms to develop (Bose and Ghosh, 2011).
- **Informative.** Neither the genotypic nor the phenotypic methods elicit an in situ biofilm structure. Although detection of the pathogenic biofilms indicates the potential for the presence of biofilms, it is not an accurate reflection of the actual microenvironment. This can lead to an inaccurate detection in the in situ environment and false diagnosis.

Whole-Cell Biosensors: Bridging the Detection Gap

As discussed above there is a strong motivation for the development of in situ detection methods with increased response time, specificity, and more informative outputs. A potential solution is the development of whole-cell biosensors, using genetically engineered biological cells to directly detect pathogenic biofilms. An essential ability for cells to function is the ability to sense and respond to a diverse range of environmental signals from environmental conditions such as temperature change to the detection of nutrients in the environment. This ability can be

broken down to two subfunctions, that of sensing and that of response (signal transduction). Whole-cell biosensors are essentially composed of the same two elements integrated into microorganisms: sensing elements detecting the analyte of interest and transducing elements that are usually coupled to the production of a detectable output, for example, expression of reporter proteins such as natural fluorescent proteins.

Some of the advantages of whole-cell biosensors are that they detect a variety of biological, organic, and nonorganic compounds. In addition, they can produce a number of detectable outputs that can be easily quantifiable, for example, fluorescent proteins measured by a fluorometer, dyes and pigments measured by spectroscopy and by eye, and electrochemical signals such as pH measured by litmus paper or a pH meter. These easily measured outputs require little expertise to use or to interpret. In addition, the timescale for biosensors is generally rapid, with signals being transduced in seconds to generate detectable signals within minutes to hours. In addition, compared to other *in vitro* biosensors like widely used enzymes or antibodies, whole-cell biosensors are easily produced by cell culture, in contrast to the costly and time-consuming purification of enzymes and antibodies.

Another advantage of whole-cell biosensors can be highlighted using the example of detecting pathogenic biofilms. Genotypic methods detect at a microscopic level the DNA genetic elements that are required for the formation of biofilms rather than the products of these genes, which can therefore lead to false positives. Phenotypic detection detects the products of biofilms but at a macroscopic level. Whole-cell biosensors offer a way to bridge the gap between these two methods, allowing sensing and evaluation at the level of microbial habitats. Whole-cell biosensors could allow the detection of new markers of biofilms to provide more effective detection methods. In recent years, there has been increased interest in the development of whole-cell biosensors, particularly within the field of synthetic biology. In the remainder of this review, the potential of synthetic biology for creating more effective biosensors for the detection of pathogenic biofilms is discussed.

Synthetic Biology: A New Generation of Biofilm Biosensors?

Synthetic biology is an application-driven field attempting to apply a rational engineering approach to the redesign of biological systems, to produce valuable and novel biological functions. Synthetic biology can be thought of as a natural evolution of biotechnology as opposed to being a separate field, although it does offer a novel and exciting approach. In the past 40 years biotechnology has undoubtedly produced many valuable biological products, yet current approaches tend to be lengthy due to the ad hoc nature of the approach. In addition, there is little lateral transfer of knowledge between projects, meaning knowledge gained in research and development in one area may not assist the post hoc development of projects in other, unrelated areas.

Synthetic biology aims to move away from this ad hoc design by providing a conceptual framework based on systematic design and rational engineering, so that redesigning biological systems would become more equivalent to that of redesigning a plane or a car. Indeed, it is the adoption of an engineering framework that has revolutionized the design and output of applications in the fields of mechanical, electrical, civil, and aeronautic engineering, and it is proposed that such an approach will do the same for biotechnology. The focus of this paper is to discuss the potential of whole-cell biosensors and the synthetic biology approach both in general and for the specific application of detection of pathogenic biofilms, the need of which was highlighted earlier.

Three major advantages of the synthetic biology approach for biosensor designs are highlighted: the advantages of the engineering approach, the development of novel sensing elements, and the complexity synthetic biology could enable.

Putting the Engineering into Genetic Engineering

Modern engineering relies on designing systems from catalogues of well-defined standard parts and higher-level devices that can be assembled into larger systems in a standardized manner. For electrical engineers, catalogues such as RS Components and Farnell provide well-defined parts and devices, each associated with data sheets of the functional characteristics. System designs can then be assessed by applying mathematical models in combination with known behavior characteristics, to computationally simulate their overall functions. This allows rapid assessment *in silico* of designs that meet the required specifications of design. Through iterations of this engineering approach or engineering cycle, efficient production of well-characterized and robust end products, from electronics to buildings, can be produced.

Synthetic biology is trying to adopt this engineering cycle and develop the tools required for high-throughput engineering of biological systems (Figure A4-2) (Gulati et al., 2009; Kitney et al., 2007). However, living systems are not computer chips and thus it is important to note that synthetic biology aims to provide a conceptual engineering approach that will allow biological engineering to be easier and more predictable. Living systems are intricate, formed from a complex network of interacting chemical components driven by the ability to self-replicate, adapt, and survive. Therefore, in living systems, context dependency and stochastic behavior are common features and therefore the engineering of predictable new cellular systems with defined functions is challenging. Nevertheless, the field of synthetic biology aims to provide a series of foundational technologies and a defined framework that will enable robust biological design and overcome these clear challenges. At present much emphasis in synthetic biology is focused on engineering single-cell organisms like *Escherichia coli* or yeast, given that our understanding of these organisms is more mature than for

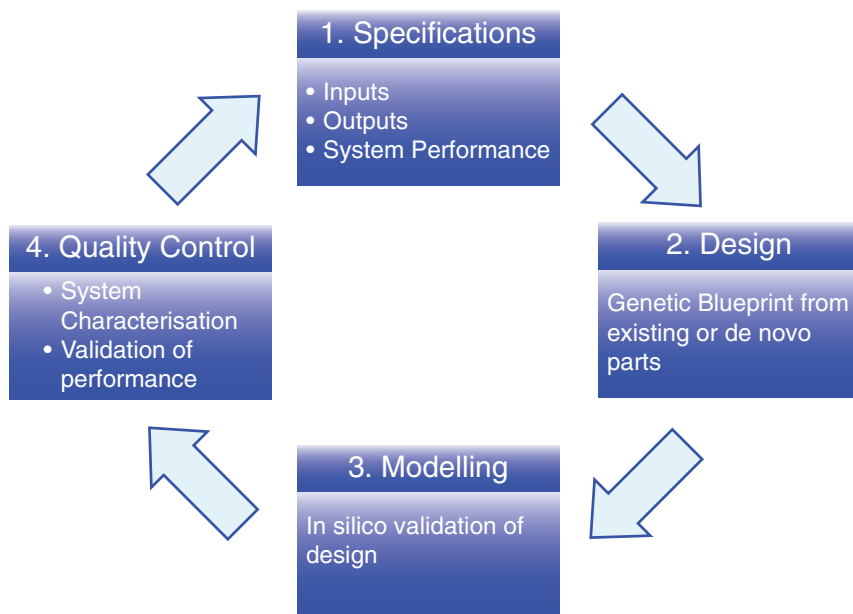


FIGURE A4-2 The engineering cycle as an approach for synthetic biology. This adaption includes the four stages of defining specifications, design of biological devices, in silico modeling of the potential designs, and characterisation of performances.

multicellular systems. With this in mind, a synthetic biology engineering cycle is discussed below.

Engineering cycle

1. *Specification* The first stage in the engineering cycle is to define the specifications required. For example, a previous whole-cell biosensor designed to detect arsenic in polluted water defined the detection range as defined by the World Health Organization recommendation of safe drinking water at 10 ppb arsenic. Other specifications can be tunable, for example, threshold, time of response, particular microbial organisms, or host chassis to be used.
2. *Design* Once the specifications have been defined, genetic devices have to be designed to give the functions of interest to host cells, often microbes. For the design of a genetic device, the principle of abstraction is applied to help separate out unnecessary layers of complexity (Endy, 2005). At the most complex layer, the design of a biological device involves the writing of a DNA nucleotide sequence to give desired func-

tion when implanted in a cell. However, an abstract view can be taken and these sequences modularized into functional parts such as promoters, gene coding sequences, ribosome binding sites, and terminators. These modules of DNA allow the functional separation of biological parts (BioParts) and allow a higher level of design, where the underlying complexity of the DNA sequence can be hidden (Endy, 2005).

Collaborative and international efforts have been made to make open-source registries to document and physically store a huge variety of BioParts and devices, most notably being the BioBricks registry (<http://partsregistry.org>) and the International Open Facility Advancing Biotechnology (BIOFAB). Two important criteria of BioParts in these registries are the adherence of standards and the documentation of characterisation data. The BioBricks foundation has proposed a “request for comment” process, encouraging the synthetic biology community to list standards for physically joining DNA and protocols such as for characterisation of BioParts.

These catalogues are beginning to provide a huge toolbox of biological parts for the engineering of biological systems, from basic BioParts such as promoters, ribosome binding sites, genes, and terminators to more complex composite devices such as toggle switches, oscillators, logic gates, and cell-to-cell communication systems. Moreover, the standards required for submission to these registries ensure compatibility and interchangeability. For example, the BioBricks registry has defined standard restriction sites flanking all parts and devices, which has allowed standardized methods of assembly where any two parts can be combined by following use of a standardized protocol. Finally, the documentation of characterisation data allows transfer of knowledge and experience gained from separate projects to be used in the evaluation of potential designs (Arkin, 2008).

3. *Modeling* After the design stage, *in silico* computational simulation of mathematical models representative of the genetic devices is performed. In the 1960s the mathematical logic in gene regulation of the *lac* operon was demonstrated (Andrianantoandro et al., 2006; Monod and Jacob, 1961). Since then, systems biology has transformed our understanding of biology using representative mathematical models based on quantitative data, most notably stochastic and ordinary differential equations. These modeling techniques, coupled with the use of experimentally defined characteristics defining model parameters, can predict the functional characteristics of a design without the need to undergo cloning or *de novo* synthesis, or experimental characterisation both of which increase overall time and cost of development. Ellis et al. (2009) demonstrated the use of computational modeling to predict the behavior of feedforward loops in yeast. To do so, a library of inducible promoters

with known minimum and maximum outputs (expression levels) were used in the device design. The characterisation data were incorporated into the modeling, the predictions of which were shown to accurately reflect the experimentally derived behaviors of these devices (Ellis et al., 2009).

4. *Quality control* The idea that a device's behavior can be fully predicted by modeling the known characteristics of individual parts is obviously an oversimplification. It is inevitable that unexpected emergent properties will result when individual parts are put together that have been characterised in isolation and different contexts such as a switch in host chassis of *E. coli* to *Bacillus subtilis* (Arkin, 2008; Serrano, 2007). Unexpected interactions can occur from the context of the other BioParts in the device or even the host chassis. For example, in mRNA the untranslated regions of promoters can form secondary structures with ribosome binding sites of mRNA sequences modifying the function of this BioPart (Arkin, 2008).

These factors necessitate a quality control step, first to assess how well the device meets the specification but also to gain a greater understanding of the device being designed. Although approaches such as BioPart characterisation and modeling might not give an absolute prediction of device function as seen in other fields such as electronics, it nonetheless reduces the number of potential designs that need to be explored and therefore increases the overall efficiency.

Novel Biosensor Targets

As mentioned earlier, biosensors are composed of two subfunctions, that of sensing and that of signal transduction. The example of quorum sensing has already been discussed, where bacteria sense the local population densities based on the concentration of autoinducers and, once a threshold is reached, gene expression is induced. In this example, transcription factors act as the sensing elements binding to autoinducers and the quorum-sensing responsive promoters act as signal-transducer elements, defining the threshold of response and converting the autoinducer concentration to transcriptional output.

The bacterial toolbox of sensing elements is mostly mRNA and protein based, both of which can form complex tertiary structures able to bind a myriad of targets. Coupled to these sensing elements, the signal-transducing elements are generally transcriptional, translational, and posttranslational. Sensing modules and transducer modules can be separate biological components such as in quorum sensing, or within the same biological molecule such as riboswitches that contain an mRNA aptamer element able to sense and convert this sensing to translational output by sequestering or releasing a proximal ribosome binding site.

When considering the design of biosensors, it is essential to identify both

the sensing and the transducing elements. In general, synthetic biologists have employed three strategies to use these elements: first, to import exogenous sensing elements into new biological host chassis; second, to use and rewire existing endogenous sensing elements to detect new signals; and finally, in the production of novel elements not found in nature.

Three examples are described in Box A4-2 to explain each of these strategies. In addition, each example provides an illustration of biosensors functioning at the level of transcription, translation, and posttranslation.

Increasing Complexity

Within the field of synthetic biology, a variety of synthetic genetic devices have been developed. Many of these devices have been inspired by those found in electronics and include toggle switches (Atkinson et al., 2003; Bayer and Smolke, 2005; Deans et al., 2007; Dueber et al., 2003; Friedland et al., 2009; Gardner et al., 2000; Ham et al., 2006, 2008; Kramer and Fussenegger, 2005; Kramer et al., 2004), memory elements (Ajo-Franklin et al., 2007; Basu et al., 2004; Friedland et al., 2009; Ham et al., 2006, 2008), pulse generators (Basu et al., 2004), time-delayed circuits (Ellis et al., 2009; Weber et al., 2007), oscillators (Atkinson et al., 2003; Danino et al., 2010; Elowitz and Leibler, 2000; Fung et al., 2005; Stricker et al., 2008; Tiggles et al., 2009), and logic gates (Anderson et al., 2007; Guet et al., 2002; Rackham and Chin, 2005; Rinaudo et al., 2007; Win and Smolke, 2008). These synthetic genetic devices are aiding the design of more complex biological systems the likes of which have not been previously seen. For the application of whole-cell biosensors, the implementation of these genetic devices could allow more complex and informative biosensors to be developed. For example, consider the use of logic gates for biosensor design. Logic gates are devices that perform a logical operation based on one or more inputs to produce a signal output. A frequently featured logic gate implemented in synthetic biology is the AND gate. An AND gate integrates two or more signals and will only give an output when all inputs are present. The principle and a biological example of an AND gate is shown in Figure A4-3.

The example of logic gates highlights the potential complexity of design that synthetic biology could offer for biosensor design. In general, multiple signal integration would allow fewer false positives by relying on more than one signal for detection and thus increasing the reliability. For example, the use of NOT gates could be used to remove known signals contributing to false positives. In addition, by being able to integrate multiple signals, logic gates could offer more complex and informative detection.

A more relevant illustration with regard to developing biosensors for detecting biofilms can be highlighted when we take into account mixed-species biofilms. Consider the case of biofilms on indwelling urinary catheters. A study of 106 biofilms found on catheters showed 14 species of bacteria to be commonly

BOX A4-2 Strategies and Examples of Biosensor Design

Transcriptional Control Using Exogenous Sensing Elements

Aleksic et al. (2007) engineered *E. coli* to detect toxic arsenic levels in drinking water, which today is a major pollutant of water in developing countries such as Bangladesh and West Bengal. To do so, exogenous transcription factors and promoters were cloned from *B. subtilis* into *E. coli* and coupled to the production of the enzyme urease, the production of which can be monitored by changes in pH. The *arsR* transcription factor and *ars* promoter were taken from *B. subtilis* (Sato and Kobayashi, 1998). In the absence of arsenic, the *arsR* represses transcription of the *ars* promoter. Upon binding of arsenic, the *arsR* repression is relieved, allowing the transcription of the chosen output. In this case a urease mRNA was the direct output that, once transcribed, is constitutively translated into the urease protein detectable by changes in pH.

The ability to import exogenous transcription factors and promoter regions between species of bacteria is relatively straightforward in theory because of the conserved mode of action of these transcription factors and conserved promoters. However, other sensing elements such as membrane protein receptors can be imported between strains, although, due to the varying nature of membranes in Gram-positive and Gram-negative species, it is only possible within these species groups. A major advantage of this approach is that it allows sensors found in strains that cannot easily be cultured to be incorporated into more commonly and well-defined strains such as *E. coli*. This is particularly advantageous when searching and identifying sensors from extremophiles that are often difficult to culture and would be impractical as whole-cell biosensors.

Translational Control Using De Novo Sensing Elements

Sinha et al. (2010) demonstrated that *E. coli* could be engineered to sense, follow, and destroy a commonly used herbicide, atrazine. To do so, de novo sensing elements had to be designed and constructed. They used the in vitro selection method, SELEX, to identify sequences of RNA that bound atrazine specifically and tightly. After identifying these RNA sequences, riboswitches were designed. Riboswitches are mRNA sequences that contain aptamer sequences that can sequester or release proximal

present (Macleod and Stickler, 2007; Stickler, 2008). Furthermore, although single-species biofilms were found, most were mixed-species biofilms—most frequently containing *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*. With the use of logic gates and multiple signal integration, synthetic biology could develop biosensors to respond not only to multiple targets but also with the ability to distinguish between species. This would allow general detection of biofilms as well as species-dependent detection that could aid correct treatment should the infection worsen.

ribosome binding site sequences upon binding or absence of a target. This allows sensing to be coupled to the translational control of any coding sequence placed downstream of the riboswitch. The atrazine riboswitch was designed so that, in the absence of atrazine, ribosome binding sites were sequestered by internal binding of proximal mRNA sequences, and in the presence and binding of atrazine, these ribosome binding sites were released, allowing translation of an output protein. In this example, the output protein was CheZ, a protein that controls motility in *E. coli*.

The design of de novo recognition elements allows the expansion of the natural "toolbox" of sensing elements available. New previously undetectable targets could be detected. This is particularly relevant for many of today's environments, where the accumulation of man-made compounds and pollutants is relatively recent and for which nature has not evolved any response or sensing mechanisms.

Posttranslational Control Using Rewired Endogenous Sensing Elements

Baumgartner et al. (1994) rewired two transmembrane signaling pathways to create a hybrid signaling pathway. A hybrid transmembrane protein was created from the sensing domains of the Trg chemoreceptor and the transducing domains (kinase/phosphatase domains) of the EnvZ osmosensor. This engineered signaling pathway allowed the sensing of the sugar-occupied ribose-binding protein in the periplasmic domain and transduction of the EnvZ kinase and phosphatase domains, triggering a cascade of signaling proteins and ultimately expression of an OmpC-LacZ fusion protein. This hybrid signaling system was further engineered by Looger et al. (2003), who computationally designed artificial periplasmic binding proteins to sense novel targets such as TNT.

The potential advantage of posttranslational biosensors is that they offer a more rapid and precise biosensor. The transduction of signals by protein-protein interactions can be transduced in microseconds and are less affected by stochastic noise, a major cause of variation in transcriptional and translational biosensors (Legewie et al., 2008; Marchisio and Rudolf, 2011; Yu et al., 2008). In addition, although design principles of signal transduction are still being elicited, studies of natural pathways have shown that optimal design principles can produce high-fidelity signals, and characteristics such as latency phases after signaling can be produced (Colman-Lerner et al., 2005; Legewie et al., 2008).

Summary

Synthetic biology is a newly developing field that aims to provide an engineering framework for the predictable construction of new biological systems. The most immediate exemplars relate to biosensors where synthetic biology has already produced several living cell biosensors that act robustly and predictably. The application of synthetic biology design tools will enable the development of a new generation of biosensors to detect pathogenic biofilms. The implementation of these designs will have a profound impact in many areas including the control of hospital-acquired infections.

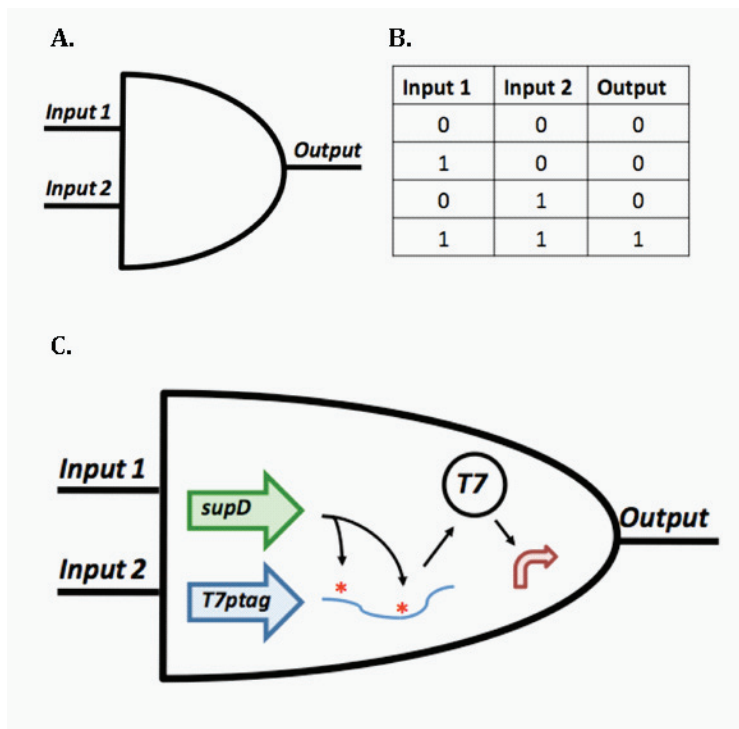


FIGURE A4-3 The principle and biological example of an AND gate (A) The symbolic representation of an AND gate and (B) a truth table describing the logic of an AND gate with regard to two inputs. Only when both inputs are present is an output produced. (C) An example of a biological AND gate previously described by Anderson et al. (2007). The AND gate is based around the interplay between two genes. One of these genes is the T7 RNA polymerase containing two amber stop codons (denoted by asterisks), meaning that translation of full-length T7 polymerase is inhibited (T7ptag). Only when the *supD* gene is transcribed are the amber stop codons translated into serines allowing full-length T7 polymerase to be translated and drive expression of the output from a T7 polymerase regulated promoter. This example represents an AND gate taking two transcriptional inputs and integrating these to a single transcriptional output.

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A5

SYNTHETIC BIOLOGY AND THE ART OF BIOSENSOR DESIGN

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Introduction

The term “biosensor” refers to a wide variety of devices. The common element is that a biological component provides highly specific recognition of a certain target analyte, and this detection event is somehow transduced to give an easily detectable, quantifiable response, preferably one that can be easily converted to an electrical signal so that the result can be fed to an electronic device

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for signal processing, data storage, etc. The biological component in many biosensors is either an enzyme, as in the glucose-oxidase-based biosensors used for blood glucose monitoring, or an antibody, as in most optical biosensors. Another class of biosensor, sometimes also referred to as a bioreporter, uses living cells as a component. These cells detect the target analyte via some more-or-less specific receptor and generate a detectable response, most commonly by induction of a reporter gene. Many such devices have been reported in the scientific literature, with detection of mercury and arsenic in the environment being particularly common applications. However, very few such devices are commercially available, the best-known examples being the mutagen-detecting devices such as the SOS-Chromotest (Environmental Bio-Detection Products, Inc.) system. Here we discuss the reasons for this gap between promise and delivery, and ways in which the emerging discipline of synthetic biology may lead to a new generation of whole-cell biosensors.

Whole-Cell Biosensors

Whole-cell biosensors, or bioreporters, are living cells that indicate the presence of a target analyte. The most commercially successful by far are nonspecific toxicity sensors based on naturally occurring luminescent bacteria such as *Vibrio harveyi*, *Vibrio (Photobacterium) fischeri*, and *Photobacterium phosphoreum*. Examples include MicroTox (Strategic Diagnostics, SDIX) and BioTox (Aboatox). In these mainly marine organisms, above a certain population density, light is produced continuously by the action of bacterial luciferase (LuxAB), which oxidizes a long-chain aldehyde such as tetradecanal in the presence of FMNH₂ and oxygen. Regeneration of the reduced flavin (catalyzed by LuxG) and the aldehyde substrate (catalyzed by LuxCDE) requires NADPH and ATP, so any toxic substance that interferes with metabolism will reduce light emission, which is easily detected using a luminometer.

However, these systems are nonspecific and are only useful for preliminary screening of environmental samples to determine whether or not a toxic substance is present. The potentially more useful class of bioreporter consists of genetically modified microorganisms in which the presence of a specific target analyte is linked to a detectable response. The genetic modification involved in these cases consists of linking the receptor for the target analyte to induction of an easily detectable reporter gene. Commonly used reporter genes are shown in Table A5-1. For recent reviews of such systems, see Belkin (2003), Daunert et al. (2000), Tecon and van der Meer (2008), and van der Meer and Belkin (2010).

Synthetic Biology and Whole-Cell Biosensors

Like “biosensor,” the term “synthetic biology” is widely used by different authors to mean different things. In this context, we are using it to refer to a

TABLE A5-1 Reporter Genes Commonly Used in Whole-Cell Biosensors

Reporter	Characteristics
<i>lacZ</i> (β -galactosidase)	Chromogenic (X-gal, <i>o</i> -nitrophenyl galactoside) and chemiluminescent substrates are available. In <i>E. coli</i> host strains with the <i>lacZ</i> Δ <i>M15</i> mutation, only a small peptide representing the missing N-terminus, designated <i>lacZ'</i> α , is required.
<i>luxAB</i> (bacterial luciferase)	Blue bioluminescence in the presence of added substrate (a long-chain aldehyde, usually decanal).
<i>luxCDABE</i> (bacterial luciferase)	As above; presence of <i>luxCDE</i> allows biosynthesis of the substrate so that it need not be added to the reaction.
Firefly or click-beetle luciferase	Bioluminescence in the presence of added substrate (D-luciferin). Quantum yield is higher than for bacterial luciferase, but the substrate is much more expensive. Luminescence is normally green, but color variants are now available.
Fluorescent proteins	Fluorescence when stimulated by ultraviolet or visible light. The original green fluorescent protein (GFP), still widely used, is stimulated best by ultraviolet; enhanced green fluorescent protein responds well to blue light, and numerous color variants are now available.

systematic approach to rationalizing genetic modification to make it more like other engineering disciplines, in terms of the use of standardized parts that can be assembled in a modular way to make a variety of different constructs. Since whole-cell biosensors are intrinsically modular, consisting of a recognition element coupled to an arbitrarily chosen reporter, synthetic biology seems well suited to the development of such devices.

This approach to synthetic biology is associated particularly with MIT, BioBricks, the Registry of Standard Biological Parts,³⁶ and iGEM (the International Genetically Engineered Machine competition).³⁷ BioBricks (Knight, 2003) are a type of standardized biological “part,” consisting of pieces of DNA which conform to a certain standard (defined by a document known as RFC10, available

³⁶ See http://partsregistry.org/Main_Page.

³⁷ For more information, see the following: iGEM 2006, University of Edinburgh, http://parts.mit.edu/wiki/index.php/University_of_Edinburgh_2006; iGEM 2007, University of Cambridge, <http://parts.mit.edu/iGEM07/index.php/Cambridge>; iGEM 2007, University of Glasgow, <http://parts.mit.edu/igem07/index.php/Glasgow>; iGEM 2007, University of Science and Technology, China, <http://parts.mit.edu/igem07/index.php/USTC>; iGEM 2008, Harvard University, <http://2008.igem.org/Team:Harvard>; iGEM 2009, University of Cambridge, <http://2009.igem.org/Team:Cambridge>; iGEM 2010, Bristol Centre for Complexity Studies, <http://2010.igem.org/Team:BCCS-Bristol>; iGEM 2010, Imperial College, London, http://2010.igem.org/Team:Imperial_College_London; and iGEM 2010, Peking University, <http://2010.igem.org/Team:Peking>.

from the BioBricks Foundation³⁸) specifying certain characteristics of the ends. Each BioBrick may be a protein-coding region, or some other component such as a promoter, ribosome binding site, transcription termination sequence, or any other piece of DNA that may be useful in making genetic constructs. The essential point of this format is that any BioBrick can, through a standardized procedure, be combined with any other BioBrick to form a new BioBrick, which can then be combined with any other BioBrick, and so on. In this way, quite large and complex constructs can be built up fairly quickly. The Registry of Standard Biological Parts, currently hosted at MIT, was established to store both the DNA of these parts and also associated information such as DNA sequence, performance characteristics, and user experience. The intention was, and is, that this library of BioBricks and associated information should become a valuable resource for synthetic biologists.

To demonstrate the potential of this approach to synthetic biology, the iGEM competition was established in 2005. Each year, interdisciplinary teams of undergraduates consisting of a mixture of biologists, engineers, and computer scientists compete over the summer vacation period to conceive, design, mathematically model, construct, and test novel genetically modified systems made using BioBricks from the Registry, as well as new BioBricks created specifically for the project. All new BioBricks made are deposited in the Registry and are available for use by future teams. iGEM projects, completed on a short timescale by undergraduate students, are generally not nearly as well characterized as systems reported in the peer-reviewed literature; however, they are often based on highly creative ideas and generally include a mathematical modeling component far in excess that usually found in biological publications. They can therefore be a very interesting way to follow possible future application areas in synthetic biology.

Since biosensors are conceptually simple devices with a clear real-world application, and many opportunities for elaboration in terms of novel input and output modalities, in vivo signal processing, and other aspects, they are a popular choice of project, and a number of interesting innovations have been reported as a result of iGEM projects. We refer to a number of these later in this report. All information relating to previous iGEM projects is available via the relevant websites.

Arsenic Biosensors

Arsenic is a particularly attractive target for whole-cell biosensors, in that it is a major groundwater contaminant in Bangladesh, West Bengal, and a number of other regions (Meharg, 2005; Smith et al., 2000). This only came to light in the 1980s, and it is a major and increasing public health issue. The problem initially arose when, to combat waterborne diarrheal diseases caused by consumption of contaminated surface water, nongovernmental organizations drilled some millions of tube wells to supply clean drinking water. Unexpectedly, it was

³⁸ See <http://www.biobricks.org/>.

discovered some years later that many of these produced water with unacceptably high levels of arsenic. The current recommended World Health Organization (WHO) limit for drinking water is 10 ppb arsenic, while Bangladesh and some other countries maintain an earlier limit of 50 ppb, but many wells exceed this by a large margin. Chronic consumption of water with high arsenic concentrations leads to arsenicosis, resulting in skin lesions and various cancers. Thus, there is a clear and present need for cheap and simple tests for monitoring arsenic levels in drinking water. Current field test kits are based around the Gutzeit method, which involves reduction of arsenate to toxic arsine gas, and the detection of this as a color spot following reaction with mercuric salts. Such tests reportedly are unreliable at low but still significant arsenic concentrations, and disposal of mercuric salts poses its own environmental issues. Thus there is a clear potential niche for a simple arsenic biosensor device (Diesel et al., 2009; M. Owens, Engineers Without Borders, personal communication).

Most early arsenic biosensors were based on the arsenic detoxification operons of *Staphylococcus* plasmid pI258 and *Escherichia coli* plasmid R773. The former consists simply of the *ars* promoter controlling genes *arsR*, *arsB*, and *arsC*, encoding the repressor, arsenite efflux pump, and arsenate reductase, respectively, whereas the latter has a relatively complex structure, *arsRDABC*, with two separate repressors, ArsR and ArsD, which control the operon with different affinities (Oremland and Stolz, 2003). ArsD is also reported to act as a metallochaperone, carrying arsenite to the efflux pump formed by ArsAB (Lin et al., 2006). Later systems were based on the simpler *E. coli* chromosomal arsenic detoxification operon (Cai and DuBow, 1996; Diorio et al., 1995), which consists of the *ars* promoter followed by *arsR*, *arsB*, and *arsC*, and the similar operon of *Bacillus subtilis* (Sato and Kobayashi, 1998), which is discussed further below. In either case, the preparation of the biosensor organism is straightforward—the reporter gene is simply inserted adjacent to the controlled promoter so that induction of the promoter results in expression of the reporter gene, giving an easily detectable signal (usually a color change, luminescence, or fluorescence). A number of systems are reportedly at or near commercialization; for example, the Aboatox BioTox Heavy Metal Assay kits, developed at the University of Turku, use *E. coli* as host, with firefly luciferase as the reporter gene. Stocker et al. (2003) described production of a set of *E. coli*-based arsenic bioreporters using β -galactosidase with a chromogenic substrate, bacterial luciferase, or green fluorescent protein (GFP) as reporter; one of these, based on the luciferase reporter gene, has been field tested in Vietnam (Trang et al., 2005) and was reported to give good results in comparison to chemical field tests. Whole-cell arsenic biosensors have been reviewed recently by Diesel et al. (2009).

The Edinburgh Arsenic Biosensor

One example which we consider in some detail is the first biosensor project to be submitted to iGEM: the arsenic biosensor submitted for iGEM 2006 by the

team from the University of Edinburgh, under the supervision of C. French and A. Elfick. (K. de Mora was a student member of this iGEM team.) The intention was to develop a device that would be suitable for field use in developing countries. It should therefore be cheap, simple to use, and should deliver an output that could be assessed by eye, without requiring expensive electrical equipment, but should also give a quantifiable response using cheap equipment where this was required. For this reason the standard reporters based on luminescence and fluorescence were not considered appropriate. Instead, it was decided that the output should be in the form of a pH change. This would give a bright and easily assessed visible response using a pH indicator chemical, and it could also give a quantitative electrical response using a cheap glass pH electrode or similar solid-state device (ISFET).

For practical reasons, it was decided to use a standard laboratory host strain of *Escherichia coli*. Such organisms are easy to manipulate, carry multiple disabling mutations that make them harmless to humans and unable to propagate in the environment, and grow well at temperatures up to 45°C. *E. coli* and related organisms naturally ferment a variety of sugars, including lactose, via the mixed acid pathway, resulting in the production of acetic, lactic, and succinic acids, which can rapidly lower the pH of the medium below 4.5. Many laboratory strains carry a deletion in the gene *lacZ*, encoding β -galactosidase, the initial enzyme of lactose degradation, so that a nonfunctional truncated LacZ protein, missing the first few amino acids, is produced. This can be complemented by a short peptide known as the alpha peptide, consisting of the first 50 to 70 amino acids of LacZ. This is encoded by a short open reading frame known as *lacZ'* α . Thus, to generate an acid response, it was only necessary to use such a lactose-defective host strain, such as *E. coli* JM109, and place the *lacZ'* α gene under the control of the *ars* promoter in a standard Registry multicopy plasmid, pSB1A2. Thus, in the presence of arsenate or arsenite, expression of the LacZ alpha peptide would be induced, complementing the truncated LacZ and allowing rapid fermentation of lactose to acids, lowering the pH. To generate an alkaline response, the urease genes, *ureABC*, of *Bacillus subtilis* (Kim et al., 2005) were chosen. (Uropathogenic strains of *E. coli* also possess urease genes, but these are longer and more complex than those of *B. subtilis*.) Expression of these genes allows conversion of urea to ammonia and carbon dioxide and can raise the pH of the medium above 10. Both acid- and alkali-producing systems were tested and were found to work well (Aleksic et al., 2007).

The original design of the arsenic biosensor submitted for iGEM 2006 was a complex system, with a multistage output. This is discussed further below. However, the practical demonstration provided consisted only of the acid-generating system, which was found to give robust and reliable responses to arsenic concentrations as low as 2.5 ppb, with the time of pH change being related to the arsenic concentration in a simple and reproducible way (Aleksic et al., 2007; Joshi et al., 2009). This construct is available from the Registry of Standard Biological Parts (BBa_J33203), as are its components, the *ars* promoter and associated *arsR* gene

(BBa_J33201) and the *lacZ'* α reporter gene (BBa_J33202). Interestingly, during early testing to determine whether buffer ions expected to be present in groundwater might interfere with the pH response, we found that bicarbonate ions actually increase the sensitivity of the response, leading to induction at much lower arsenate levels (Joshi et al., 2009). The reason for this is not clear, but it may be due to altered speciation or uptake of arsenate (de Mora et al., 2011).

The original concept for this biosensor system involved use of a universal pH indicator solution which gives a strong color response—blue in alkaline conditions, green in neutral conditions, and red in acidic conditions—coupled with quantitation via a glass pH electrode. However, it became apparent that the red component of the universal pH indicator, as well as pure methyl red, were rapidly bleached in the presence of living cells under the conditions used. This was therefore replaced with bromothymol blue, which is blue under alkaline conditions and yellow under acid conditions, with pKa around 7.3 (Figure A5-1).

For quantitative monitoring of multiple samples simultaneously, as might be useful in a local or regional testing laboratory, an inexpensive system was developed based on the use of freeze-dried cells together with a webcam; following aseptic addition of groundwater samples to freeze-dried cells and sterile medium, the webcam would monitor the color of multiple tubes simultaneously, and software would extract the pixels representing the tubes and monitor the color of each over time. From these data, the time of color change could be extracted, and this was found to correlate well with arsenic levels in model groundwaters

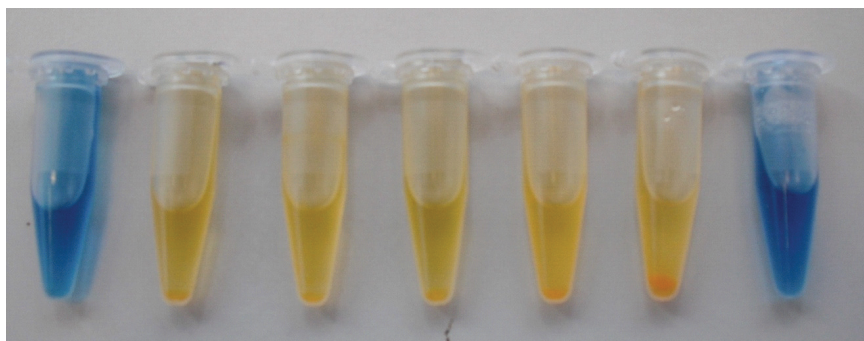


FIGURE A5-1 Demonstration of the Edinburgh pH-based arsenic biosensor, *Escherichia coli* JM109/pSB1A2-BBa_J33203 with bromothymol blue as pH indicator, following static overnight incubation. From left to right: arsenic-free control; 5, 10, 25, 50, and 100 ppb arsenic as sodium arsenate; and cell-free control with 100 ppb arsenate. Note the increasing size of the cell pellet in tubes with increasing arsenic concentrations. Color change occurs more rapidly in samples with increasing arsenic concentration (not shown). SOURCE: C. French, unpublished.

and also in real arsenic-contaminated groundwater samples from Hungary (de Mora et al., 2011).

This system seems well suited for use in water quality laboratories at a local or regional level. However, our original aim was to develop a system that could be used by relatively unskilled users in the field, so that local people could easily monitor the quality of their own well water. This poses several further issues. One technical issue is that any contamination with lactose-degrading bacteria will lead to false-positive results. To avoid this, water samples must be sterilized prior to introduction into the test device. To overcome this problem, we envisage a disposable plastic device containing freeze-dried cells and medium components, with an integral sterile filter through which the water sample is introduced. A second potential complicating factor is the temperature dependence of the rapidity of color change. This requires further investigation. Storage lifetime under relevant conditions also requires further research. However, we are confident that these issues can be overcome, and that this system can form the basis of a simple, cheap, sensitive, and reliable field test for arsenic concentration in groundwater.

One nontechnical issue which must first be addressed is the regulatory and safety issues associated with use of live genetically modified bacteria outside of a contained laboratory context. As noted above, the host strains used are disabled and are unable to colonize humans or to propagate in the environment in competition with wild-type bacteria (Chart et al., 2001). Nevertheless, depending on the jurisdiction, there are many regulatory hurdles associated with the use of genetically modified microorganisms in poorly contained applications. This is discussed further below. In the meantime, we are focusing our attention on development of a device suitable for use in local or regional laboratories. A company, Lumin Sensors, has been formed to explore these possibilities (www.luminsensors.com).

In the remainder of this paper, we consider some of the ways synthetic biology can improve the performance of whole-cell biosensors and lead to the development of a new generation of devices with improved performance.

Better Biosensors Through Synthetic Biology

Alternative Host (Chassis) Organisms

Synthetic biology generally involves the introduction of a new genetic system into a host organism, which in the context of synthetic biology is often called a “chassis.” The great majority of whole-cell biosensors reported in the literature have used *E. coli* as a host organism, due to familiarity, ease of manipulation, and availability of a wide variety of vector systems and other tools. However, *E. coli* has a number of characteristics which may mean that it is not necessarily the ideal chassis for any given purpose. For example, as a Gram-negative bacterium, it produces and sheds lipopolysaccharide (endotoxin), a powerful activator of the innate immune system; hence, it is generally unsuitable for any *in vivo* uses. Its

outer membrane prevents large analytes such as peptides from approaching the cell membrane. Unlike many other bacteria, it generally does not secrete most proteins effectively into the medium, unless specific modifications are introduced to allow it. Most significantly in terms of biosensor applications, *E. coli* does not naturally produce dormant states such as spores, meaning that freeze drying is likely to be required for storage and distribution. While freeze drying of bacterial cells is a well-understood process, this adds an extra level of complexity and expense to the manufacturing process. Some other potential hosts have much better characteristics in this regard. Most attention has been paid to *Bacillus subtilis*, a low-GC Gram-positive soil bacterium. *B. subtilis* is used as a model Gram-positive bacterium; its physiology is therefore well studied, and a number of vector systems and other tools are available, though in both characteristics it lags well behind *E. coli*. In contrast to *E. coli*, *B. subtilis* is naturally competent during a certain stage of its life cycle, and, unlike *E. coli*, will happily take up large pieces of linear DNA and integrate them onto its chromosome by homologous recombination. Also, and more importantly, *B. subtilis* naturally forms a dormant resting state known as endospores (Errington, 2003). When conditions become unfavorable for growth, each cell undergoes an asymmetrical cell division resulting in a large “mother cell” and a small “forespore.” The mother cell engulfs the forespore and produces layers of protein coats to surround it, while the forespore produces small acid-soluble proteins and calcium dipicolinate, which act to protect its nucleic acids. When this process is complete, the mother cell lyses to release the mature endospore. The spores can simply be harvested and dried for storage and distribution. Endospores are extremely tolerant to heat, drying, and other stresses. There are well-attested reports of endospores surviving for hundreds and even thousands of years in dry conditions (Nicholson et al., 2000), as well as more controversial reports of survival for many millions of years in unusual contexts such as in the guts of insects preserved in amber (Cano and Borucki, 1995).

B. subtilis possesses an arsenic detoxification operon similar to that found on the *E. coli* chromosome (Sato and Kobayashi, 1998); thus, arsenic biosensors can be prepared in a similar way to that described above, either by adding the *ars* promoter and *arsR* regulatory gene to a reporter gene on a multicopy plasmid, or by introduction of such a reporter gene to the chromosome downstream of the *ars* promoter. As a demonstration of principle, we have constructed such a system, designated a “Bacillosensor,” using the plasmid vector pTG262 and the reporter gene *xylE* of *Pseudomonas putida*, which encodes catechol-2,3-dioxygenase. This enzyme acts on the cheap substrate catechol to produce a bright yellow compound, 2-hydroxy-*cis,cis*-muconic semialdehyde. This system was found to be sensitive to arsenic levels well below the WHO recommended limit of 10 ppb (Figure A5-2). Spores could be boiled for 2 minutes prior to use in the assay; this would not only kill competing organisms but also activate the spores for rapid germination. The ability to remove most contaminating organisms from

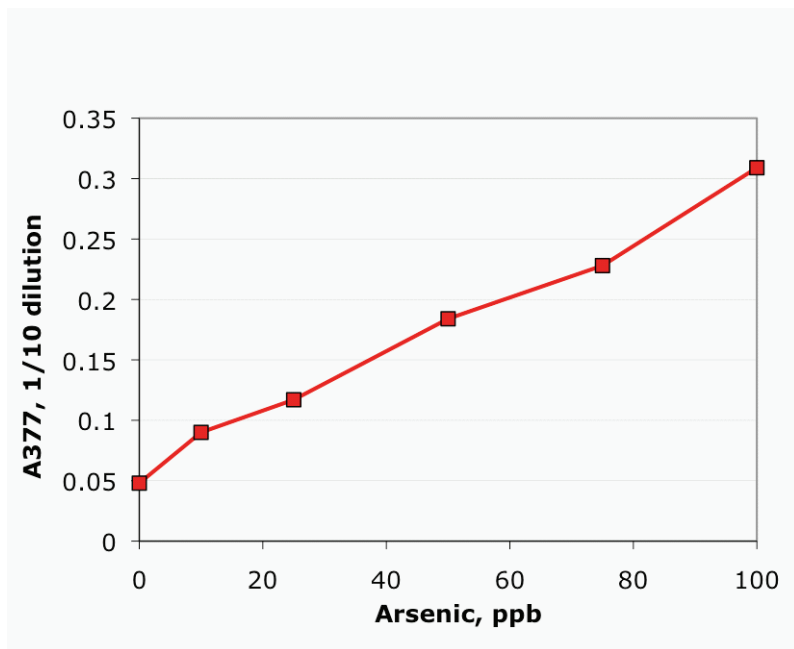


FIGURE A5-2 Detection of arsenic by *B. subtilis* 168/pTG262-*arsR-xylE*: absorbance at 377 nm vs. arsenic concentration (ppb arsenic as sodium arsenate). The vector and BioBrick components used to make this device (BBa_J33206, BBa_J33204) are available from the Registry of Standard Biological Parts. Conditions of the assay were as described by Joshi et al. (2009).

SOURCE: L. Montgomery and C. French, unpublished.

the sample simply by heat treatment offers a considerable potential advantage for field use of such devices, eliminating the need for filter sterilization or similar treatments.

Several reports in the literature have also described bioreporters based on *B. subtilis* and related organisms. Tauriainen et al. (1997, 1998) reported the use of *B. subtilis* as a host for firefly luciferase-based bioreporters for arsenic, antimony, cadmium, and lead but did not specifically describe the use of endospores in the assays. More recently, Date et al. (2007) reported the construction of bioreporters for arsenic and zinc based on endospores of *B. subtilis* and *Bacillus megaterium*, with β -galactosidase plus a chemiluminescent substrate, or enhanced green fluorescent protein, as reporter genes. The genetically modified spores could be stored at room temperature for at least 6 months. The same authors later described incorporation of such endospore-based bioreporters into microfluidic devices (Date et al., 2010, discussed further below). Fantino et al. (2009) reported the construc-

tion of a device designated “Sposensor,” incorporating *B. subtilis* endospores engineered to produce β -galactosidase in response to the target analyte. Systems responsive to zinc and bacitracin were demonstrated using a chromogenic substrate with spores dried on filter paper discs.

Another potentially interesting host is the yeast *Saccharomyces cerevisiae*. Again, this is a model organism, well studied, and for which numerous vector systems and other genetic modification tools are available. It can be stored and distributed in a “dry active” state (Baronian, 2003). However, as a eukaryote, *S. cerevisiae* has more complex regulatory systems than bacteria such as *E. coli* and *B. subtilis*, and to date there have been relatively few reports of its use as a host for bioreporter applications. One example is a nonspecific toxicity reporter described by Välimaa et al. (2008), using *S. cerevisiae* modified to produce firefly luciferase; as in the MicroTox system described above, toxic substances reduced the level of luminescence observed. *S. cerevisiae* has also been used as a platform for analyte-specific biosensors. For example, Leskinen et al. (2003) reported construction of a yeast-based bioreporter for copper ions, with firefly luciferase under control of the copper-responsive CUP1 promoter. This was used in environmental analysis for bioavailable copper (Peltola et al., 2005). Some further examples are described by Baronian (2003). With further development, analyte-specific yeast-based biosensors could be a useful addition to the biosensor toolkit.

Detection of Extracellular Analytes

In the examples discussed so far, the signal has been generated internally, either as a stress response (as in the case of the SOS-Chromotest) or else by binding of an intracellular protein, such as ArsR, to an analyte that has been internalized (arsenate is probably taken up in error by the phosphate uptake machinery). For medical applications, it would be advantageous to be able to detect and respond to analytes such as peptides, which do not naturally enter bacterial or fungal cells. However, bacteria are able to sense and respond to extracellular analytes via “two-component” systems. In these cases, one component is a sensor kinase that spans the cell membrane, and the second is a response regulator protein that binds DNA to activate or repress transcription from a given promoter. The extracellular analyte binds to the extracellular domain of the sensor kinase, and this increases or decreases the kinase activity of the intracellular domain. This alters the tendency of the kinase domain to phosphorylate the response regulator protein, which in turn alters its propensity to bind to and activate or repress the promoter(s) in question. Most bacteria possess multiple two-component sensor systems responding to a variety of different stimuli, including osmotic strength of the extracellular medium, extracellular phosphate levels, and the presence of various small molecules such as sugars and amino acids. One interesting subgroup consists of two-component sensor systems in Gram-positive bacteria such as *Bacillus*, *Streptococcus*, and *Enterococcus*, which sense and respond to the pres-

ence of short-peptide pheromones produced by other cells of the same species, a phenomenon analogous to the more familiar N-acyl homoserine lactone-based quorum-sensing systems of Gram-negative bacteria. One might imagine that such systems could be modified, perhaps by rational engineering or directed evolution, to respond instead to some peptide of analytical interest.

There are two interesting points regarding two-component sensor systems. One is that these systems show a surprising degree of modularity. A number of reports have described cases where the extracellular domain of one sensor kinase has been fused to the intracellular domain of another, giving a hybrid protein that responds to the normal stimulus of the first sensor kinase by activating the normal response regulator of the second. One well-known example is the report of Levskaya et al. (2005) describing fusion of the extracellular domain of the light-sensing domain of a cyanobacterial phytochrome, Cph1, to the intracellular domain of an *E. coli* osmotic stress sensor, EnvZ; the hybrid protein, Cph8, responded to red light by activating the response regulator, OmpR, which normally responds to EnvZ. When a promoter controlled by OmpR was fused to a pigment-producing gene, the resulting genetically modified cells responded to red light by producing a pigment, allowing “bacterial photographs” to be made by focusing images onto a plate of the bacteria (Levskaya et al., 2005). Another example is fusion of the extracellular domain of a chemotaxis receptor, Trg, which responds to the presence of ribose, among other chemoattractants, by controlling the cell’s motility apparatus, to the intracellular domain of EnvZ (Baumgartner et al., 1994). (In this case, the interaction is indirect: ribose binds to periplasmic ribose binding protein, which then interacts with the extracellular domain of Trg.) In the presence of the hybrid protein, designated Trz, an OmpR-controlled promoter (specifically, the *ompC* promoter) was found to respond to the presence of ribose. Recent structural studies have begun to offer some insight into the basis of communication between extracellular and intracellular domains in two-component sensor kinases (Casino et al., 2010), which may allow more rational engineering of such hybrid proteins.

The second point is that it is possible to engineer the recognition elements of such sensors to respond to nonnatural target molecules. For example, Looger et al. (2003) reported rational reengineering of the ribose binding protein, which binds ribose and interacts with the extracellular domain of the hybrid Trz sensor kinase mentioned above, so that it would respond to lactate or to 2,4,6-trinitrotoluene (TNT). In the presence of these molecules, OmpR-controlled promoters were reported to be activated. This opens the possibility that it might be possible to use such a platform as a “universal bioreporter” by generating a library of reengineered sensor kinases or associated binding proteins to respond to any analyte of interest. If such rational reengineering proved challenging in the general case, another interesting possibility would be to attempt fusion of the extracellular domain of such a sensor to some binding molecule such as a nanobody (variable region of a camelid heavy-chain-only antibody) or scFv fragment of an antibody,

so that any analyte to which an antibody could be raised could be detected by a bioreporter. Other protein-based scaffolds for specific recognition are also under development (Hosse et al., 2006; Nuttall and Walsh, 2008) and might also be suitable for such applications. Alternatively, extracellular domains able to bind a desired target analyte might be selected from a library of mutants by a technique such as phage display or cell surface display (Benhar, 2001); this might also enable simple screening for clones which were able not only to bind to the analyte of interest but also to generate the appropriate response on binding.

An interesting, but rather application-specific, approach to the detection of extracellular analytes via two-component sensing systems was proposed by the 2010 iGEM team of Imperial College, London. In this case, the objective was to detect cercaria (larvae) of the parasite *Schistosoma* in water. It was proposed that a protease produced by the parasite could be detected by cleavage of an engineered substrate to release the autoinducer peptide of *Streptococcus pneumoniae*; this peptide would then be detected by the two-component sensor system ComDE of *S. pneumoniae* expressed in *B. subtilis*. A similar system might be used to detect other proteases or hydrolytic enzymes of biological interest.

Another possible platform for detection of extracellular peptides would be the yeast mating peptide sensing system. In *Saccharomyces cerevisiae*, mating peptides (α and α') are detected by G-protein coupled receptors, Ste2 and Ste3, which initiate an intracellular signalling pathway (Bardwell, 2004; Naider and Becker, 2004). As noted above, *S. cerevisiae* is arguably an underexploited platform for biosensor development, with much potential for investigation. One interesting example of a yeast-based sensor system was reported by Chen and Weiss (2005). In this case, a cytokinin (plant hormone) was detected by yeast cells expressing *Arabidopsis thaliana* cytokinin receptor Atcre1, a two-component-type sensor kinase, which apparently is fortuitously able to interact with the endogenous yeast response regulator Ypd1 in the absence of Ypd1's normal sensor kinase partner, Sln1 (Inoue et al., 2001). The engineered "receiver" cells expressed GFP from a Ypd1-activated promoter in the presence of cytokinin.

Animal cells have numerous and varied extracellular receptors but, with current techniques, probably lack the necessary robustness to be generally useful as a biosensor platform except possibly for specialized clinical uses.

Modulation of Sensitivity and Dynamic Range

The critical parameters in the performance of a sensor are the sensitivity (the lowest analyte concentration to which a detectable response is seen) and the dynamic range (in this context, the range of analyte concentrations over which the analyte concentration can be estimated based on the response; that is, between the sensitivity limit and the concentration at which the response saturates). In simple whole-cell biosensors such as those described above, a hyperbolic or sigmoidal response is seen, and it may be that the response curve initially obtained is not in

the desired range for the planned application. Obviously, one critical parameter is the affinity of the receptor for the analyte; however, in practice, many other factors are important, making analysis rather complicated (for a more detailed discussion of such issues, see van der Meer et al., 2004). For example, in the case of arsenic biosensors, arsenate is first taken up by the cell (probably mistakenly, via the phosphate uptake system). The characteristics of uptake determine the ratio between extracellular and intracellular arsenate concentrations. It then interacts with the ArsR repressor with a certain affinity, and interferes with the binding of the repressor to the *ars* promoter; both of these interactions have a characteristic affinity. Within a given cell, the numbers of arsenate anions, ArsR repressor molecules, and promoter sites are also all limited, so the relative numbers of these will also strongly affect the interaction. Finally, when the repressor is not bound to the promoter, and the promoter is thus “active,” RNA polymerase will bind the promoter with a certain affinity, and begin transcription with a certain efficiency. The actual level of arsenate repressor and reporter protein molecules generated will be strongly affected by the rate of messenger RNA synthesis, the rate of mRNA degradation, the affinity of ribosomes for the ribosome binding sites on the mRNA, and the degradation rate of the proteins. Thus, many steps intervene between the extracellular arsenate concentration and the level of the reporter protein. This means that, in practice, it is possible to modulate the sensitivity and dynamic range of the sensor without actually altering the receptor at all, simply by making minor changes to factors such as the strength of the ribosome binding sites.

This type of experiment is greatly facilitated by the modular, composable nature of BioBricks and the availability of a library of ribosome binding sites of different strengths in the Registry of Standard Biological Parts. To give one trivial example, we assembled an alternative version of the simple arsenic biosensor construct BBa_J33203, the differences being that the reporter gene was moved to a position between the promoter and the *arsR* gene encoding the repressor, and the native ribosome binding site of *arsR* was replaced by a strong synthetic ribosome binding site, BBa_J15001. The response characteristics of this modified construct were quite different from those of the original construct (Figure A5-3). While the mechanism of this was not investigated, one plausible explanation would be an increased level of expression of the ArsR repressor due to the stronger ribosome binding site.

More profound reorganizations of the arsenic recognition system have also been investigated. For example, a second copy of the ArsR-binding site was introduced between *arsR* and the reporter gene, with the aim of decreasing background expression in the absence of arsenic. This led to considerably improved induction characteristics (Stocker et al., 2003). It was further reported that modification of the activity or synthesis rate of the reporter enzyme (cytochrome c peroxidase or β -galactosidase) led to strong changes in the system response to given arsenic concentrations (Wackwitz et al., 2008), allowing the generation of an array of

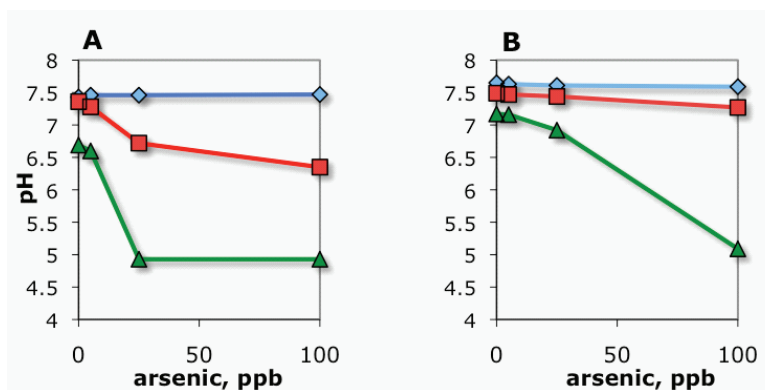


FIGURE A5-3 Altered response characteristics of a whole-cell arsenic biosensor through reassembly of the components. (A) Original Edinburgh arsenic biosensor, consisting of the *E. coli* chromosomal *ars* promoter and *arsR* gene (BBa_J33201) followed by *lacZ'* α (BBa_J33202). Both *arsR* and *lacZ'* α have their native ribosome binding site. (B) Reassembled operon consisting of *ars* promoter (BBa_J15301), strong synthetic ribosome binding site (BBa_J15001), *lacZ'* α coding sequence (BBa_J15103), ribosome binding site (BBa_J15001), and *arsR* coding sequence (BBa_J15101). Diamonds, time zero; squares, 6 hours; triangles, 24 hours. The vector was pSB1A2 and host was *E. coli* JM109 in all cases. Assay conditions were as described by Joshi et al. (2009). All components and assembled constructs are available from the Registry of Standard Biological Parts. SOURCE: X. Wang and C. French, unpublished.

reporter strains with different response characteristics (discussed further below). In a similar investigation, using mercury biosensors, the 2010 Peking University iGEM team investigated the effects of placing the regulatory gene *merR* under the control of a variety of promoters of different strengths and found that this resulted in a wide variety of different sigmoidal response curves. A similar effect was achieved by screening a library of mutants with altered MerR-binding sites in the mercury-responsive promoter. Thus, a variety of simple modifications to the system can be used to achieve alterations in the sensitivity and dynamic range of such sensors. The composable nature of BioBricks, together with other assembly strategies used in synthetic biology, make it easy to generate and screen a large number of such systems to find a set with the desired characteristics.

It is also possible to use rational design principles to modify the dynamic range of a sensor, for example, by amplifying a weak transcriptional signal. One way to do this is through the use of genetic “amplifiers.” One set of such devices, submitted and tested by iGEM teams from the University of Cambridge in 2007 and 2009, consists of bacteriophage activator-promoter pairs. Rather than the

analyte-responsive promoter deriving the reporter gene directly, the promoter drives expression of an activator protein, which activates a second promoter, which controls the reporter gene. This gives a genetic “amplification” effect. A small library of cross-reactive activators and promoters allows a mix-and-match approach to select a pair that gives the desired response characteristics. In these projects, the promoters came from bacteriophages P2 (promoters P_{F} , P_O , P_P , and P_V) and P4 (promoters P_{sid} and P_{LL}), and the activators from bacteriophages P2 (Ogr), P4 (δ protein), PSP3 (Pag), and ϕ R73 (δ protein) (Julien and Calendar, 1996). Fifteen promoter-activator combinations were characterized, allowing the biosensor designer to choose a pair with the desired response characteristics.

The availability of a number of similar biosensors with different response characteristics, as described above, allows the preparation of “bar graph”-like arrays of sensors to obtain a quantitative output over a wider range of analyte concentrations than any single sensor could achieve. This was proposed by Wackwitz et al. (2008), who used the term “traffic light biosensors” to describe such devices. In principle, such devices should not require calibration (van der Meer and Belkin, 2010).

Another issue with induction-based whole-cell biosensors is the time required for induction; few systems in the literature show detectable responses in less than 30 minutes or so, and several hours is more typical. For many applications, it would be advantageous to obtain a faster response. One ingenious approach to this problem was presented by the Imperial College iGEM team of 2010. In this case, the reporter enzyme is presynthesized in the cell in an inactive form. The analyte-responsive promoter drives expression of a protease, which cleaves and activates the reporter. Since each molecule of protease can rapidly activate multiple molecules of the reporter protein, this can potentially give a much faster response. In the case of the Imperial College iGEM project, the reporter, catechol-2,3-dioxygenase (Xyle), was synthesised as an inactive fusion with GFP, joined by a linker which could be cleaved by site-specific TEV protease. Detection of the target analyte, in this case a peptide released by a protease of the parasite *Schistosoma*, led to induction of TEV protease expression and consequent cleavage of the linker, allowing rapid formation of active catechol-2,3-dioxygenase tetramers.

In Vivo Signal Processing and Multiplex Output

In addition to tuning of response characteristics, it is possible to introduce more complex forms of in vivo signal processing. One simple example is a genetic inverter, in which a promoter that would normally activate transcription is instead used to repress it. This is accomplished by having the analyte-responsive promoter drive production of a repressor, which represses expression from a second promoter driving the reporter gene. Three well-characterized repressor-promoter pairs are widely used in such systems: the *LacI/lac* promoter pair, bac-

terioophage λ cI/P_L or P_R pair, and the TetR-*tet* promoter pair (see, for example, Elowitz and Leibler, 2000). These repressor-promoter pairs are analogous to insulated wires used in an electronic circuit to communicate between different parts of the device. For complex devices, it would clearly be useful to have more than three such “wires” available. One interesting approach to this was reported by the USTC iGEM team of 2007 (following work described by Sartorius et al., 1989). In this case, mutations were made to the bases of the *lac* operator site involved in binding of the repressor LacI, and also to the amino acids of LacI involved in this binding. The libraries of mutant *lac* promoters and LacI repressors were then analyzed to determine which pairs interacted efficiently. From this experiment, multiple repressor-promoter pairs were chosen that did not crosstalk. Furthermore, these were used to generate biological equivalents of several logic gates. The simplest of these is the NOT gate, in which activation of one promoter leads to repression of another. This is simply achieved by having the first promoter drive expression of a repressor which represses the second, as described above. More complex gates include NAND and NOR. In the former case, binding of two different repressors is required to inactivate the second promoter; in the latter case, binding of either of two repressors is sufficient to achieve this effect. From combinations of such gates, more complex circuits can be assembled. Such logic systems can also be extended to systems consisting of several different types of engineered cell, with the cells communicating via quorum-sensing signals (Tamsir et al., 2010).

The term “traffic-light sensors,” discussed above, is also applied to a class of devices that have been proposed and modeled, but, so far as we know, never demonstrated in practice, in which discrete, different outputs are activated at different analyte levels by in vivo signal processing within a single bioreporter organism. The originally proposed iGEM 2006 Edinburgh arsenic biosensor fell into this category, giving an alkaline response at very low arsenate concentrations, a neutral pH at moderate arsenate concentrations, and an acidic response at dangerously high arsenate levels (Aleksic et al., 2007). This was to be achieved through the use of two separate repressors, with different affinities for arsenate, controlling two different reporters: urease for an alkaline response, and β -galactosidase for the acidic response. Response of the high-affinity repressor-promoter pair was inverted via a repressor, so that the presence of a low concentration of arsenate led to production of a repressor that switched off production of urease, whereas higher levels of arsenate switched on production of β -galactosidase. A similar arrangement of two different repressor systems can be used to generate a genetic “band detector,” which responds only to analyte concentrations within a certain concentration range.

Visual Outputs

While the majority of reported arsenic sensors use luminescence or fluorescence as output, it might be advantageous in some cases to have an output that can be easily detected by eye. The use of enzymes such as β -galactosidase and catechol-2,3-dioxygenase, together with chromogenic substrates, offers one route to achieving this end. An alternative is the pH-based approach used in the Edinburgh arsenic biosensor described above; this is useful in that pH changes, together with standard pH indicators, give very strong and easily detected color changes, but they can also be quantified using an inexpensive pH electrode. In some cases it might be preferable to have cells produce an endogenous pigment in response to the target analyte. Several such examples have been reported. Fujimoto et al. (2006) described a system in which the photosynthetic bacterium *Rhodovulum sulfidophilum* was engineered to place the endogenous carotenoid pigment gene *crtA* under the control of the *E. coli ars* promoter, so that the presence of arsenite led to a change in cell pigmentation from yellow to red. Subsequently, Yoshida et al. (2008) reported a similar system based on *Rhodopseudomonas palustris*. The combinatorial nature of synthetic biology components, together with the possibility of multistage outputs discussed above, opens the possibility of systems in which a range of discrete colors is produced for different levels or combinations of analytes. To facilitate the construction of such devices, the University of Cambridge iGEM team, 2009, presented a set of modular BioBrick components that could generate a variety of carotenoid and indole-based pigments using different combinations of components from the carotenoid and violacein biosynthetic pathways (Figure A5-4). As with all of the iGEM entries discussed in this paper, these genetic modules are freely available from the Registry of Standard Biological Parts.



FIGURE A5-4 *Escherichia coli* cells producing a variety of pigments. From left to right, the first four tubes are derived from the carotenoid biosynthesis pathway, the last three from the violacein biosynthesis pathway. All of these pigment-producing pathways are available in BioBrick format from the Registry of Standard Biological Parts.

SOURCE: University of Cambridge, iGEM 2009.

Integration of Biological and Electronic Components

For data processing and storage, it is advantageous if biosensor outputs can easily be converted into electrical signals that can be read by a computer. A number of integrated biological-electrical devices have been reported. One example is the Bioluminescent Bioreporter Integrated Circuit (Nivens et al., 2004; Vijayaraghavan et al., 2007), in which luminescence emitted by bacterial luciferase is detected by a Complementary Metal-Oxide Semiconductor (CMOS) microluminometer incorporated with signal processing circuitry and other relevant components on a “biochip.” Several recent reports have described the integration of bioreporter cells into microfluidic devices. For example, Diesel et al. (2009) reported incorporation of an *E. coli* GFP-based arsenic bioreporter into a microfluidic device. Fluorescence from approximately 200 cells within the detection cavity was imaged using a camera. Date et al. (2010) reported the integration of *Bacillus* endospore-based biosensors for arsenic and zinc (described above) into a centrifugal microfluidic platform, in which pumping is provided by rotation of a disc-shaped substrate. Luminescence and fluorescence measurements were performed using separate laboratory instruments with fiber-optic probes held above the detection chambers of the microfluidic device. Storage and germination of endospores, and sensitive detection of the analytes, were reported.

Whereas these devices use “conventional outputs (fluorescence and luminescence), other reporter systems can give an electrical output directly, which may be more convenient for incorporation into simple devices for field use. One example is the Edinburgh arsenic biosensor described above. The pH response can easily be converted to a voltage signal using a standard glass pH electrode, or an equivalent solid-state device (ion selective field effect transistor). An alternative is the generation of an amperometric signal via a device similar to a microbial fuel cell. Bacteria respire by transferring electrons from a donor (such as a sugar) to an acceptor (such as oxygen). This electron transfer occurs at the cell membrane. Many bacteria can instead transfer electrons to or from an electrode, generating an electrical current; others can do so in the presence of electron shuttle molecules known as mediators (Lovley, 2006). An amperometric signal can be generated by placing synthesis of such a mediator, or of an essential component of the electron transfer apparatus, under the control of an analyte-responsive promoter. Both of these approaches have been described by iGEM teams. The iGEM 2007 entry from the University of Glasgow described a system for biosynthesis of pyocyanin, a redox-active metabolite of *Pseudomonas aeruginosa*, which could act as a mediator to transport electrons between the bacterial respiratory chain and an electrode. The iGEM 2008 entry from Harvard University took advantage of a naturally “electricigenic” bacterium, *Shewanella oneidensis*, by controlling synthesis of one of the outer membrane proteins, MtrB, required for efficient transfer of electrons to an electrode. Either of these approaches can allow controllable generation of an electrical current induced by the presence of a target analyte.

Regulatory Issues Related to Field Use of Whole-Cell Biosensors

The Edinburgh arsenic biosensor described above was designed to be used in the field in the form of a contained disposable device. Some other bioreporters have been designed for more direct use in the field. One of the earliest examples to be tested was that of Ripp et al. (2000), reportedly the first genetically modified microorganisms to be approved for field testing for a bioremediation application in the United States, in which a strain of *Pseudomonas fluorescens* modified to produce a luminescent signal in the presence of aromatic hydrocarbons was introduced into the soil in a contaminated site, and its persistence monitored over a 2-year period. While this organism was designed to persist in the environment for a prolonged period, there are other cases where the bioreporter only needs to survive for a few hours—just long enough to report on the conditions in which it finds itself. One well-known example is the Microbial Mine Detection System developed at Oak Ridge National Laboratories. This consisted of a strain of *Pseudomonas* modified to produce GFP in the presence of explosives leaking from land mines (discussed by Habib, 2007). The positions of the land mines could then be mapped using an ultraviolet lamp. This system was reportedly field tested with good results. Another example was reported by the iGEM team of the Bristol Centre for Complexity Studies (BCCS) in 2010. In this case, the bioreporter consisted of *E. coli* cells modified to indicate the presence of nitrate by producing a fluorescent response. These cells, encapsulated in a hydrophilic gel, were to be spread over the soil and left for several hours to express the fluorescent protein, after which the nitrate levels in the field could be mapped by a mobile device, providing information useful in agriculture.

On consideration of the examples described above, we can distinguish between four different cases:

1. bioreporters designed to be used within the confines of a laboratory (e.g., the SOS-Chromotest);
2. bioreporters designed to be used in a contained device, but outside of a laboratory (e.g., the Edinburgh arsenic biosensor);
3. bioreporters designed to be exposed directly to the environment, but not to survive longer than required to report the levels of the target analyte (e.g., the MMDS and Bristol nitrate sensor); and
4. bioreporters designed to survive and persist in the field for long periods (e.g., the bioremediation monitoring system of Ripp et al., 2000).

Unfortunately, current regulatory regimes in the United Kingdom and European Union do not appear to distinguish between cases 2, 3, and 4. Case 1 is unproblematic; such “contained use” applications are dealt with in the United Kingdom by the Health and Safety Executive under the Genetically Modified Organisms (Contained Use) Regulations 2000, whereas “uncontained uses” are dealt with by the Department for the Environment, Food and Rural Affairs un-

der the Genetically Modified Organisms (Deliberate Release) Regulations 2002 (which implement EU Directive 2001/18/EC). Whereas this is wholly appropriate for case 4, where such organisms are designed to persist in the environment, no provision seems to be made for cases 2 or 3, where organisms may be specifically designed not to survive in the environment, thereby minimizing any threat to ecosystems. This is a serious inhibiting factor in the use of genetically modified whole-cell biosensors or bioreporters outside of the laboratory, as expensive field trials with extensive postrelease monitoring are required. We feel that it would be useful if future legislation took this into account. In the United States, the situation is less clear to us; the relevant federal legislation appears to be the Toxic Substances Control Act (Sayre and Seidler, 2005). In the absence of specific legal and regulatory expertise, we will refrain from further comment. However, it is clear that until these regulatory issues are addressed, it will not be possible to use genetically modified bioreporter organisms on a large scale in the field, despite their obvious potential.

Conclusions

Sensitive and highly specific response to various molecules is one of the core functions of biological systems. As such, whole-cell biosensors offer a versatile and widely applicable method for detecting the presence of a wide range of analytes. The techniques of synthetic biology offer numerous possible improvements in terms of response tuning, *in vivo* signal processing, and direct interface with electronic devices for further signal processing and output. However, regulatory issues will need to be clarified before such devices can fulfill their true potential as highly sensitive, inexpensive sensors for field use.

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Disclosures

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A6

**SYSTEMS ANALYSIS OF ADAPTIVE IMMUNITY BY
UTILIZATION OF HIGH-THROUGHPUT TECHNOLOGIES³⁹***Sai T. Reddy*^{40,41} and *George Georgiou*^{40,41,42,43}

A new generation of high-throughput technologies for quantitative and clonal analysis of adaptive immune responses have been developed. Functional analysis of lymphocyte populations has been accomplished via microfluidic assay systems. Additionally, lymphocyte receptor repertoires have been characterized on proteomic and genomic levels with multiplexed protein microarrays and high-throughput DNA sequencing. These tools are providing an unprecedented level of information depth on the distribution of adaptive immune cell (B and T cell) functionalities and repertoires, which develop upon activation following vaccination, pathogenic infection, or in disease states. These various high-throughput technologies have unlocked the potential to transform immunology into an information-rich science that will enable rapid expansion of the field of experimental systems immunology.

Introduction

Adaptive immunity plays an indispensable role in maintaining vertebrate host protection against a constant barrage of pathogens. Adaptive immunity is fundamentally reliant upon the generation of an incredible diversity of lymphocyte

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receptors (B cell receptors, or BCRs; and T cell receptors, or TCRs); furthermore, the development of lymphocyte subsets, which display a plethora of functions, act synergistically to counteract pathogenic invasion. Receptor diversity in lymphocyte populations is generated by the genetic recombination of noncontiguous germline elements: variable (V), diversity (D), and joining (J) gene segments. Primary and secondary diversification via germline and junctional recombination, combinatorial pairing, and somatic hypermutation (for B cells only) combine to generate theoretical diversities of human antibodies and TCRs of $\sim 10^{13}$ and $\sim 10^{18}$, respectively (Janeway, 2005), far outnumbering the physiological number of lymphocytes in an average human ($\sim 10^{10}$). In addition to molecular diversity, there is an assortment of lymphocyte cellular subclasses (e.g., CD4+ and CD8+ T cells, Tregs, Th17 cells) and differentiation states (e.g., naïve, memory, and plasma B cells) that synergize to produce a multipronged attack on pathogens, leading to infection resolution and long-term immunological memory. Finally, the anatomical compartments (e.g., lymph nodes, blood, bone marrow) in which lymphocyte activation, homing, and trafficking take place add to the complex interconnected nature of adaptive immunity.

Conventional approaches for measuring adaptive immune responses have so far relied on cursory biological measurements, such as antibody titers to an antigen or lymphocyte assays that measure a single (ELISPOT) or multiple cytokines (intracellular staining, ICS) expressed by individual cells (Janetzki et al., 2004; Papagno et al., 2007). However, these techniques are surrogate measures and provide only a partial picture of the state of the immune system; for example, antibody titers do not provide specific molecular information on the monoclonal antibody components that comprise polyclonal responses in serum or secretory fluids. Likewise, ELISPOT or ICS assays can interrogate only a subset of lymphocytes based on functional properties; furthermore, they are sample-destructive thus precluding the simultaneous cloning and analysis of the respective BCR and TCR repertoires. Very importantly, existing assays typically capture the mean of a particular response or cellular phenotype and afford limited quantification. The understanding of immunological processes at greater resolution is not only important from a scientific point of view but can have major ramifications for human health. Detailed and quantitative deconvolution of the humoral immune response can assist in human immunotherapy by the identification of neutralizing or cytotoxic antibodies or of antigens overexpressed in a disease state (Gnjatic et al., 2010; Law et al., 2008; Yu et al., 2008). High-resolution information on adaptive immune responses to immunization may also greatly aid in assessing the efficacy of experimental vaccines (Germain et al., 2010; Querec et al., 2009), a process that currently relies on longitudinal studies of neutralizing antibody titers as the primary readout. In this review, we highlight recent advances in the high-throughput analysis of functional properties, proteomic interactions, and genomic repertoires of lymphocyte populations following an adaptive immune response (Table A6-1).

TABLE A6-1 High-throughput technologies used to study adaptive immunity and the scale of information generated.

High-throughput technology	Applications	Data Scale	Refs.
Single B cell-cloning	Antibody discovery; repertoire analysis	$\sim 10^2$	Tiller et al., 2008; Smith et al., 2009; Wrammert et al., 2008; Meijer et al., 2006; Poulsen et al., 2007; Wiberg et al., 2006
Microengraved devices	Single cell functional analysis, antibody and TCR specificity; antibody discovery; T cell cytokine reactivity; transcriptional profiling	$\sim 10^5$	Tokimitsu et al., 2007; Jin et al., 2009; Love et al., 2006; Ogunniyi et al., 2009; Story et al., 2008; Song et al., 2010; Han et al., 2010; Flatz et al., 2011; Kwong et al., 2009
Protein microarrays	Antigen discovery from pathogens, cancer and autoimmune disease; serological antibody profiling; vaccine development	$\sim 10^3$ – 10^4	Davies et al., 2005; Felgner et al., 2009; Kunnath-Velayudhan et al., 2010; Robinson et al., 2002; Gnjjatic et al., 2009; Reddy et al., 2011; Legutki et al., 2010
Next generation DNA sequencing	Antibody and TCR repertoire analysis, immunological biomarker discovery, clinical monitoring, antibody library quality control, monoclonal antibody discovery	$\sim 10^6$ – 10^7	Weinstein et al., 2009; Jiang et al., 2011; Boyd et al., 2009; Freeman et al., 2009; Robins et al., 2009, 2010; Warren et al., forthcoming; Glanville et al., 2009; Ge et al., 2010; Ravn et al., 2010; Reddy et al., 2010

Single Cell Analysis of Lymphocyte Populations

Single cell PCR-cloning of immunoglobulin genes

Until recently, the determination of the surface-anchored immunoglobulins/antibodies (BCRs) expressed by animal or human cells has relied on B cell immortalization techniques such as hybridoma technology or viral transduction (Becker et al., 2010; Kwakkenbos et al., 2009; Lanzavecchia and Sallusto, 2009); however, these techniques are compatible with only some stages of B cell maturation and furthermore, due to their low efficiencies (merely 1–3% of B cells interrogated) they are unable to generate a comprehensive picture of humoral repertoires. For example, terminally differentiated antibody-secreting plasma cells are not amenable to immortalization. Consequently, the antibodies isolated by immortalization of memory B cells may neither correspond to nor exhibit the same therapeutic potency as the population of circulating antibodies.

These problems can be addressed by single-cell cloning. Briefly, a desired B cell population is plated at limiting dilution in microtiter well plates and

then the variable heavy (V_H) and light (V_L) chains of surface immunoglobulins (BCRs) or of secreted antibodies from antibody secreting cells are amplified by PCR using primer sets. The V_H and V_L genes are then inserted into suitable vectors for expression from heterologous hosts, resulting in recombinant antibody fragments (e.g., scFv, FAB) or full-length IgGs. Subsequent antigen-specificity and binding affinity of antibodies are determined by standard ELISA techniques (Tiller et al., 2008). In one notable example, flow cytometry was used to isolate human plasmablasts from the peripheral blood of patients immunized with the influenza vaccine. The cells were sorted into 96 well plates at limiting dilution to yield approximately one cell per well. Nested RT-PCR and cDNA amplification of V_L and V_H genes was performed to amplify V genes in wells occupied by cells, followed by cloning into eukaryotic expression vectors (Smith et al., 2009). This approach was used to study the dynamics and antigen specificity of transient plasmablasts that arise three to seven days after vaccination and decay within a month. It was observed that at the peak of the response, nearly 80% of the plasmablasts expressed influenza-specific antibodies and the respective antibody repertoires were pauciclonal, as they were dominated by only a few sequences (Wrämmert et al., 2008). Alternative techniques have been developed that rely on single B-cell cloning by linking V_L and V_H genes during RT-PCR followed by subsequent expression and screening in bacterial systems. For example, peripheral blood plasma cells were isolated from patients receiving tetanus-toxoid (TT) immunizations; single-cell PCR and screening was performed which was then followed by extensive bioinformatic analysis of V_L and V_H pairing (Meijer et al., 2006). In another study, Andersen and co-workers isolated tetanus toxoid-specific antibodies from immunized patients and showed by biochemical characterization that they display affinity and kinetic binding constants close to the proposed limits for protective immunity (Poulsen et al., 2007). These single-cell cloning methods have been used by the company Symphogen (Denmark) to develop recombinant polyclonal antibodies, potentially valuable as human therapeutics. Multiple neutralizing antibodies were expressed under carefully controlled bioprocess conditions to produce a 'recombinant polyclonal' mixture that mimics the diversity, specificity, and binding affinity of natural polyclonal human immune responses (Wiberg et al., 2006).

Single cell microarrays for functional profiling of lymphocytes

Recently, large-scale analysis of lymphocyte populations has been enabled by methods in soft lithography and microengraving. These techniques have generated lymphocyte functional profiles. For example, microfluidic devices were deployed for the large-scale detection of antigen-specific B lymphocytes at the single-cell level and the subsequent cloning of their respective V genes. Kishi and co-workers used deep reactive ion etching on silicon to produce chips containing arrays of 10 mm diameter and 20 mm deep wells to trap single B lympho-

cytes at a density $>200,000$ cells per chip. The well surfaces were coated with anti-immunoglobulin antibodies, which allowed for capture of antibodies from antibody-secreting cells isolated from mouse or human B cells (Tajiri et al., 2007; Tokimitsu et al., 2007). Next, antigen was added to discover cells producing specific antibodies, which was followed by single-cell RT-PCR and cloning into a eukaryotic system for the generation of recombinant monoclonal antibodies (Jin et al., 2009). Alternatively, Love et al. used photolithography and intaglio printing to generate microarrays from poly(dimethylsiloxane). The dimensions of the arrays were compatible for imaging on a microscope slide, therefore allowing fluorescence-based imaging of B lymphocytes expressing antigen-specific antibodies (Love et al., 2006; Ogunniyi et al., 2009). Microengraved devices were used to assay the molecular profile of individual B cells isolated from mice following immunization; multiparametric analysis was performed to determine antibody specificities, isotypes, and apparent affinities (Story et al., 2008). The high throughput afforded by the microwell chip arrays described above notwithstanding, these methods rely on single-cell PCR and thus suffer from practical cloning limitations, therefore making it challenging to obtain a complete picture of the repertoire.

In addition to B cells, the functional profiles of T cells can also be measured by single cell microarray technologies (Song et al., 2010). Multidimensional activation profiles were generated from human peripheral blood T cells; cytokine secretion rates from individual cells were measured with very high sensitivity (0.5–4 molecules/s) (Han et al., 2010). In another recent study, single cell gene expression profiling was performed on CD8 T cell populations following various prime-boost HIV DNA vaccine schemes in mice. Multiparameter transcriptional analysis from single T cells was used to measure mRNA expression of 91 different genes related to phenotype, regulation, survival, and function, allowing discrimination of central memory and effector memory T cell subsets; furthermore providing quantitative parameters associated with vaccine efficacy and protective immunity (Flatz et al., 2011). The Heath group has developed a system for detecting antigen-specific T cells using nucleic acid cell sorting. Peptide-major histocompatibility complex tetramers (p/MHC) were site-specifically attached to DNA oligomers, which were annealed to complimentary strands printed on a glass slide. These p/MHC microarrays allowed for multiplexed sorting of antigen-specific T cells from heterogenous cell suspensions; this system can be exploited for clinical lymphocyte detection, such as detection of T cells from cancer patients (Kwong et al., 2009). These examples illustrate that single-cell methods are rapidly expanding the ability to generate high-throughput data for quantitative molecular analysis and functional profiling of adaptive immune responses.

Microarrays for Studying Protein–Protein Interactions in the Adaptive Immune Response

Systems-level analysis of gene expression using DNA microarrays has had a tremendous impact on cellular and molecular biology; the potential now exists

for proteomic microarrays to have a similar influence on immunological research (Michaud et al., 2003; Prechl et al., 2010). A substantial amount of work has been done to apply multiplexed high-throughput protein microarrays for the serological profiling and analysis of antibody responses; this research has ranged from infectious disease to cancer to autoimmune disease (Kingsmore, 2006).

Technologies for high-throughput printing of pathogen proteomes (with each protein expressed individually) onto protein microarrays have been developed. A typical approach consists of high-throughput PCR amplification of each predicted open-reading frame, which is followed by in vivo homologous recombination into a T7 expression vector. An *Escherichia coli*-based cell-free in vitro expression system is used to generate crude lysates possessing recombinant proteins, which are then printed onto nitrocellulose membranes resulting in a pathogen proteome microarray. In one example, the 185 viral proteins that comprise the vaccinia virus proteome were expressed individually and used to prepare protein arrays, which then were used to analyse the specificity of serum antibodies from vaccinia virus-vaccinated subjects; this allowed for high-throughput evaluation of humoral immunity (Davies et al., 2005). In an even more impressive study, protein microarrays comprising the 1205 proteins encoded by *Burkholderia pseudomallei* were constructed and probed with sera from infected and healthy patients. Such antibody profiling studies can reveal immunodominant antigens, which can be exploited as biomarkers for diagnostic purposes or to guide vaccine development (Felgner et al., 2009). Utilizing this same protein microarray methodology, it was found that in tuberculosis patients, circulating antibodies react with 10% of the *Mycobacterium tuberculosis* proteome. Further analysis identified that during active infection the humoral response was highly biased towards only 0.5% of the proteome and particularly towards extracellular proteins, suggesting that the humoral immune response to *M. tuberculosis* correlates with the evolution of the infection (Kunnath-Velayudhan et al., 2010).

In addition to pathogenic proteins, autologous cellular antigens expressed by malignant cells or in autoimmune disorders can also elicit robust antibody responses. These autoantibodies serve as potential immunological biomarkers valuable for diagnosis and possibly for treatment. In an early study, a protein array was constructed by the immobilization of 196 different human proteins onto a glass slide and used for the detection of autoantibodies (Robinson et al., 2002). More recently, Old and co-workers constructed an array of 329 cancer antigens that was used to detect antibodies produced in cancer patients but not in healthy volunteers (Gnjatic et al., 2009). In a follow-up study, they expanded their analysis and used an array consisting of >8000 human proteins to probe the antibody specificities in sera from over 150 individuals, including healthy volunteers and ovarian and pancreatic cancer patients; this led to the identification of autoantigen signatures for these two malignancies (Gnjatic et al., 2010). In a recent study, a combinatorial library of unnatural synthetic molecules was used to screen for ligands reactive with antibodies abundant in serum (Reddy et al., 2011). The underlying concept of this study was that synthetic molecules act as a 'shape library', such that they are outside of the chemical space range occupied

by natural biomolecules. Microarrays of peptoids (N-substituted oligoglycines) were used to identify reactive IgGs in a mouse model of multiple sclerosis and in human patients with Alzheimer's disease, thus demonstrating the potential to identify immunological serum antibody biomarkers without previous knowledge of antigen. In a similar approach, a microarray consisting of 10,000 random peptides was used to determine the serological antibody binding profile in mice and humans immunized with influenza. Although peptides were random sequences, this analysis still allowed for discrimination of pre-immune and post-immune samples, suggesting a novel platform for the discovery of immunological signatures present in antibody responses (Legutki et al., 2010).

High-Throughput DNA Sequencing of Adaptive Immune Repertoires

The emergence of 'next generation' high-throughput DNA sequencing platforms has been applied to the analysis of immune repertoires. The sequencing of immune repertoires at unprecedented levels of depth has opened up a realm of possibilities; these include answering basic questions on repertoire diversity and selection, monitoring repertoire diversity for clinical applications, and advancing strategies in monoclonal antibody discovery and engineering. The challenge of sequencing variable regions of immune receptors is that there is significant redundancy in the sequences encoding the four framework regions but extensive diversity in the three hypervariable complementarity determining regions (CDRs). As a result, sequencing platforms such as SOLiD (Applied Biosystems) that generate short reads (<100 bp) cannot be used to assemble the complete variable genes. Therefore, typically, the 454 (Roche) technology which yields reads >350 bp has been used to sequence the entire variable genes but at a relatively lower throughput (up to 10^6 reads per plate). Alternatively, the Illumina technology which currently has a maximum read length of ~200 bp (using paired-end sequencing) has been used to sequence the most hypervariable domain of the V gene (CDR3) at high depth (> 10^6) (Fischer, 2011).

In one of the first examples, Quake and co-workers used 454 GS-FLX to generate 640 million bp of antibody V gene sequences from zebrafish, thus providing the first ever glimpse of the complete antibody diversity in an organism. Zebrafish were shown to utilize between 50% and 86% of all possible germline VDJ combinations. Additionally convergence was observed such that several fish had identical antibody sequences (Weinstein et al., 2009). In a follow-up work, Jiang et al. showed that in early stages of zebrafish development, the VDJ repertoire is highly stereotypical, but becomes more diversified as it matures, suggesting both deterministic and stochastic elements shaped the antibody repertoire (Jiang et al., 2011). Boyd, Fire, and colleagues recently explored the application of antibody repertoire sequencing for clinical studies. In this work, the B lymphocyte clonality of V_H genes was determined from the blood of healthy patients and from various tissues (e.g., bone marrow, lymph nodes) of patients with haematological

malignancies. This study demonstrated that high-resolution analysis of immunoglobulin repertoires and their clonality offers a potential strategy for diagnosis and therapeutic monitoring in cancer (Boyd et al., 2009).

Similarly the human TCR repertoire has been analyzed by Illumina-based high-throughput sequencing; high-resolution analyses were performed on CDR3 length distribution frequency and germline usage in TCR β chains from peripheral blood T cells (Freeman et al., 2009; Robins et al., 2009). In an expanded study, the TCR β CDR3 regions from naïve and memory CD8 $^+$ T cells isolated from seven patients were sequenced revealing a strong preferential usage of certain V–J combinations, with a bias towards a reduction in nontemplated nucleotide insertions. A computational analysis indicated a substantial overlap in the CD8 $^+$ CDR3 repertoire between any two individuals, approaching ~ 7000 fold greater than what would be expected based on the theoretical diversity of the TCR repertoire (Robins et al., 2010). In another similar study, the TCR β CDR3 regions from three different individuals were sequenced; comparisons between these repertoires (at the amino acid level) also revealed substantial overlap (e.g., two donors had 14.4% common sequences) (Warren et al., forthcoming). These studies suggest that overlap may be indicative of CDR3 TCRs that share specificity to common pathogens; the presence of such sequences could potentially be used as biomarkers for detection and diagnosis in infectious and autoimmune disease.

High-throughput DNA sequencing and analysis of immune repertoires has also started to make an impact on the field of monoclonal antibody discovery and engineering. For example, the antibody repertoire diversity in a phage display human combinatorial library was precisely quantified using 454 sequencing and bioinformatic analysis; it was found that nearly all germline families were present and that a substantial amount of diversity was generated by somatic mutations in CDR1 and 2 (Glanville et al., 2009). Similarly, a synthetic antibody library constructed by polynucleotide annealing and extension (amplification, PCR-free) was subjected to deep-sequencing for accurate determination of diversity and quality control (Ge et al., 2010). The Illumina platform was used to sequence CDR3 regions from an antibody fragment phage display library following rounds of antigen panning; this sequence-based approach allowed for identification and rescue of clones that were lost by subsequent rounds of screening (Ravn et al., 2010). In addition to *in vitro* libraries, high-throughput sequencing has been utilized for sequencing of antibody repertoires in immunized animals. Recently, 454 sequencing was performed on the antibody repertoires derived from bone marrow plasma cells of protein-immunized mice; it was found that V_H and V_L repertoires were highly polarized with the most abundant sequences representing 1–10% of the entire repertoire (Reddy et al., 2010). The most abundant V_H and V_L genes were paired simply by relative frequency and then constructed by high-throughput automated gene synthesis. Following recombinant expression, it was found that $>75\%$ (21/27) of antibodies were antigen-specific, representing the first ever example of monoclonal antibody discovery without screening.

Conclusions

The development of high-throughput technologies has led to a substantial amount of progress in the understanding of adaptive immune responses, which has allowed for a shift towards information-rich, systems-based approaches to immunological research (Table A6-1). Techniques that utilize single-cell analysis have generated high content genetic and functional data from various lymphocyte populations in mice and humans. Additionally, protein microarray technologies have been extensively used for humoral serological profiling and identification of antigens from a variety of pathogens and disease states. Finally, high-throughput DNA sequencing of adaptive immune repertoires has facilitated substantial advances in our understanding of immunological diversity and its subsequent exploitation for clinical and technological applications. These are only the beginning stages; the next steps will be to leverage these high-throughput technologies for the integration of biophysical, biochemical, genomic, and proteomic data sets, which will require the incorporation of statistical and mathematical modelling. This rapidly expanding field of experimental systems immunology will enable a more comprehensive understanding of adaptive immunity.

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A7

THE NEW SCIENCE OF SOCIOMICROBIOLOGY AND THE REALM OF SYNTHETIC AND SYSTEMS ECOLOGY

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The Emergence of Sociomicrobiology as an Interest Area in Microbiology

Up until the later part of the past century, microbiologists believed that with rare exceptions bacteria did not practice sociality; this was in spite of the fact that growing bacteria as colonies on agar plates was and still remains a cornerstone technology for the discipline. Just the terminology “colonial” or “colony growth” implies some sort of social interactions. But with the few exceptional scientists working on myxobacteria, a special group of social bacteria, the eyes of microbiology did not see social interactions among individual bacteria. The long-standing view that bacteria were by-and-large asocial creatures began to crumble in the 1990s with rapid developments in the areas of quorum sensing and biofilm research. Now it seems apparent that the same selective pressures that led to the evolution of sociality in animals are forces for evolution of sociality in bacteria, and we see and appreciate social behavior in bacteria (Camilli and Bassler, 2006; Parsek and Greenberg, 2005). I have no proof but I believe that contributing to our past resistance to sociomicrobiology is the fact that bacteria are at once single cells and single individuals. They have served biology as wonderful models for cellular activities. We have not focused as much on the bacterial cell as an individual member of a species as we have on their existence as single-celled organisms.

My laboratory has focused on three areas of sociomicrobiology. The first is quorum sensing, which can be defined as a cell-to-cell communication system that enables individuals to sense the local population density and regulate expression of specific genes in response to population density. This is the primary topic

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of this paper. The second is biofilm biology. Biofilms are organized assemblages of bacteria embedded in a self-produced extracellular matrix. As a consequence of the biofilm lifestyle, the assembled bacteria exist in a heterogeneous environment and bacteria in different regions of a biofilm have different functions, which confer different traits to the group. As a consequence of the biofilm lifestyle, at least a subpopulation of the assemblage is protected from environmental stresses and in fact biofilm infections are difficult or impossible to cure by antibiotic treatment (Costerton et al., 1999). Because biofilm infections are very prevalent medical problems there is considerable interest in developing novel therapies that are effective in killing biofilm bacteria. To name just a few, infections of any implanted medical device are biofilm infections, cystic fibrosis lung infections are biofilm infections, heart valve infections are biofilms, and at least some chronic middle ear infections are biofilms (Costerton et al., 1999). Third, we have also worked on conspecific territoriality exhibited by swarms of bacteria moving over surfaces (Gibbs and Greenberg, 2011; Gibbs et al., 2008).

The emergence of sociomicrobiology as a new subdiscipline is important for several reasons. First, there is an idea that we might be able to develop novel antivirulence therapeutics, which target quorum sensing in bacterial species that control virulence gene expression by cell-to-cell signaling. In fact there are many investigators working to identify potent quorum-sensing and biofilm inhibitors, and we ourselves have undertaken such programs together with collaborators in the pharmaceutical industry (Banin et al., 2008; Muh et al., 2006a,b), but this is not the primary motivation of our work. This is in part because it is my belief that we do not yet understand enough about the biology of quorum sensing to predict how, when, or where its inhibition might be of therapeutic value. Second, it is now clear that if one strives to really understand bacteria, social aspects of their biology cannot be ignored. Third, now that we understand that bacteria are social and we understand sociality at an unprecedented level of molecular detail in bacteria like *Pseudomonas aeruginosa*, these model organisms have become wonderful tools to study fundamental questions about the costs and benefits of cooperation, the selective pressures that lead to cooperative behaviors, and the advantages of controlling cooperative behaviors by communication. Finally, our detailed understanding of quorum-sensing signal synthesis and signal reception, as is discussed below, has led to the widespread use of quorum-sensing regulatory circuits in synthetic biology (Marguet et al., 2010, and references therein).

Quorum Sensing in *Proteobacteria*

In the late 1960s and early 1970s there was a very modest literature describing pheromone production and activity in bacteria (Eberhard, 1972; Tomasz, 1965). It was not until the 1980s that work on quorum sensing in marine luminescent bacteria led to the idea that these sorts of gene regulatory activities function as intercellular communication systems that coordinate group activities.

Not until the 1990s did we begin to understand the prevalence of quorum sensing in bacteria. Although there are many different types of bacterial cell-to-cell signaling systems considered as quorum-sensing systems, our work focuses on acyl-homoserine lactone quorum-sensing systems prevalent but not universal among the Proteobacteria. Our original model system was a marine bacterium, *Vibrio fischeri*, which controls a small set of about 25 or fewer genes, including genes for light production, by a transcriptional activator called LuxR, and 3-oxo-hexanoyl-homoserine lactone (3OC6-HSL), which is produced by the *luxI* gene product (Antunes et al., 2007; Engebrecht et al., 1983). This quorum-sensing system allows *V. fischeri* to discriminate between its free-living low-population-density lifestyle and its high-density host-associated lifestyle (Figure A7-1). It exists in the light organs of specific marine animals where it produces light, which serves the mutualistic symbiosis. Of note, 3OC6-HSL moves in and out of cells by passive diffusion. In this way the environmental signal concentration is a reflection of cell density. The *luxI* gene itself is controlled by quorum sensing—it is activated by LuxR and 3OC6-HSL. In terms of biology, this provides hysteresis to the system. The population density required to activate quorum-controlled genes is much higher than the density required to shut down an activated system.

We later turned our attention to the opportunistic pathogen *P. aeruginosa*. We learned that there were two acyl-HSL circuits, the C4-HSL-RhlI-RhlR circuit and the 3OC12-HSL-LasI-LasR circuits, which together are required for activation of about 325 genes (Figure A7-2) (Pearson et al., 1994, 1995; Schuster et al., 2003). Other investigators showed that at least under certain experimental conditions quorum-sensing mutants were impaired in virulence (Pearson et al., 2000; Tang et al., 1996), and thus the belief was that, as for *V. fischeri* quorum sensing, *P. aeruginosa* quorum sensing allowed discrimination between host and free-living states. However, it is not clear from the evidence that this is in fact the case. Among the 300-plus quorum-controlled genes, those coding for extracellular products like exoenzymes or coding for functions required for the production of exoproducts like hydrogen cyanide or pyocyanin are grossly overrepresented.

In a social context these sorts of extracellular products can be considered public goods or resources produced by individuals and shared by all members of the group. Thus we believe quorum sensing serves to coordinate cooperative behaviors. Investigators have devised experiments where growth of *P. aeruginosa* requires quorum sensing. This can be accomplished by providing protein as the sole source of carbon and energy. Growth requires quorum-sensing-induced production of the exoprotease elastase. In this setting, LasR quorum-sensing mutants will emerge and become a stable, significant minority of the overall population. These cheaters or freeloaders do not bear the cost of contributing to the public good (elastase in this case), but they benefit from use of the public goods (Sandoz et al., 2007). It is obvious that light production by *V. fischeri* is a shared behavior and we believe this also represents quorum control of cooperation. As far as I am aware, there are no biological systems sensitive enough to detect the light pro-

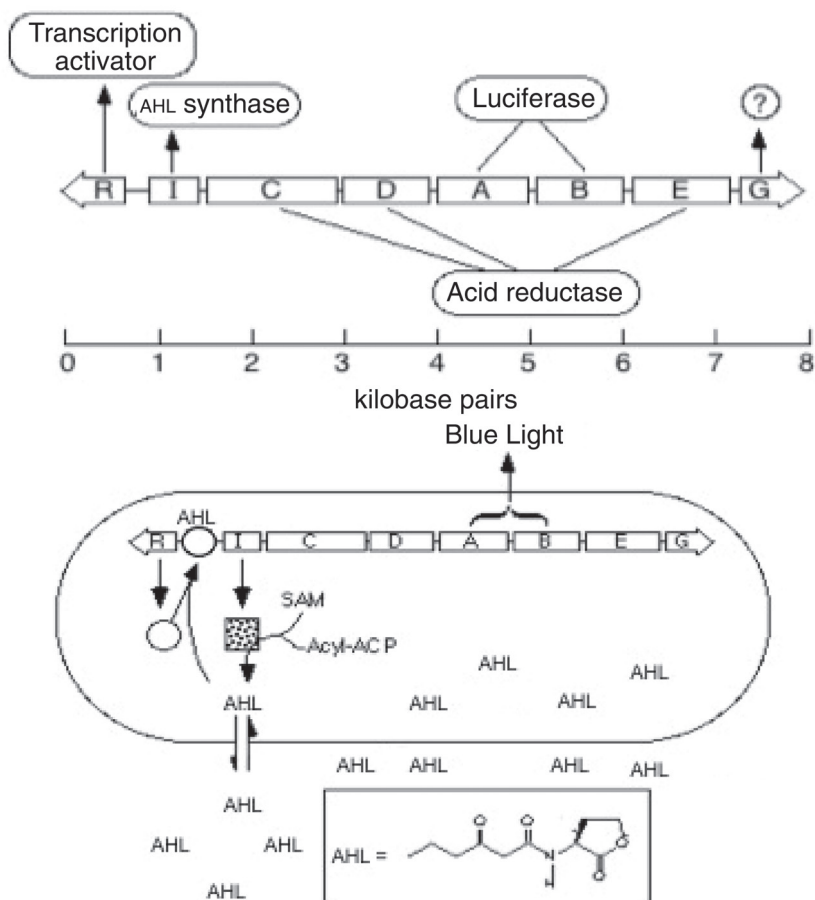


FIGURE A7-1 Quorum sensing in *Vibrio fischeri*. (Top) The *lux* gene cluster. The *luxR* gene encodes a 3OC6-HSL (AHL)-dependent transcriptional activator of the *luxI-G* operon. The *luxI* product is the AHL synthase; *luxC*, *D*, and *E* form a complex responsible for generation of one of the substrates for the luciferase reaction, *luxA* and *B* encode the two subunits of luciferase, and the function of *luxG* remains unknown. (Bottom) Cartoon of a *V. fischeri* cell producing the diffusible AHL signal. At low cell densities the luminescence operon is transcribed at a basal level. At high cell densities the AHL signal can reach a sufficient concentration and bind to the cellular LuxR protein, which will then activate transcription of the luminescence operon. The substrates for AHL synthesis are shown, as is the molecule. Note the positive autoregulation of *luxI*, a common feature of R-I circuits.

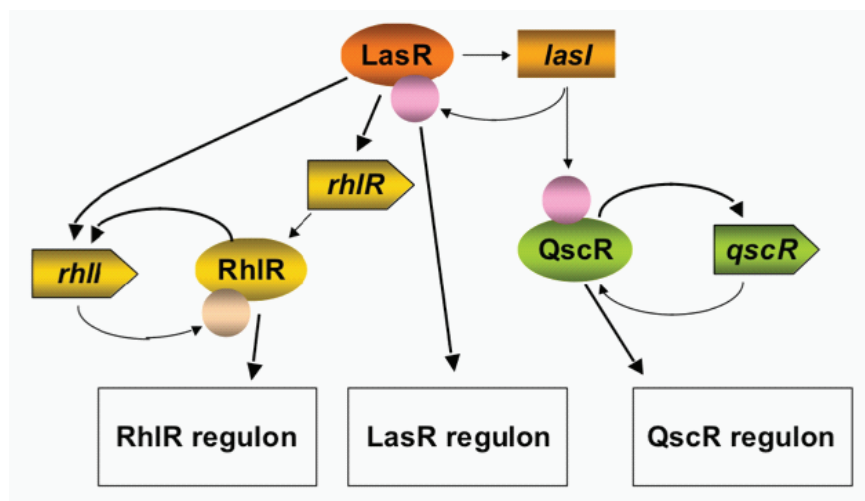


FIGURE A7-2 Diagram of the acyl-HSL quorum-sensing regulatory circuit in *P. aeruginosa*. The *lasI* and *R* genes are linked on the chromosome, as are the *rhlR* and *I* genes. The *qscR* gene is not linked tightly to any other quorum-sensing regulator. LasI-R sit atop a quorum-sensing cascade. The *rhl* quorum-sensing system is among the functions controlled by LasI-R and transcription of *qscR* is positively autoregulated but requires production of 3OC12-HSL by LasI. Pink ball, 3OC12-HSL; orange ball, C4-HSL.

duced by one or a few *V. fischeri* cells (maximum emission is about 1,000 photons per second per cell). But the light produced by large groups of cells can easily be observed. For example, we can see the light produced by colonies of *V. fischeri* growing on a Petri plate. So here are two examples where it seems apparent that quorum-sensing functions control and coordinate cooperative behaviors. Is this universal for LuxR-LuxI-acyl-HSL-type systems? Of course we do not know the answer to this question and one might imagine that such regulatory elements have been adapted by different bacteria for different purposes.

As a general principle, the LuxR-LuxI type of regulatory circuits appears to represent elements of true communication. That is, the signal and the signal receptor are coevolved (Keller and Surette, 2006). One finds that a disturbing anthropomorphic trend has crept into the field and the term “bacterial language” is sometimes used to describe quorum-sensing systems. I do not believe any linguist would find evidence for syntax and grammar in the bacterial world.

With the discovery that quorum-sensing-controlled virulence of bacteria like *P. aeruginosa*, the number of scientists studying acyl-HSL signaling grew exponentially. Generally speaking, studies of quorum sensing and control of social activity in bacteria are important for the several reasons listed earlier in this paper. We might be able to develop novel antivirulence therapeutics that target

quorum sensing in bacteria like *P. aeruginosa*. Sociality is an important aspect of bacterial biology. Bacteria are now viewed as experimental models for fundamental studies about the costs and benefits of cooperation, the selective pressures that lead to cooperative behaviors, and the advantages of controlling cooperative behaviors by communication. In addition, our detailed understanding of signal synthesis by Lux-type proteins and signal reception by LuxR-type transcription factors has led to their widespread use in the area of synthetic biology (Marguet et al., 2010). This was a largely unintended consequence of our work, and I believe there are three reasons for the popularity of these systems in building synthetic regulatory circuits. First, the acyl-HSLs are diffusible signaling ligands (Kaplan and Greenberg, 1985). Thus, the complication of transport systems for the ligand need not concern the synthetic biologist. Second, the *V. fischeri* system (Engebrecht et al., 1983), the *P. aeruginosa* systems, and many other acyl-HSL quorum-sensing circuits have positive autoregulatory loops. Signal production proceeds at a low level until it has accumulated sufficiently to activate quorum-controlled genes, one of which is the gene for its own synthesis. The signal concentration then increases very rapidly. Work in the area of synthetic biology has focused on this phenomenon as a method to reduce noise in regulatory circuits or build gene expression oscillators. Finally, we have identified many different systems and we know of dozens of acyl-HSL signals—the signal specificity of a given LasR homolog resides in the nature of the acyl side group. Thus, multiple systems can be strung together in a single bacterium with each controlling, for example, expression of fluorescent proteins that emit at different wavelengths. Examples showing the diversity of acyl-HSL signals identified in different bacterial species are shown in Figure A7-3.

New Twists to the Acyl-Homoserine Lactone Signaling Story

The *P. aeruginosa* quorum-sensing circuitry described in Figure A7-2 shows, in addition to LasR-LasI and RhIR-RhII, a LuxR homolog, QscR. There is no cognate acyl-HSL synthase gene for QscR and it has been termed an orphan quorum-sensing signal receptor (Chugani et al., 2001). These orphans, which have also been called solos (Subramoni and Venturi, 2009), are quite commonly found in sequenced proteobacterial genomes. We know that QscR responds to the LasI-produced 3OC12-HSL, and it controls a set of genes that partially overlaps with the genes controlled by LasR and RhIR (Lee et al., 2006; Lequette et al., 2006). There has been recent interest in studying orphans (Subramoni and Venturi, 2009). There are orphan LuxR homologs in *Salmonella* and in pathogenic *E. coli*, neither of which have any *luxI* homologs. Evidence indicates that the *E. coli* and *Salmonella* LuxR orphans respond to acyl-HSLs made by other bacterial species in mixed microbial communities (Soares and Ahmer, 2011; Sperandio, 2010).

Most of the acyl-HSL signals that have been identified are fatty acyl-HSLs with fatty acyl groups of varying carbon length and with a limited number of

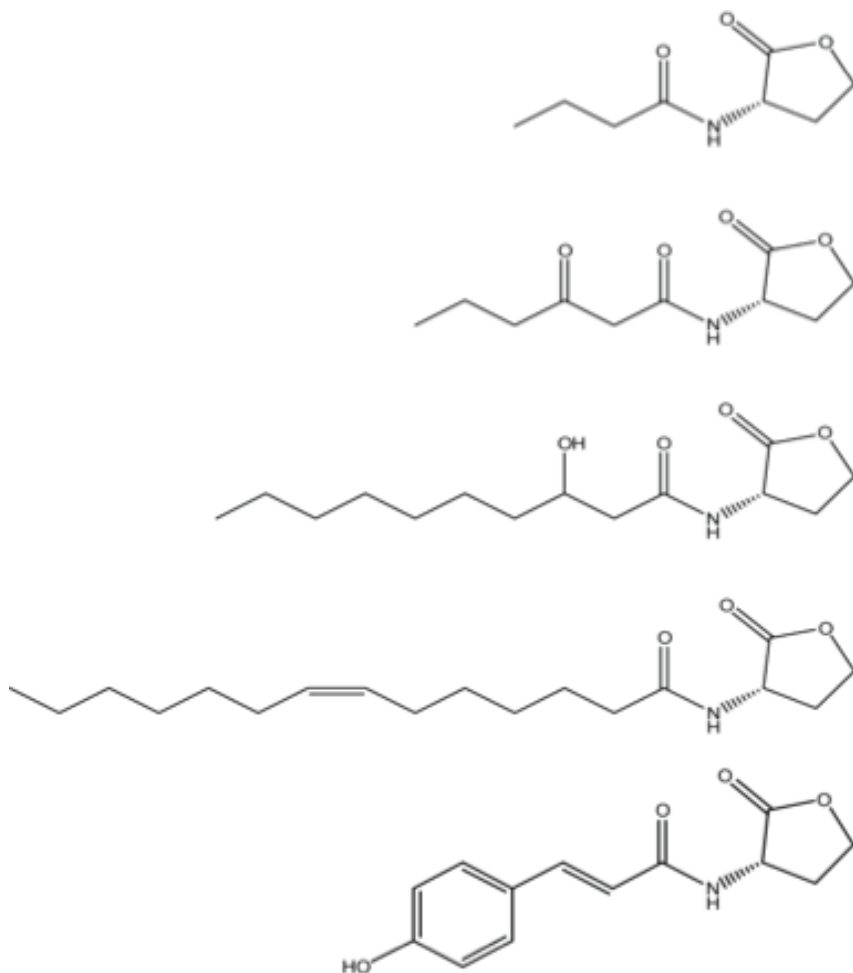


FIGURE A7-3 Some examples of acyl-HSL quorum-sensing signals. From top to bottom; the *P. aeruginosa* RhII signal butanoyl-HSL, the *V. fischeri* LuxI signal, 3-oxo-hexanoyl-HSL, The *B. mallei* BmaI signal, 3-hydroxy-decanoyl-HSL, and the *Rhodopseudomonas palustris* RpaI signal, *para*-coumaroyl-HSL.

substitutions on the fatty acyl carbon chain. We recently described examples of two bacteria that use LuxI-LuxR homologs to produce and respond to aryl-HSLs. Both are photosynthetic; *Rhodopseudomonas palustris* uses *p*-coumaroyl-HSL as a quorum-sensing signal and photosynthetic baradyrhizobia uses cinnamoyl-HSL (Ahlgren et al., 2011; Schaefer et al., 2008). These discoveries broaden the possibilities in terms of signal molecules. Furthermore, the photosynthetic bradyrhizobia produce nanomolar amounts of cinnamoyl-HSL and respond to picomolar

amounts. This is of interest because the fatty acyl-HSL systems that have been described involve responses to nanomolar signal levels and the signals are usually produced at levels in the micromolar range. The bradyrhizobium system is shifted to much lower levels for production and detection. The significance of the low production and ultrasensitivity is not clearly understood. However, it is clear that, by assuming acyl-HSLs will be produced at higher levels, we likely have overlooked at least some acyl-HSL signaling systems.

Concluding Comments

This paper seeks to introduce an emerging field, sociomicrobiology, and to point out opportunities for synthetic biology and therapeutic development in the area of sociomicrobiology. The paper emphasizes quorum sensing in Proteobacteria and provides examples where quorum sensing serves to allow individuals of a species to communicate. This rudimentary form of chemical communication serves to coordinate certain cooperative behaviors. Whether this is a universal characteristic of acyl-HSL signaling systems remains to be seen. There are also a variety of other small-molecule signals produced by bacteria and many of these are considered quorum-sensing signals (Camilli and Bassler, 2006). If and how these intercellular signals are controlling cooperation also remains to be seen. Finally, this Forum has covered topics related to synthetic and systems biology, and opportunities in the area of human health and disease. We need to remain cognizant that bacterial cells are individual organisms involved in complex social interactions. So not only do we need to think about systems and synthetic biology from a cellular perspective, but we need to think about systems and synthetic ecology too.

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A8

CREATION OF A BACTERIAL CELL CONTROLLED BY A CHEMICALLY SYNTHESIZED GENOME⁴⁵

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We report the design, synthesis, and assembly of the 1.08-mega-base pair *Mycoplasma mycoides* JCVI-syn1.0 genome starting from digitized genome sequence information and its transplantation into a *M. capricolum* recipient cell to create new *M. mycoides* cells that are controlled only by the

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Materials and Methods

Figs. S1 to S6

Tables S1 to S7

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synthetic chromosome. The only DNA in the cells is the designed synthetic DNA sequence, including “watermark” sequences and other designed gene deletions and polymorphisms, and mutations acquired during the building process. The new cells have expected phenotypic properties and are capable of continuous self-replication.

In 1977, Sanger and colleagues determined the complete genetic sequence of phage ϕ X174 (Sanger et al., 1977), the first DNA genome to be completely sequenced. Eighteen years later, in 1995, our team was able to read the first complete genetic sequence of a self-replicating bacterium, *Haemophilus influenzae* (Fleischmann et al., 1995). Reading the genetic sequence of a wide range of species has increased exponentially from these early studies. The ability to rapidly digitize genomic information has increased by more than eight orders of magnitude over the past 25 years (Venter, 2010). Efforts to understand all this new genomic information have spawned numerous new computational and experimental paradigms, yet our genomic knowledge remains very limited. No single cellular system has all of its genes understood in terms of their biological roles. Even in simple bacterial cells, do the chromosomes contain the entire genetic repertoire? If so, can a complete genetic system be reproduced by chemical synthesis starting with only the digitized DNA sequence contained in a computer?

Our interest in synthesis of large DNA molecules and chromosomes grew out of our efforts over the past 15 years to build a minimal cell that contains only essential genes. This work was inaugurated in 1995 when we sequenced the genome of *Mycoplasma genitalium*, a bacterium with the smallest complement of genes of any known organism capable of independent growth in the laboratory. More than 100 of the 485 protein-coding genes of *M. genitalium* are dispensable when disrupted one at a time (Hutchison et al., 1999; Glass et al., 2006; Smith et al., 2008). We developed a strategy for assembling viral-sized pieces to produce large DNA molecules that enabled us to assemble a synthetic *M. genitalium* genome in four stages from chemically synthesized DNA cassettes averaging about 6 kb in size. This was accomplished through a combination of in vitro enzymatic methods and in vivo recombination in *Saccharomyces cerevisiae*. The whole synthetic genome [582,970 base pairs (bp)] was stably grown as a yeast centromeric plasmid (YCp) (Gibson et al., 2008b).

Several hurdles were overcome in transplanting and expressing a chemically synthesized chromosome in a recipient cell. We needed to improve methods for extracting intact chromosomes from yeast. We also needed to learn how to transplant these genomes into a recipient bacterial cell to establish a cell controlled only by a synthetic genome. Because *M. genitalium* has an extremely slow growth rate, we turned to two faster-growing mycoplasma species, *M. mycoides* subspecies *capri* (GM12) as donor, and *M. capricolum* subspecies *capricolum* (CK) as recipient.

To establish conditions and procedures for transplanting the synthetic ge-

nome out of yeast, we developed methods for cloning entire bacterial chromosomes as centromeric plasmids in yeast, including a native *M. mycoides* genome (Lartigue et al., 2009; Benders et al., 2010). However, initial attempts to extract the *M. mycoides* genome from yeast and transplant it into *M. capricolum* failed. We discovered that the donor and recipient mycoplasmas share a common restriction system. The donor genome was methylated in the native *M. mycoides* cells and was therefore protected against restriction during the transplantation from a native donor cell (Lartigue et al., 2007). However, the bacterial genomes grown in yeast are unmethylated and so are not protected from the single restriction system of the recipient cell. We overcame this restriction barrier by methylating the donor DNA with purified methylases or crude *M. mycoides* or *M. capricolum* extracts, or by simply disrupting the recipient cell's restriction system (Lartigue et al., 2009).

We now have combined all of our previously established procedures and report the synthesis, assembly, cloning, and successful transplantation of the 1.08-Mbp *M. mycoides* JCVI-syn1.0 genome, to create a new cell controlled by this synthetic genome.

Synthetic Genome Design

Design of the *M. mycoides* JCVI-syn1.0 genome was based on the highly accurate finished genome sequences of two laboratory strains of *M. mycoides* subspecies *capri* GM12 (Lartigue et al., 2009; Benders et al., 2010).⁴⁹ One was the genome donor used by Lartigue et al. [GenBank accession CP001621] (2007). The other was a strain created by transplantation of a genome that had been cloned and engineered in yeast, YCpMmyc1.1- Δ *typeIIIres* [GenBank accession CP001668] (Lartigue et al., 2009). This project was critically dependent on the accuracy of these sequences. Although we believe that both finished *M. mycoides* genome sequences are reliable, there are 95 sites at which they differ. We began to design the synthetic genome before both sequences were finished. Consequently, most of the cassettes were designed and synthesized based on the CP001621 sequence.⁴⁹ When it was finished, we chose the sequence of the genome successfully transplanted from yeast (CP001668) as our design reference (except that we kept the intact *typeIIIres* gene). All differences that appeared biologically significant between CP001668 and previously synthesized cassettes were corrected to match it exactly.⁴⁹ Sequence differences between our synthetic cassettes and CP001668 that occurred at 19 sites appeared harmless and so were not corrected. These provide 19 polymorphic differences between our synthetic genome (JCVI-syn1.0) and the natural (nonsynthetic) genome (YCpMmyc1.1) that we have cloned in yeast and use as a standard for genome transplantation from yeast (Lartigue et al., 2009). To further differentiate between the synthetic

⁴⁹ Supporting material is available on *Science* Online.

genome and the natural one, we designed four watermark sequences (fig. S1) to replace one or more cassettes in regions experimentally demonstrated [watermarks 1 (1246 bp) and 2 (1081 bp)] or predicted [watermarks 3 (1109 bp) and 4 (1222 bp)] to not interfere with cell viability. These watermark sequences encode unique identifiers while limiting their translation into peptides. Table S1 lists the differences between the synthetic genome and this natural standard. Figure S2 shows a map of the *M. mycoides* JCVI-syn1.0 genome. Cassette and assembly intermediate boundaries, watermarks, deletions, insertions, and genes of the *M. mycoides* JCVI syn1.0 are shown in fig. S2, and the sequence of the transplanted mycoplasma clone sMmYCP235-1 has been submitted to GenBank (accession CP002027).

Synthetic Genome Assembly Strategy

The designed cassettes were generally 1080 bp with 80-bp overlaps to adjacent cassettes.⁵⁰ They were all produced by assembly of chemically synthesized oligonucleotides by Blue Heron (Bothell, Washington). Each cassette was individually synthesized and sequence-verified by the manufacturer. To aid in the building process, DNA cassettes and assembly intermediates were designed to contain Not I restriction sites at their termini and recombined in the presence of vector elements to allow for growth and selection in yeast (Gibson et al., 2008b).⁵⁰ A hierarchical strategy was designed to assemble the genome in three stages by transformation and homologous recombination in yeast from 1078 1-kb cassettes (Fig. A8-1) (Gibson, 2009; Gibson et al., 2008a).

Assembly of 10-kb synthetic intermediates

In the first stage, cassettes and a vector were recombined in yeast and transferred to *Escherichia coli*.⁵⁰ Plasmid DNA was then isolated from individual *E. coli* clones and digested to screen for cells containing a vector with an assembled 10-kb insert. One successful 10-kb assembly is represented (Fig. A8-2A). In general, at least one 10-kb assembled fragment could be obtained by screening 10 yeast clones. However, the rate of success varied from 10 to 100%. All of the first-stage intermediates were sequenced. Nineteen out of 111 assemblies contained errors. Alternate clones were selected, sequence-verified, and moved on to the next assembly stage.⁵⁰

Assembly of 100-kb synthetic intermediates

The pooled 10-kb assemblies and their respective cloning vectors were transformed into yeast as above to produce 100-kb assembly intermediates.⁵⁰

⁵⁰ Supporting material is available on *Science* Online.

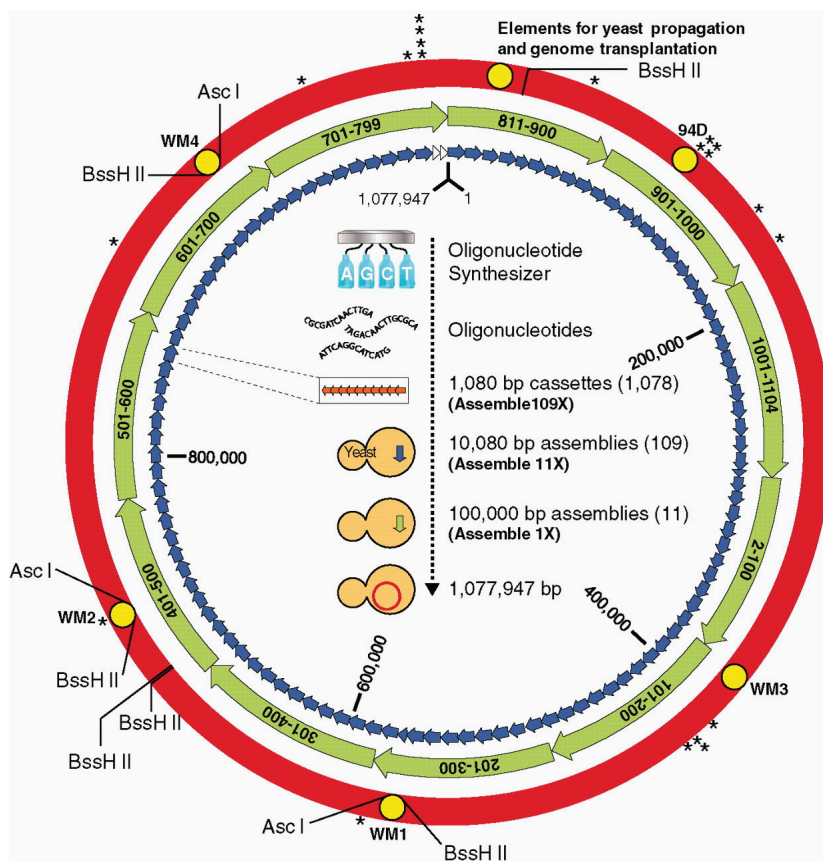


FIGURE A8-1 The assembly of a synthetic *M. mycoides* genome in yeast. A synthetic *M. mycoides* genome was assembled from 1078 overlapping DNA cassettes in three steps. In the first step, 1080-bp cassettes (orange arrows), produced from overlapping synthetic oligonucleotides, were recombined in sets of 10 to produce 109 ~10-kb assemblies (blue arrows). These were then recombined in sets of 10 to produce 11 ~100-kb assemblies (green arrows). In the final stage of assembly, these 11 fragments were recombined into the complete genome (red circle). With the exception of two constructs that were enzymatically pieced together in vitro (Gibson et al., 2009) (white arrows), assemblies were carried out by in vivo homologous recombination in yeast. Major variations from the natural genome are shown as yellow circles. These include four watermarked regions (WM1 to WM4), a 4-kb region that was intentionally deleted (94D), and elements for growth in yeast and genome transplantation. In addition, there are 20 locations with nucleotide polymorphisms (asterisks). Coordinates of the genome are relative to the first nucleotide of the natural *M. mycoides* sequence. The designed sequence is 1,077,947 bp. The locations of the Asc I and BssH II restriction sites are shown. Cassettes 1 and 800-810 were unnecessary and removed from the assembly strategy. (Supporting material is available on *Science Online*.) Cassette 2 overlaps cassette 1104, and cassette 799 overlaps cassette 811.

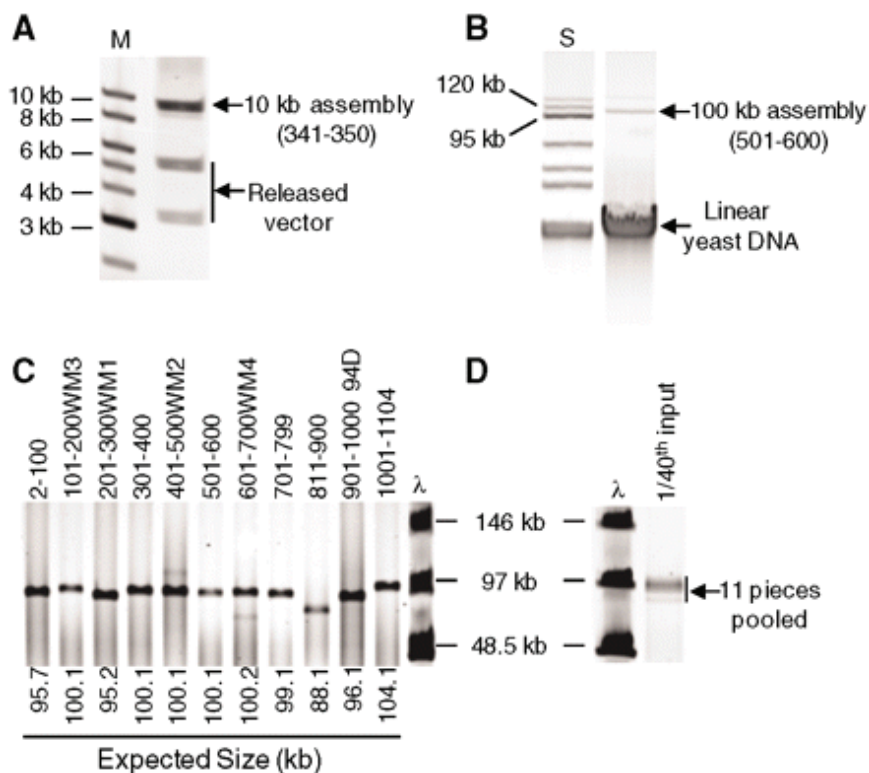


FIGURE A8-2 Analysis of the assembly intermediates. (A) Not I and Sbf I double restriction digestion analysis of assembly 341-350 purified from *E. coli*. These restriction enzymes release the vector fragments (5.5 and 3.4 kb) from the 10-kb insert. Insert DNA was separated from the vector DNA on a 0.8% E-gel (Invitrogen). M indicates the 1-kb DNA ladder (New England Biolabs; NEB). (B) Analysis of assembly 501-600 purified from yeast. The 105-kb circles (100-kb insert plus 5-kb vector) were separated from the linear yeast chromosomal DNA on a 1% agarose gel by applying 4.5 V/cm for 3 hours. S indicates the BAC-Tracker supercoiled DNA ladder (Epicentre). (C) Not I restriction digestion analysis of the 11 ~100-kb assemblies purified from yeast. These DNA fragments were analyzed by FIGE on a 1% agarose gel. The expected insert size for each assembly is indicated. λ indicates the lambda ladder (NEB). (D) Analysis of the 11 pooled assemblies shown in (C) following topological trapping of the circular DNA and Not I digestion. One-fortieth of the DNA used to transform yeast is represented.

Our results indicated that these products cannot be stably maintained in *E. coli*, so recombinant DNA had to be extracted from yeast. Multiplex polymerase chain reaction (PCR) was performed on selected yeast clones (fig. S3 and table S2). Because every 10-kb assembly intermediate was represented by a primer pair in this analysis, the presence of all amplicons would suggest an assembled 100-kb

intermediate. In general, 25% or more of the clones screened contained all of the amplicons expected for a complete assembly. One of these clones was selected for further screening. Circular plasmid DNA was extracted and sized on an agarose gel alongside a supercoiled marker. Successful second-stage assemblies with the vector sequence are ~105 kb in length (Fig. A8-2B). When all amplicons were produced following multiplex PCR, a second-stage assembly intermediate of the correct size was usually produced. In some cases, however, small deletions occurred. In other instances, multiple 10-kb fragments were assembled, which produced a larger second-stage assembly intermediate. Fortunately, these differences could easily be detected on an agarose gel before complete genome assembly.

Complete genome assembly

In preparation for the final stage of assembly, it was necessary to isolate microgram quantities of each of the 11 second-stage assemblies.⁵¹ As reported (Devenish and Newlon, 1982), circular plasmids the size of our second-stage assemblies could be isolated from yeast spheroplasts after an alkaline-lysis procedure. To further purify the 11 assembly intermediates, they were treated with exonuclease and passed through an anion-exchange column. A small fraction of the total plasmid DNA (1/100) was digested with Not I and analyzed by field-inversion gel electrophoresis (FIGE) (Fig. A8-2C). This method produced ~1 mg of each assembly per 400 ml of yeast culture (~10¹¹ cells).

The method above does not completely remove all of the linear yeast chromosomal DNA, which we found could substantially decrease the yeast transformation and assembly efficiency. To further enrich for the 11 circular assembly intermediates, ~200 ng samples of each assembly were pooled and mixed with molten agarose. As the agarose solidifies, the fibers thread through and topologically “trap” circular DNA (Dean et al., 1973). Untrapped linear DNA can then be separated out of the agarose plug by electrophoresis, thus enriching for the trapped circular molecules. The 11 circular assembly intermediates were digested with Not I so that the inserts could be released. Subsequently, the fragments were extracted from the agarose plug, analyzed by FIGE (Fig. A8-2D), and transformed into yeast spheroplasts.⁵¹ In this third and final stage of assembly, an additional vector sequence was not required because the yeast cloning elements were already present in assembly 811-900.

To screen for a complete genome, multiplex PCR was carried out with 11 primer pairs, designed to span each of the 11 100-kb assembly junctions (table S3). Of 48 colonies screened, DNA extracted from one clone (sMmYCp235) produced all 11 amplicons. PCR of the wild-type positive control (YCpMmyc1.1) produced an indistinguishable set of 11 amplicons (Fig. A8-3A). To further demonstrate the complete assembly of a synthetic *M. mycoides* genome, intact DNA

⁵¹ Supporting material is available on *Science Online*.

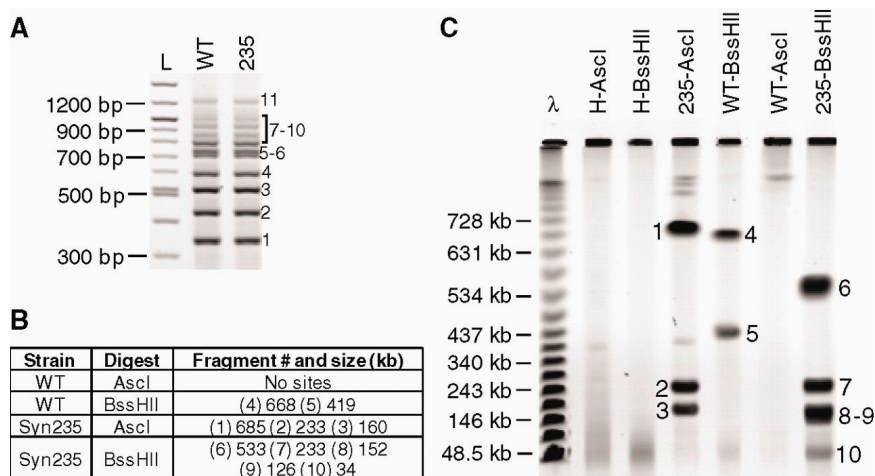


FIGURE A8-3 Characterization of the synthetic genome isolated from yeast. (A) Yeast clones containing a completely assembled synthetic genome were screened by multiplex PCR with a primer set that produces 11 amplicons; one at each of the 11 assembly junctions. Yeast clone sMmYCp235 (235) produced the 11 PCR products expected for a complete genome assembly. For comparison, the natural genome extracted from yeast (WT, wild type) was also analyzed. PCR products were separated on a 2% E-gel (Invitrogen). L indicates the 100-bp ladder (NEB). (B) The sizes of the expected Asc I and BssH II restriction fragments for natural (WT) and synthetic (Syn235) *M. mycoides* genomes. (C) Natural (WT) and synthetic (235) *M. mycoides* genomes were isolated from yeast in agarose plugs. In addition, DNA was purified from the host strain alone (H). Agarose plugs were digested with Asc I or BssH II, and fragments were separated by clamped homogeneous electrical field (CHEF) gel electrophoresis. Restriction fragments corresponding to the correct sizes are indicated by the fragment numbers shown in (B).

was isolated from yeast in agarose plugs and subjected to two restriction analyses: Asc I and BssH II.⁵² Because these restriction sites are present in three of the four watermark sequences, this choice of digestion produces restriction patterns that are distinct from that of the natural *M. mycoides* genome (Figs. A8-1 and A8-3B). The sMmYCp235 clone produced the restriction pattern expected for a completely assembled synthetic genome (Fig. A8-3C).

Synthetic Genome Transplantation

Additional agarose plugs used in the gel analysis above (Fig. A8-3C) were also used in genome transplantation experiments.⁵² Intact synthetic *M. mycoides*

⁵² Supporting material is available on *Science Online*.

genomes from the sMmYCp235 yeast clone were transplanted into restriction-minus *M. capricolum* recipient cells, as described (Lartigue et al., 2009). Results were scored by selecting for growth of blue colonies on SP4 medium containing tetracycline and X-gal at 37°C. Genomes isolated from this yeast clone produced 5 to 15 tetracycline-resistant blue colonies per agarose plug, a number comparable to that produced by the YCpMmyc1.1 control. Recovery of colonies in all transplantation experiments was dependent on the presence of both *M. capricolum* recipient cells and an *M. mycooides* genome.

Semisynthetic Genome Assembly and Transplantation

To aid in testing the functionality of each 100-kb synthetic segment, semisynthetic genomes were constructed and transplanted. By mixing natural pieces with synthetic ones, the successful construction of each synthetic 100-kb assembly could be verified without having to sequence these intermediates. We cloned 11 overlapping natural 100-kb assemblies in yeast by using a previously described method (Leem et al., 2003). In 11 parallel reactions, yeast cells were cotransformed with fragmented *M. mycooides* genomic DNA (YCpMmyc1.1) that averaged ~100 kb in length and a PCR-amplified vector designed to overlap the ends of the 100-kb inserts. To maintain the appropriate overlaps so that natural and synthetic fragments could be recombined, the PCR-amplified vectors were produced via primers with the same 40-bp overlaps used to clone the 100-kb synthetic assemblies. The semisynthetic genomes that were constructed contained between 2 and 10 of the 11 100-kb synthetic subassemblies (Table A8-1). The

TABLE A8-1 Genomes that have been assembled from 11 pieces and successfully transplanted. Assembly 2-100, 1; assembly 101-200, 2; assembly 201-300, 3; assembly 301-400, 4; assembly 401-500, 5; assembly 501-600, 6; assembly 601-700, 7; assembly 701-799, 8; assembly 811-900, 9; assembly 901-1000, 10; assembly 1001-1104, 11. WM, watermarked assembly.

Genome assembly	Synthetic fragments	Natural fragments
Reconstituted natural genome	None	1-11
2/11 semisynthetic genome with one watermark	5 WM, 10	1-4, 6-9, 11
8/11 semisynthetic genome without watermarks	1-4, 6-8, 11	5, 9, 10
9/11 semisynthetic genome without watermarks	1-4, 6-8, 10-11	5, 9
9/11 semisynthetic genome with three watermarks	1, 2 WM, 3 WM, 4, 6, 7 WM, 8, 10-11	5, 9
10/11 semisynthetic genome with three watermarks	1, 2 WM, 3 WM, 4, 5 WM, 6, 7 WM, 8, 10-11	9
11/11 synthetic genome, 811-820 correction of <i>dnaA</i>	1, 2 WM, 3 WM, 4, 5 WM, 6, 7 WM, 8, 9-11	None
11/11 synthetic genome, 811-900 correction of <i>dnaA</i>	1, 2 WM, 3 WM, 4, 5 WM, 6, 7 WM, 8, 9-11	None

production of viable colonies produced after transplantation confirmed that the synthetic fraction of each genome contained no lethal mutations. Only one of the 100-kb subassemblies, 811-900, was not viable.

Initially, an error-containing 811-820 clone was used to produce a synthetic genome that did not transplant. This was expected because the error was a single-base pair deletion that creates a frameshift in *dnaA*, an essential gene for chromosomal replication. We were previously unaware of this mutation. By using a semisynthetic genome construction strategy, we pinpointed 811-900 as the source for failed synthetic transplantation experiments. Thus, we began to reassemble an error-free 811-900 assembly, which was used to produce the sMmYCp235 yeast strain. The *dnaA*-mutated genome differs by only one nucleotide from the synthetic genome in sMmYCp235. This genome served as a negative control in our transplantation experiments. The *dnaA* mutation was also repaired at the 811-900 level by genome engineering in yeast (Noskov et al., 2010). A repaired 811-900 assembly was used in a final-stage assembly to produce a yeast clone with a repaired genome. This yeast clone is named sMmYCP142 and could be transplanted. A complete list of genomes that have been assembled from 11 pieces and successfully transplanted is provided in Table A8-1.

Characterization of the Synthetic Transplants

To rapidly distinguish the synthetic transplants from *M. capricolum* or natural *M. mycoides*, two analyses were performed. First, four primer pairs that are specific to each of the four watermarks were designed such that they produce four amplicons in a single multiplex PCR reaction (table S4). All four amplicons were produced by transplants generated from sMmYCp235, but not YCpMmyc1.1 (Fig. A8-4A). Second, the gel analysis with Asc I and BssH II, described above (Fig. A8-3C), was performed. The restriction pattern obtained was consistent with a transplant produced from a synthetic *M. mycoides* genome (Fig. A8-4B).

A single transplant originating from the sMmYCp235 synthetic genome was sequenced. We refer to this strain as *M. mycoides* JCVIsyn1.0. The sequence matched the intended design with the exception of the known polymorphisms, eight new single-nucleotide polymorphisms, an *E. coli* transposon insertion, and an 85-bp duplication (table S1). The transposon insertion exactly matches the size and sequence of IS1, a transposon in *E. coli*. It is likely that IS1 infected the 10-kb subassembly following its transfer to *E. coli*. The IS1 insert is flanked by direct repeats of *M. mycoides* sequence, suggesting that it was inserted by a transposition mechanism. The 85-bp duplication is a result of a nonhomologous end joining event, which was not detected in our sequence analysis at the 10-kb stage. These two insertions disrupt two genes that are evidently nonessential. We did not find any sequences in the synthetic genome that could be identified as belonging to *M. capricolum*. This indicates that there was a complete replacement of the *M. capricolum* genome by our synthetic genome during the trans-

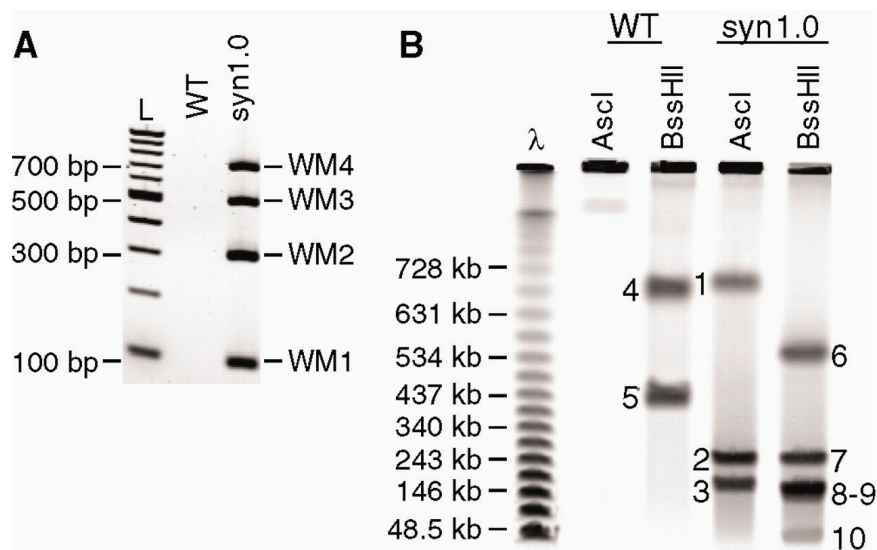


FIGURE A8-4 Characterization of the transplants. (A) Transplants containing a synthetic genome were screened by multiplex PCR with a primer set that produces four amplicons, one internal to each of the four watermarks. One transplant (syn1.0) originating from yeast clone sMmYCp235 was analyzed alongside a natural, nonsynthetic genome (WT) transplanted out of yeast. The transplant containing the synthetic genome produced the four PCR products, whereas the WT genome did not produce any. PCR products were separated on a 2% E-gel (Invitrogen). (B) Natural (WT) and synthetic (syn1.0) *M. mycooides* genomes were isolated from *M. mycooides* transplants in agarose plugs. Agarose plugs were digested with Asc I or BssH II and fragments were separated by CHEF gel electrophoresis. Restriction fragments corresponding to the correct sizes are indicated by the fragment numbers shown in Fig. A8-3B.

plant process. The cells with only the synthetic genome are self-replicating and capable of logarithmic growth. Scanning and transmission electron micrographs (EMs) of *M. mycooides* JCVI-syn1.0 cells show small, ovoid cells surrounded by cytoplasmic membranes (Fig. A8-5, C to F). Proteomic analysis of *M. mycooides* JCVI-syn1.0 and the wild-type control (YCpMmyc1.1) by two-dimensional gel electrophoresis revealed almost identical patterns of protein spots (fig. S4) that differed from those previously reported for *M. capricolum* (Lartigue et al., 2007). Fourteen genes are deleted or disrupted in the *M. mycooides* JCVI-syn1.0 genome; however, the rate of appearance of colonies on agar plates and the colony morphology are similar (compare Fig. A8-5, A and B). We did observe slight differences in the growth rates in a color-changing unit assay, with the JCVI-syn1.0 transplants growing slightly faster than the MmcyYCp1.1 control strain (fig. S6).

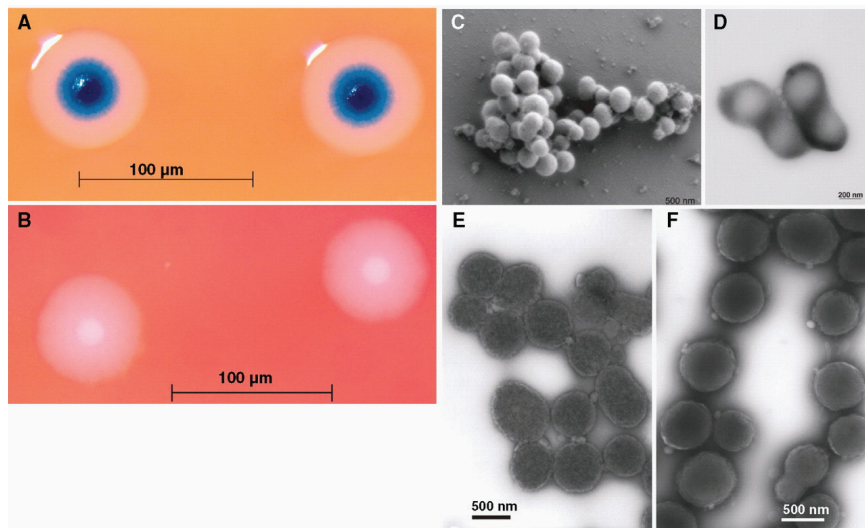


FIGURE A8-5 Images of *M. mycoides* JCVI-syn1.0 and WT *M. mycoides*. To compare the phenotype of the JCVI-syn1.0 and non-YCp WT strains, we examined colony morphology by plating cells on SP4 agar plates containing X-gal. Three days after plating, the JCVI-syn1.0 colonies are blue because the cells contain the *lacZ* gene and express β -lactosidase, which converts the X-gal to a blue compound (A). The WT cells do not contain *lacZ* and remain white (B). Both cell types have the fried egg colony morphology characteristic of most mycoplasmas. EMs were made of the JCVI-syn1.0 isolate using two methods. (C) For scanning EM, samples were postfixed in osmium tetroxide, dehydrated and critical point dried with CO₂, and visualized with a Hitachi SU6600 SEM at 2.0 keV. (D) Negatively stained transmission EMs of dividing cells with 1% uranyl acetate on pure carbon substrate visualized using JEOL 1200EX CTEM at 80 keV. To examine cell morphology, we compared uranyl acetate-stained EMs of *M. mycoides* JCVI-syn1.0 cells (E) with EMs of WT cells made in 2006 that were stained with ammonium molybdate (F). Both cell types show the same ovoid morphology and general appearance. EMs were provided by T. Deerinck and M. Ellisman of the National Center for Microscopy and Imaging Research at the University of California at San Diego.

Discussion

In 1995, the quality standard for sequencing was considered to be one error in 10,000 bp, and the sequencing of a microbial genome required months. Today, the accuracy is substantially higher. Genome coverage of 30 to 50 \times is not unusual, and sequencing only requires a few days. However, obtaining an error-free genome that could be transplanted into a recipient cell to create a new cell controlled only by the synthetic genome was complicated and required many quality-control steps. Our success was thwarted for many weeks by a single-base

pair deletion in the essential gene *dnaA*. One wrong base out of more than 1 million in an essential gene rendered the genome inactive, whereas major genome insertions and deletions in nonessential parts of the genome had no observable effect on viability. The demonstration that our synthetic genome gives rise to transplants with the characteristics of *M. mycoides* cells implies that the DNA sequence on which it is based is accurate enough to specify a living cell with the appropriate properties.

Our synthetic genomic approach stands in sharp contrast to various other approaches to genome engineering that modify natural genomes by introducing multiple insertions, substitutions, or deletions (Itaya et al., 2005; Itaya, 1995; Mizoguchi et al., 2007; Chun et al., 2007; Wang et al., 2009). This work provides a proof of principle for producing cells based on computer-designed genome sequences. DNA sequencing of a cellular genome allows storage of the genetic instructions for life as a digital file.

The synthetic genome described here has only limited modifications from the naturally occurring *M. mycoides* genome. However, the approach we have developed should be applicable to the synthesis and transplantation of more novel genomes as genome design progresses (Khalil and Collins, 2010). We refer to such a cell controlled by a genome assembled from chemically synthesized pieces of DNA as a “synthetic cell,” even though the cytoplasm of the recipient cell is not synthetic. Phenotypic effects of the recipient cytoplasm are diluted with protein turnover and as cells carrying only the transplanted genome replicate. Following transplantation and replication on a plate to form a colony (>30 divisions or >10⁹-fold dilution), progeny will not contain any protein molecules that were present in the original recipient cell (Lartigue et al., 2007).⁵³ This was previously demonstrated when we first described genome transplantation (Lartigue et al., 2007). The properties of the cells controlled by the assembled genome are expected to be the same as if the whole cell had been produced synthetically (the DNA software builds its own hardware).

The ability to produce synthetic cells renders it essential for researchers making synthetic DNA constructs and cells to clearly watermark their work to distinguish it from naturally occurring DNA and cells. We have watermarked the synthetic chromosome in this and our previous study (Gibson, et al., 2008b).

If the methods described here can be generalized, design, synthesis, assembly, and transplantation of synthetic chromosomes will no longer be a barrier to

⁵³A mycoplasma cell, with a mass of about 10⁻¹³ g, contains fewer than 10⁶ molecules of protein. (If it contains 20% protein, this is equivalent to 2 × 10⁻¹⁴ g of protein per cell. At a molecular mass of 120 daltons per amino acid residue, each cell contains (2 × 10⁻¹⁴)/120 = 1.7 × 10⁻¹⁶ mol of peptide residues. This is equivalent to (1.7 × 10⁻¹⁶) × (6 × 10²³) = 1 × 10⁸ residues per cell. If the average size of a protein is 300 residues, then a cell contains about 3 × 10⁵ protein molecules.) After 20 cell divisions the number of progeny exceeds the total number of protein molecules in the recipient cell. So, following transplantation and replication to form a colony on a plate, most cells will contain no protein molecules that were present in the original recipient cell.

the progress of synthetic biology. We expect that the cost of DNA synthesis will follow what has happened with DNA sequencing and continue to exponentially decrease. Lower synthesis costs combined with automation will enable broad applications for synthetic genomics.

We have been driving the ethical discussion concerning synthetic life from the earliest stages of this work (Cho et al., 1999; Garfinkel et al., 2007). As synthetic genomic applications expand, we anticipate that this work will continue to raise philosophical issues that have broad societal and ethical implications. We encourage the continued discourse.

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A9

SYNTHETIC BIOLOGY “FROM SCRATCH”

Gerald F. Joyce⁵⁴

The Darwinian Basis of Life

The previous presentations in this session described a mix of top-down and bottom-up approaches to synthetic biology. This presentation is intended to take us all the way down to the very bottom as we discuss synthetic biology “from scratch,” that is, life from scratch. Life is something we all know when we see it, right? Consider the giant bacterium *Titanospirillum velox*, which we all would agree is alive. Yet in a recent publication by Richard Hoover that appeared in the online *Journal of Cosmology* (Hoover, 2011), he describes what he believes is an extraterrestrial analog of *Titanospirillum*, found within a certain class of carbonaceous meteorites. Much of the popular media fell for it, abetted by a Fox News report showing a micrograph of *Titanospirillum*, rather than the mineralic artifacts within the meteorite that most experts agree have nothing to do with life.

But surely the experts must know life when they see it, right? And therefore the experts must know how to define life. Indeed some scientists have advanced a definition, or at least a working definition, of life. Some, including me, have pointed to the so-called NASA working definition of life, which is a self-sustained chemical system that is capable of undergoing Darwinian evolution (Joyce, 1994). In several of the presentations at this workshop, evolution has been referred to as the distinguishing, if not defining, feature of life. The Darwinian paradigm is the only one we know that explains how biological complexity can sustain itself against the vagaries of a changing environment. Darwinian evolution has a rigorous scientific foundation, but the word “life” is not a scientific term. As Andrew Ellington would say, it is a term for poets and philosophers, and scientists, let alone a government agency such as NASA, should not be in the business of trying to define it.

Without becoming bogged down in definitions, we can agree that life is all about Darwinian evolution, and that scientists understand what Darwinian evolution is all about. The key principles of Darwinian evolution are, first, heritable variation of form and function among a population of individuals; second, competition for finite resources by those individuals; and third, preferential reproduction of variants that operate most effectively in the competitive environment. In *The Origin of Species*, Charles Darwin made the observation: “Owing to this struggle for life, any *variation* ... if it be in any degree *profitable* to an individual of any species, in its infinitely complex relations to other organic beings and to external nature ... will generally be *inherited* by its offspring” (Darwin, 1859;

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italics added). If life has a slogan, if not a formal definition, it would be *inherit profitable variation*. I, for one, would be comfortable abiding by this slogan.

In considering synthetic biology from scratch, the focus is on the evolution of functional molecules, rather than organisms. The principles of directed molecular evolution are the same as those for the Darwinian evolution of organisms, again captured by the slogan *inherit profitable variation*. In chemical terms, Darwinian evolution involves three processes: (1) reproduction of information-carrying molecules (*inherit*), (2) selection of molecules that meet some fitness criteria (*profitable*), and (3) maintenance of chemical diversity among the population of molecules (*variation*).

Over the past two decades, the technology of direct molecular evolution has become very powerful, but also routine. There are many methods for introducing molecular variation, both for generating initial combinatorial libraries and for maintaining variation in a population. Individuals that meet some fitness test can be separated based on a high-throughput screen or a selection procedure. Through screening, one or more highly advantageous variants can be identified and subsequently mutagenized to provide a second-generation combinatorial library. This process of iterative high-throughput screening can be a powerful discovery tool, although it does not fully capture the power of Darwinian evolution, which requires that the population as a whole be subject to repeated rounds of selection and randomization. Maintaining a diverse population is key to exploring the fitness landscape because it allows both dominant and subdominant individuals to give rise to novel variants. The subdominant individuals, following the acquisition of a few beneficial mutations, may yield descendants that are more advantageous compared to the previously dominant individuals. Ideally, the loss of variation due to selection should be compensated by the introduction of novel variation throughout the course of evolution.

There are many methods for selecting profitable molecules. For example, molecules can be selected based on their specific chromatographic mobility, their ability to withstand exposure to some physical condition, their ability to bind to a target ligand (aptamers), or their ability to catalyze a particular chemical transformation (enzymes). More complex selection criteria can be imposed, such as a combination of both positive and negative selection, conditional selection, and the selection for multiple attributes.

Finally, there are various methods for reproducing the profitable molecules in order to bring about the inheritance of selectively advantageous traits. If the selected molecules are DNA or RNA, then it is straightforward to achieve their amplification by using the appropriate polymerase enzyme(s), resulting in large numbers of progeny. If the selected molecules are proteins, which cannot be amplified directly, then one must amplify nucleic acid molecules that encode and are physically linked to the corresponding proteins. Techniques such as phage display, ribosome display, and compartmentalized self-replication make it possible to amplify an ensemble of genes that encode a corresponding set of proteins. The same principle of genetic encoding can be used to amplify other

informational macromolecules, such as peptide analogs, polysaccharides, and even multicomponent organic molecules.

All of the amplification methods discussed above are not part of a self-sustained evolving system because they rely on informational macromolecules that are not themselves subject to evolution within the system. Protein polymerases, phage particles, and ribosomes all are the products of Darwinian evolution in biology. They are employed as operators in laboratory evolution systems, but their information content is not subject to evolution within those systems.

The experimental pursuit of life from scratch began in 1953 with the work of Stanley Miller, then a graduate student at the University of Chicago, who sought to cook up a prebiotic soup from entirely abiotic ingredients (Miller, 1953). None of those ingredients had information content derived from Darwinian processes. It has been almost 60 years since Miller's classic experiments, and life has yet to be produced starting from a prebiotic soup. However, considerable progress has been made toward synthesizing life using other methods, and it now appears that the production of life from scratch will be achieved in the near future. Some research efforts along these lines have attempted, as Miller did, to recapitulate the historical origins of life on Earth. Other efforts take inspiration from the origins of life on Earth, but aim for a second origin that would occur under decidedly artificial laboratory conditions. Considerable attention has been directed toward the critical role that RNA is thought to have played in the early history of life on Earth, during an era referred to as the "RNA world" (Atkins et al., 2011). RNA continues to play a central role in contemporary biology, which further motivates the goal of constructing RNA-based life from scratch.

Self-Sustained Darwinian Evolution

An explicit aim of my own research program is to construct a system of RNA molecules that undergo self-sustained Darwinian evolution. In fact this goal was recently achieved, although the system still lacks the complexity and inventiveness of what one might regard as life. The self-sustained evolving system employs populations of RNA enzymes that catalyze the RNA-templated joining of RNA substrates. The enzymes contain ~55 essential nucleotides and can be made to join pairs of RNA substrates of almost any sequence (Rogers and Joyce, 2001). If the substrates, once joined, form additional copies of the enzymes, then self-replication can be achieved. The newly formed enzymes behave similarly, resulting in exponential growth (Paul and Joyce, 2002). However, the process cannot be sustained indefinitely and is informationally restricted by the requirement that the original and newly formed enzymes must have the same sequence.

An improved version of the replication system employs two different RNA enzymes that catalyze each other's synthesis, enabling their cross-replication and sustained exponential growth (Kim and Joyce, 2004; Lincoln and Joyce, 2009). Each enzyme of the cross-replicating pair contains two substrate-binding domains that recognize corresponding oligonucleotide substrates through Watson-Crick pairing.

During cross-replication, the “Watson” enzyme joins two pieces of RNA to form the “Crick” enzyme, while the “Crick” enzyme joins two pieces of RNA to form the “Watson” enzyme. Information is passed back and forth between these two enzymes in the form of particular sequences within the two substrate-binding domains.

Following optimization of the cross-replication system, it now is possible to achieve 100-fold amplification in just a few hours at a constant temperature and in the absence of any biological materials (Lincoln and Joyce, 2009). The only informational macromolecules in the system are the enzymes and their components, which themselves are subject to Darwinian evolution within the system. The only other components are MgCl_2 , a buffer to maintain pH, and H_2O . Evolution can occur because there are many potential variants of the cross-replicating enzymes that must compete for a finite supply of substrates and can undergo mutation through recombination of the two substrate-binding domains.

Beginning with a small seed of the cross-replicating enzymes, amplification occurs with exponential growth, limited only by the amount of substrates that are available. The amplification profile follows the logistic growth equation [enzyme] $_t = a / (1 + be^{-ct})$, where a is the maximum extent of amplification, b is the degree of sigmoidicity, and c is the exponential growth rate. This equation also describes population growth for biological organisms constrained by the carrying capacity of their local environment.

Cross-replication of the RNA enzymes can be sustained indefinitely by continuing to supply the necessary substrates. This is most conveniently achieved through a serial transfer procedure, whereby a small aliquot is taken from a spent reaction mixture and transferred to a new reaction vessel that contains a fresh supply of substrates. The new reaction mixture contains only those enzymes that were carried over in the aliquot, and these enzymes immediately resume exponential amplification in the new mixture. Within a period of 24 hours, an overall amplification factor of $>10^9$ can be achieved (Lincoln and Joyce, 2009).

Self-sustained exponential amplification provides the growth engine for Darwinian evolution, but it is the diversity of enzymes in the population, their differential reproductive fitness, and their capacity for mutation that provides the opportunity to evolve macromolecular information. Profitable variation in the system emerges through particular combinations of substrates that form cross-replicators with high reproductive fitness. The genetic basis for this variation is represented by the two “loci”—the two substrate-binding domains—that can exist as any of a large number of possible “alleles.” Each allele can be made to encode a different corresponding phenotypic trait, embodied by the functional domain that is physically linked to that allele. If there are n potential variants of the first allele and m potential variants of the second allele, then the combinatorial complexity of the system is $n \times m$.

As an example, a population of cross-replicating enzymes was constructed with 12 different alleles at each of the two loci, providing a combinatorial complexity of $12 \times 12 = 144$. Each variant allele was linked to a different form of the catalytic center of the enzyme, which resulted in differential reproductive fitness

for various combinations of alleles at the two loci (Lincoln and Joyce, 2009). The evolution process was seeded with 12 different cross-replicating pairs that, due to mutation, could give rise to any of the other 132 combinations. Evolution was allowed to proceed in a self-sustained manner for 100 hours, with an overall amplification factor of 10^{25} . During this time the starting 12 replicators decreased in abundance as novel variants arose and came to dominate the population. Three of these novel variants together accounted for about half of the population members after 100 hours. The basis for their selective advantage was shown to be their relatively fast amplification rate and their propensity to cross-mutate to form additional copies of each other.

Toward Inventive Evolution

The capacity for the invention of novel function within the context of Darwinian evolution depends on both the genetic complexity of the system and the functional richness of the corresponding phenotypes. A population of 144 replicators is represented by only ~ 7 bits of genetic information. This is much less than the genetic complexity of even the simplest biological systems, which have an information content of 2 bits per base pair of genetic material. In principle, the synthetic RNA-based evolving system could have an information content of 30 bits, considering the 15 total base pairs within the two genetic loci. This would provide a molecular diversity of $4^{15} = 10^9$. However, it would not be possible to manage such high diversity because of the vast number of substrate molecules that would need to be present in the reaction mixture. These would slow replication as each enzyme must find its cognate substrates from among the complex mixture.

Current research efforts in our laboratory aim to maximize the genetic complexity of the self-sustained evolving system within the practical limits of both generating and harvesting molecular diversity. We constructed a population of cross-replicating enzymes with 64 different alleles for each of the two genetic loci, providing a combinatorial complexity of $64 \times 64 = 4,096$. In this test population, each variant allele was linked to the same functional sequence. This was done to assess the extent to which differences in genotype alone would result in differential fitness, something that should be minimized to allow the broadest exploration for novel phenotypes. Starting with this library, 10^6 -fold selective amplification was carried out; then individuals were cloned from the population and sequenced. Indeed two sources of genotype-related bias were identified, and these biases were eliminated from subsequent populations that were constructed.

Although the replicative function is the most important aspect of phenotype, replication can be made contingent on other functions so that fitness reflects the ability to execute those other functions. There is a stem-loop region of the RNA enzyme that supports the structure of the catalytic center and is generic in sequence, so long as it forms a stable secondary structure. This stem loop can be replaced by a ligand-binding (aptamer) domain, configured so that in the absence

of the ligand the domain is unstructured, whereas in the presence of the ligand the domain adopts a folded state that supports the active structure of the enzyme. In this way replication can be made contingent upon recognition of the target ligand (Lam and Joyce, 2009).

As two examples, the supporting stem loop of the enzyme was replaced by an aptamer that recognizes either theophylline or FMN (Lam and Joyce, 2009). In the absence of the ligand there is no replication, but in the presence of the ligand there is sustained exponential growth. Furthermore, the exponential growth rate depends on the concentration of the ligand relative to the K_d (equilibrium binding constant) of the ligand-binding domain. This method for quantitative, ligand-dependent exponential amplification may have applications in biosensing and molecular diagnostics. It is analogous to quantitative PCR for the measurement of nucleic acid targets, but it operates at a constant temperature and can be generalized to non-nucleic acid targets, including proteins, drugs, and metabolites that can be recognized by an aptamer (Lam and Joyce, 2011).

The self-sustained evolution system could, in principle, be used to discover novel aptamers. A genetically encoded random-sequence domain could be placed adjacent to the catalytic center such that ligand recognition would result in selective amplification of the functional molecules. This would require sufficient genetic complexity to encode a population containing enough variants to include molecules with the desired function. Our most recently constructed populations of cross-replicating enzymes have 256 different alleles for each of the two loci, providing a combinatorial complexity of $256 \times 256 = 65,536$. This still is likely to be insufficient to derive novel aptamers within the context of self-sustained evolution, unless the target ligands are compounds that have a strong propensity to bind to RNA.

The populations with a combinatorial complexity of 256×256 were constructed by two different methods. The first involved serial production of all $256 + 256 = 512$ allelic variants, synthesizing individual DNA templates to link each genotype-phenotype combination, then transcribing the templates to generate the corresponding RNAs. The advantage of this approach is that each variant is stored in a separate location, allowing one to prepare custom sublibraries. The disadvantage is that, at cost of synthesis of $\sim \$10$ per variant, the method cannot be extended to much more complex populations. The second approach for constructing the populations involved a novel split-and-pool method that makes it possible to synthesize the entire population in parallel. The parallel method enables the construction of populations with as many as 10^9 different members, although, as discussed above, it would be difficult to manage such high-diversity populations throughout the course of a self-sustained evolution experiment.

With increasing population complexity it becomes important to consider the nature of the code that relates particular genetic sequences to their corresponding functional sequences. This code can be chosen arbitrarily, but evolutionary optimization likely will benefit if more closely related genetic sequences correspond to more closely related functional sequences. The code need not have a collinear

3:1 relationship, as is the case for the genetic code in biology which relates trinucleotide codons within mRNA to individual amino acids within proteins. Furthermore, the same code need not apply for all genotype positions, although this simplification clearly has great selective advantage for natural biology. In synthetic biology, the experimenter must decide what genetic code to employ based on theoretical and practical considerations.

In preparing the populations of 256×256 combinatorial complexity, two different “sparse” codes were implemented, whereby each nucleotide within the genotype region encodes one or more nucleotides within the phenotype region. For the serially constructed population, each of four genetic nucleotides encodes three noncontiguous nucleotides in the functional region, with a different codon relationship for each genetic position. For the parallel library, each of four genetic nucleotides encodes either one or two contiguous nucleotides in the functional region, again with a different codon relationship for each genetic position. Experimental studies are under way to assess the operational characteristics of these different codes.

When practicing synthetic biology from scratch the experimenter makes the rules and allows Darwinian evolution to play the game. The capacity of the system to invent novel function is the most important measure of its robustness. Inventiveness should be as broad as possible so that the system can adapt to unanticipated changes in its environment. Life on Earth, although vulnerable to extreme changes of environmental conditions, has demonstrated extraordinary resiliency and inventiveness in adapting to highly disparate niches. Perhaps the most significant invention of life is a genetic system that has an extensible capacity for inventiveness, something that likely will not be achieved soon for synthetic biological systems. However, once informational macromolecules are given the opportunity to inherit profitable variation through self-sustained Darwinian evolution, they just may take on a life of their own.

Acknowledgments

Jeff Rogers developed the RNA enzyme that provides the basis for replication, Natasha Paul provided the first demonstration of a self-replicating RNA enzyme, Dong-Eun Kim converted the system to a cross-replication format, Tracey Lincoln first achieved self-sustained Darwinian evolution of RNA enzymes, Bianca Lam made replication contingent upon recognition of a target ligand, and Michael Robertson and Jonathan Sczepanski constructed increasingly complex populations of the cross-replicating RNA enzymes. This work was supported by grants from the National Aeronautics and Space Administration (NNX10AQ91G), the National Institutes of Health (GM065130), the National Science Foundation (MCB-0948161), and the Defense Advanced Research Projects Agency (DSO BAA-09-63).

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A10

MANUFACTURING MOLECULES THROUGH
METABOLIC ENGINEERING⁵⁵Jay D. Keasling^{56,57,58}

Metabolic engineering has the potential to produce from simple, readily available, inexpensive starting materials a large number of chemicals that are currently derived from nonrenewable resources or limited natural resources. Microbial production of natural products has been achieved by transferring product-specific enzymes or entire metabolic pathways from rare or genetically intractable organisms to those that can be readily engineered, and production of unnatural specialty chemicals, bulk chemicals,

⁵⁵ From Keasling, J. D. 2010. Manufacturing molecules through metabolic engineering. *Science* 330 (6009): 1355-1358. Reprinted with permission from AAAS.

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and fuels has been enabled by combining enzymes or pathways from different hosts into a single microorganism and by engineering enzymes to have new function. Whereas existing production routes use well-known, safe, industrial microorganisms, future production schemes may include designer cells that are tailor-made for the desired chemical and production process. In any future, metabolic engineering will soon rival and potentially eclipse synthetic organic chemistry.

The term “metabolic engineering” was coined in the late 1980s–early 1990s (Bailey, 1991). Since that time, the range of chemicals that can be produced has expanded substantially, in part due to notable advances in fields adjacent to metabolic engineering: DNA sequencing efforts have revealed new metabolic reactions and variants of enzymes from many different organisms; extensive databases of gene expression, metabolic reactions, and enzyme structures allow one to query for desired reactions and design or evolve novel enzymes for reactions that do not exist; new genetic tools enable more precise control over metabolic pathways; new analytical tools enable the metabolic engineer to track RNA, protein, and metabolites in a cell to identify pathway bottlenecks; and detailed models of biology aid in the design of enzymes and metabolic pathways. Yet even with these substantial developments, microbial catalysts are not as malleable as those in synthetic organic chemistry, and metabolic engineers must weigh many trade-offs in the development of microbial catalysts: (i) cost and availability of starting materials (e.g., carbon substrates); (ii) metabolic route and corresponding genes encoding the enzymes in the pathway to produce the desired product; (iii) most appropriate microbial host; (iv) robust and responsive genetic control system for the desired pathways and chosen host; (v) methods for debugging and debottlenecking the constructed pathway; and (vi) ways to maximize yields, titers, and productivities (Fig. A10-1). Unfortunately, these design decisions cannot be made independently of each other: Genes cannot be expressed, nor will the resulting enzymes function, in every host; products or metabolic intermediates may be toxic to one host but not another host; different hosts have different levels of sophistication of genetic tools available; and processing conditions (e.g., growth, production, product separation and purification) are not compatible with all hosts. Even with these many challenges, metabolic engineering has been successful for many applications, and with continued developments more applications will be possible.

Starting Materials, Products, and Metabolic Routes

One area where metabolic engineering has a sizable advantage over synthetic organic chemistry is in the production of natural products, particularly active pharmaceutical ingredients (APIs), some of which are too complex to be chemically synthesized and yet have a value that justifies the cost of developing a

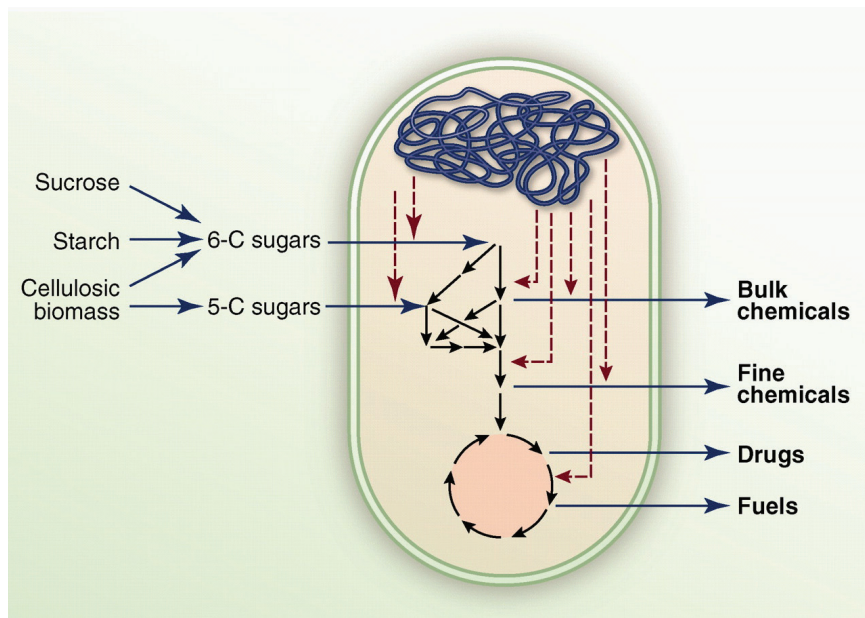


FIGURE A10-1 Conversion of sugars to chemicals by means of microbial catalysts.

genetically engineered microorganism. The cost of starting materials is generally a small fraction of their cost, and relatively little starting material is necessary so availability is not an issue. Most APIs fall into three classes of natural products, and many of the biosynthetic pathways for their precursors have been reconstituted in heterologous hosts.

Alkaloids are nitrogen-containing, low molecular weight compounds found primarily in and derived from plants and widely used as drugs. Two recent studies conclude that the large group of benzyl isoquinoline alkaloids (BIAs) will one day be producible in *Escherichia coli* and *Saccharomyces cerevisiae* (Hawkins and Smolke, 2008). Unfortunately, the BIAs are only one of four major alkaloid groups, all of which are produced through different pathways. As the metabolic pathways for other alkaloids are discovered in their natural producers, many more of these valuable molecules could be produced microbially.

Polyketides and nonribosomal peptides (NRPs) have found broad use as APIs, veterinary agents, and agrochemicals. Naturally occurring polyketides and NRPs are produced by a number of bacteria and fungi using large, modular enzymes. Their titers and yields in the native producers have been improved through traditional strain engineering and advanced metabolic engineering. More recently, some of the most valuable molecules have been produced with engineered industrial hosts (Pfeifer et al., 2001). Recombination of various synthase modules

allows one to produce a nearly infinite range of chemicals (Menzella et al., 2005; Siewers et al., 2010), opening up the possibility that they may one day be used to produce fine and bulk chemicals. Isoprenoids have found use as fragrances and essential oils, nutraceuticals, and pharmaceuticals. Many isoprenoids have been produced microbially, including carotenoids and various plant-derived terpenes (Martin et al., 2003; Ro et al., 2006; Leonard et al., 2010), taking advantage of terpene synthases to form the most complicated part of the molecules and hydroxylases to introduce hydroxyl group that can be subsequently functionalized chemically or biologically (Ro et al., 2006; Chang et al., 2007). Isoprenoids are one of the few classes of natural products where there are alternative precursor production pathways. An example of using metabolic engineering and synthetic chemistry together to produce an API is the semisynthesis of the antimalarial drug artemisinin with *S. cerevisiae* engineered to produce artemisinic acid, the most complex part of the molecule, and synthetic chemistry to produce artemisinin from the microbially sourced artemisinic acid (Martin et al., 2003; Ro et al., 2006; Chang et al., 2007). Beyond producing natural products, laboratory evolution or rational engineering of terpene cyclases, terpene hydroxylases, and a host of other terpene-functionalizing enzymes (Leonard et al., 2010; Yoshikuni et al., 2006; Schmidt-Dannert et al., 2000; Dietrich et al., 2009) and combinatorial expression of these evolved enzymes in a heterologous host will enable the production of unnatural terpenes, some of which might be more effective than the natural product for the treatment of human disease.

Although individual metabolic pathways have been developed to produce natural products derived from a single pathway, there is an opportunity to synthesize multisubstituent APIs (e.g., Taxol) or other molecules from the products of multiple biosynthetic pathways. This will require simultaneous expression of multiple precursor pathways in a single microorganism, as well as “ligases” that can assemble multiple substituents together into a single molecule. The benefit would be the synthesis of complicated molecules that might not otherwise be produced.

Although not as valuable as pharmaceuticals, many fine chemicals have been produced economically from natural and engineered microorganisms, including amino acids, organic acids, vitamins, flavors, fragrances, and nutraceuticals. For fine chemicals, profit margins are generally much lower than for APIs and may be affected by substrate availability and cost. Some of these molecules are sufficiently complicated that they cannot be produced economically by any route other than biological production, whereas others have chemical routes. For some important products (fragrances, flavors, amino acids), heterologous hosts have been engineered to enhance their production. Yet we have barely begun to investigate what will be possible to produce.

In contrast, bulk chemicals such as solvents and polymer precursors are rarely produced from microorganisms, because they can be produced inexpensively from petroleum by chemical catalysis. Due to fluctuations in petroleum prices and recognition of dwindling reserves, trade imbalances, and political considerations, it is now possible to consider production of these inexpensive

chemicals from low-cost starting materials such as starch, sucrose, or cellulosic biomass (e.g., agricultural and forest waste, dedicated energy crops, etc.) with a microbial catalyst. For example, 1,3-propanediol (1,3-PDE), a useful intermediate in the synthesis of polyurethanes and polyesters, is now being produced from glucose by *E. coli* engineered with genes from *Klebsiella pneumoniae* and *S. cerevisiae* (Nakamura and Whited, 2003). There is an opportunity to produce many other bulk chemicals (e.g., polymer precursors) by using metabolically engineered cells, but the key will be to produce the exact molecule needed for existing products rather than something “similar but green” that will require extensive product testing before it can be used.

By far the highest-volume (and lowest-margin) application for engineered metabolism is the production of transportation fuels. For many of the same reasons that it is desirable to produce petroleum-derived chemicals using biological systems, it is desirable to produce transportation fuels from readily available, inexpensive, renewable sources of carbon. There is a long history of using microorganisms to produce alcohols, primarily ethanol and butanol. Although much of the work on these alcohols was done by traditional strain mutagenesis and selection, more recent work focused on engineering yeasts and bacteria to produce ethanol or butanol from a variety of sugars while eliminating routes to side products and improving the tolerance of the host to the alcohol (Steen et al., 2008). Larger, branched-chain alcohols can be produced by way of the Ehrlich pathway. By incorporating broad substrate-range 2-keto acid decarboxylases and alcohol dehydrogenases, several microbes have now been engineered to produce these fuels (Donaldson et al., 2007; Atsumi et al., 2008). These alcohols are generally considered better fuels than ethanol and butanol and can also be used to produce a variety of commodity chemicals.

Recent advances in metabolic pathway and protein engineering have made it possible to engineer microorganisms to produce hydrocarbons with properties similar or identical to those of petroleum-derived fuels and thus compatible with our existing transportation infrastructure. Linear hydrocarbons (alkanes, alkenes, and esters) typical of diesel and jet fuel can be produced by way of the fatty acid biosynthetic pathway (Steen et al., 2010; Beller et al., 2010; Schirmer et al., 2010). For diesel in cold weather and jet fuel at high altitudes, branches in the chain are beneficial—regularly branched and cyclic hydrocarbons of different sizes with diverse structural and chemical properties can be produced via the isoprenoid biosynthetic pathway (Withers et al., 2007; Renninger and McPhee, 2008). Both the fatty acid-derived and the isoprenoid-derived fuels diffuse (or are pumped) out of the engineered cells and phase separate in the fermentation, making purification simple and reducing fuel cost.

Although the pathways described above produce a wide range of fuel-like molecules, there are many other molecules that one might want to produce, such as short, highly branched hydrocarbons (e.g., 2,2,4-trimethyl pentane or isooctane) that would be excellent substitutes for petroleum-derived gasoline. Additionally, most petroleum fuels are mixtures of large numbers of components

that together create the many important properties of the fuels. It should be possible to engineer single microbes or microbial consortia to produce a mixture of fuels from one of the biosynthetic pathways or from multiple biosynthetic pathways. Indeed, some enzymes produce mixtures of products from a single precursor—maybe these enzymes could be tuned to produce a fuel mixture ideal for a particular engine type or climate.

To make these new fuels economically viable, we must tap into inexpensive carbon sources (namely, sugars from cellulosic biomass). Given the variety of sugars in cellulosic biomass, the fuel producer must be able to consume both five- and six-carbon sugars. Because many yeasts do not consume five-carbon sugars, recent developments in engineering yeast to catabolize these sugars will make production of these fuels more economically viable (Wisselink et al., 2009). Engineering fuel-producing microorganisms to secrete cellulases and hemicellulases to depolymerize these sugar polymers into sugars before uptake and conversion into fuels has the potential to substantially reduce the cost of producing the fuel.

Hosts and Expression Systems

From the applications cited above, it should be evident that the product, starting materials, and production process all affect host choice. Some of the most important qualities one must consider when choosing a host are whether the desired metabolic pathway exists or can be reconstituted in that host; if the host can survive (and thrive) under the desired process conditions (e.g., ambient versus extremes of temperature, pH, ionic strength, etc.); if the host is genetically stable (both with the introduced pathway and not susceptible to phage attack); and if good genetic tools are available to manipulate the host. Widely used, heterologous hosts include *E. coli*, *S. cerevisiae*, *Bacillus subtilis*, and *Streptomyces coelicolor*, to name a few. Although *E. coli* and *S. cerevisiae* excel in the genetic tools available, *E. coli* has the disadvantage of being susceptible to phage attack. And while *B. subtilis* and *S. coelicolor* have the ability to easily express polyketide synthases, they have fewer genetic tools available than either *S. cerevisiae* or *E. coli*. Although minimal, bacterial hosts may have scientific interest (Gibson et al., 2010), minimal hosts that require addition of many nutrients or that cannot cope with stresses in processing will probably not find a niche in industrial chemical or fuel production where cost is critical. Thus, it is essential to have genetic tools for existing industrial hosts that can grow on simple, inexpensive carbon sources and salts or on an inexpensive, undefined medium with minimal additions (Wang et al., 2009; Pósfai et al., 2006).

The key issue necessitating good genetic tools is the introduction of foreign genes encoding the metabolic pathway and control over their expression to maximize yields and titers. The genes encoding the transformational enzymes in metabolically engineered cells do not need to be highly expressed, but must be produced in catalytic amounts sufficient to adequately transform the metabolic

intermediates into the desired products at a sufficient rate. Expression of the desired genes at too high a level will rob the cell of metabolites that might otherwise be used to produce the desired molecule of interest, particularly important for production of low-margin chemicals, while underexpressed genes will create pathway bottlenecks. Furthermore, because intermediates of a foreign metabolic pathway can be toxic to the heterologous host (Martin et al., 2003), which results in decreased production of the desired final compound, it is essential that the relative levels of the enzymes be coordinated.

Central to any genetic manipulation is the vector used to carry and/or harbor the transforming DNA in the host. Important features of the cloning vector include segregational stability, minimal and consistent copy number in all cells of a culture, and the ability to replicate and express large sequences of DNA. There is growing recognition that one or only a few copies of a gene are needed, particularly for metabolic engineering applications. With the ability to vary promoter (Jensen and Hammer, 1998) and ribosome binding strength (Salis et al., 2009), as well as the stabilities of the mRNA (Smolke et al., 2000) and the resulting protein produced from it, there are many factors other than copy number that can be manipulated to alter enzyme production.

Promoters play an essential role in controlling biosynthetic pathways. Inducible promoters are one of the easiest and most effective ways to regulate gene expression, but it is essential that the promoter be induced consistently in all cells of a culture (Khlebnikov et al., 2001). Constitutive promoters (Jensen and Hammer, 1998) and promoters that respond to a change in growth condition or to an important intermediary metabolite (Farmer and Liao, 2000) allow for inexpensive, inducer-free gene expression, which is particularly important where cost is an issue (Fig. A10-2). Although there are many inducible promoters for

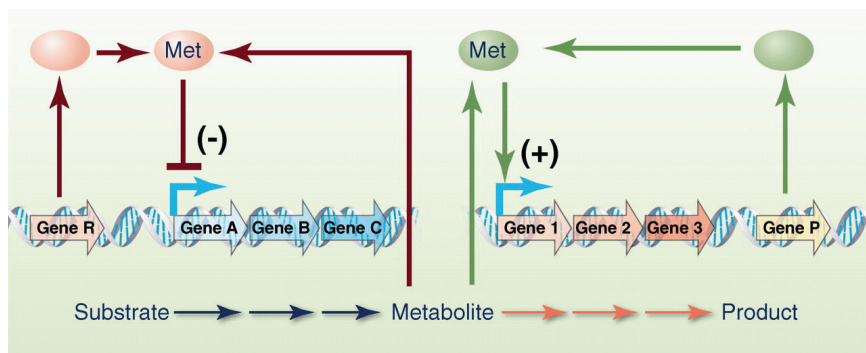


FIGURE A10-2 Use of synthetic regulators to modulate metabolic pathways that have a toxic intermediate. Regulatory proteins or RNAs bind the toxic metabolite and down-regulate the biosynthetic pathway and up-regulate the consumption pathway.

bacteria, the small number of inducible promoters for yeast and other potential industrial hosts makes regulation of metabolic pathways in those organisms more challenging than in bacteria.

Because production of complicated molecules often requires several enzymes, it is desirable to coordinate expression of the genes encoding these enzymes to prevent accumulation of toxic intermediates and bottlenecks in biosynthetic pathways. There are many ways to coordinate expression of multiple genes, such as using a non-native RNA polymerase or transcription factor to induce multiple promoters (Alper and Stephanopoulos, 2007); grouping multiple, related genes into operons; varying the ribosome binding strength for the enzymes encoded in the operon (Salis et al., 2009); or controlling segmental mRNA stability of each coding region to regulate the amount of each enzyme produced (Pfleger et al., 2006). One of the limitations to expressing multiple genes in yeast is the lack of internal ribosomal entry sequences (IRESs) that are available for higher eukaryotes. The development of yeast IRESs would allow one to express genes encoding metabolic pathways without the need for a promoter for each gene.

Debottlenecking, Debugging, and Process Optimizing

Even with a kit full of tools, building a biosynthetic pathway is made difficult without accurate blueprints. In almost all areas of engineering, there are models and simulation tools that allow one to predict which components to assemble to obtain a larger system with a desired function or characteristic. Similar biological design tools are in their infancy. However, metabolic models that incorporate cell composition and gene regulation have become relatively predictive and may one day be used to design metabolic pathways and predict the level of gene expression needed to achieve a particular flux through a reaction or pathway (Edwards et al., 2001).

Regardless of how sophisticated the design tools and how good the blueprint, there will always be “bugs” in the engineered system. Analogous to software debuggers that allow one to find and fix errors in computer code, the development of similar tools for biological debugging would reduce development times for optimizing engineered cells. For the development of microbial chemical factories, functional genomics can serve in the role of debugging routines (Park et al., 2007), because imbalances in a metabolic pathway often elicit a stress response in central metabolism (due to protein overproduction or accumulation of toxic intermediates or end products) (Martin et al., 2003; Kizer et al., 2008). Information from one or more of these techniques can be used to diagnose the problem and modify expression of genes in the metabolic pathway or in the host to improve titer and/or productivity.

Many desirable chemicals will be toxic to the producer, particularly at the high titers needed for industrial-scale production. Taking advantage of the cell's

native stress response pathways can be an effective way to alleviate part or all of the toxicity (Alper et al., 2006). Even better, transporters could be used to pump the desired product outside the cell, reducing intracellular toxicity and purifying the product from the thousands of contaminating intracellular metabolites (Dunlop et al., 2010).

Designer Cells for Designer Chemicals

One can envision a future when a microorganism is tailor-made for production of a specific chemical from a specific starting material, much like chemical engineers build refineries and other chemical factories from unit operations (Fig. A10-3). The chemical and physical characteristics of the product and starting materials would be considered in the design of the organism to minimize both production and purification costs (e.g., operating the engineered cell at the boiling point of volatile, toxic products to drive production and reduce product toxicity). The cell envelope would be designed to be resistant to the specific desired chemical, and the cell wall would be designed to make the organism tolerant to industrial processing conditions. Specific, engineered transporters would be incorporated into the membrane to pump the desired product out of the cell and keep it out and to import the desired starting material. The biosynthetic pathway would be constructed from a parts registry containing all known enzymes by means of retrosynthesis software (Prather and Martin, 2008), and done so to maximize yield and minimize the time required to grow the organism and produce the desired chemical from the desired starting material. In the event that an enzyme does not exist for a particular reaction or set of reactions, one would use computer-aided design (CAD) software to design the desired enzyme (Siegel et al., 2010).

Once the cell has been designed in the computer, the genetic control system would be designed to control expression of all the genes at the correct time and at the appropriate levels. Redundancies in the genetic control system would be engineered to ensure that design parameters are maintained regardless of the transient changes the cell encounters during the production process. Simulations and scenario planning would test various designs, including genetic control system failure. Safety for the plant operators and the environment would be an essential design criterion. When the genetic controls were fully designed and tested, the chromosome(s) would be designed and constructed. Gene location, modularity, and ease of construction are but a few of the important considerations in designing the chromosome. The chromosome would be ordered from a commercial DNA manufacturer. Depending on the state of the technology at the time, the chromosome would arrive in pieces and be assembled in the constructed envelope or would be completely assembled at the factory and sent to another location to be introduced into the ghost cell. One can even envision a day when cell manufacturing is done by different companies, each specializing in certain aspects of

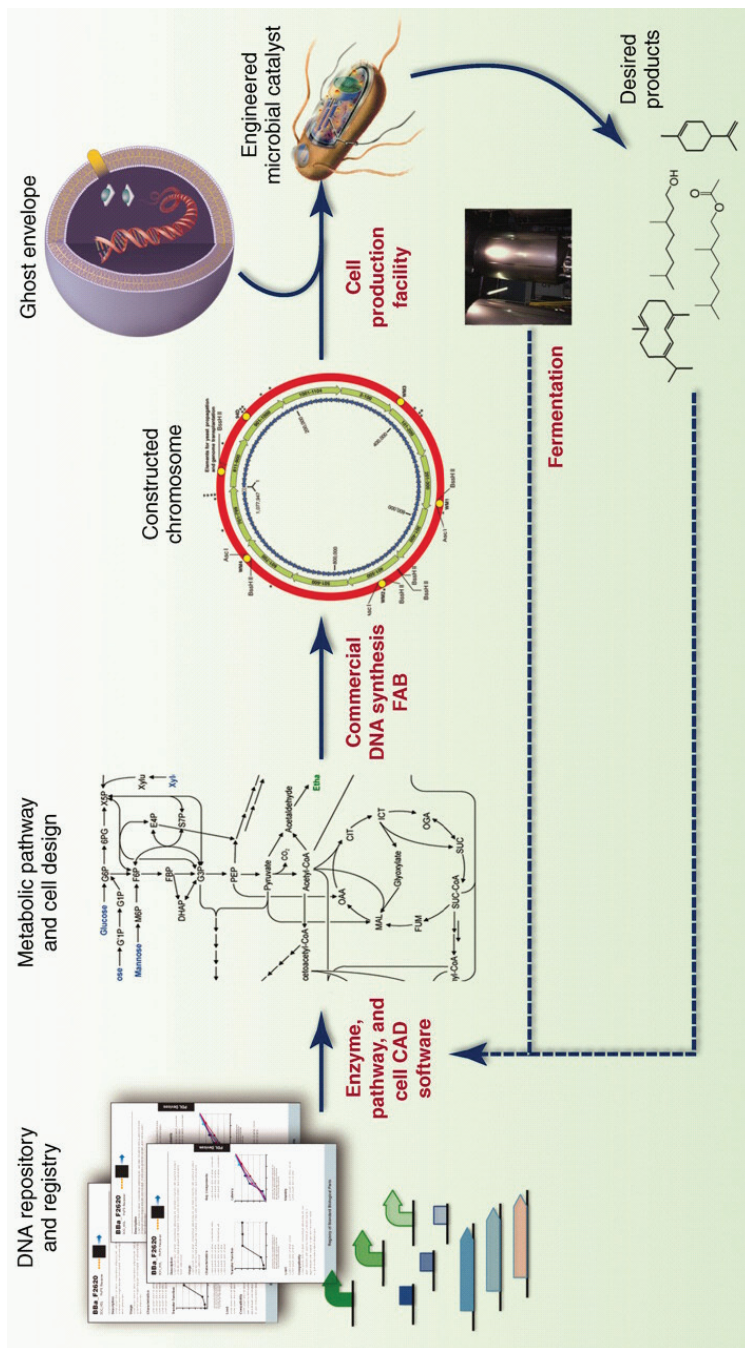


FIGURE A10-3 The future of engineered biocatalysts, enzymes, and genetic controls are designed from characteristic parts (enzymes, promoters, etc.) by means of pathway and enzyme CAD software. The chromosomes encoding those elements are synthesized at a FAB and incorporated into a ghost envelope to obtain the new catalyst. The design of the engineered catalyst is influenced by the desired product and the production process.

the synthesis—one company constructs the chromosome, one company builds the membrane and cell wall (the “bag”), one company fills the bag with the basic molecules needed to boot up the cell.

Until this future arrives, manufacturing of molecules will be done with well-known, safe, industrial microorganisms that have tractable genetic systems. Continued development of tools for existing, safe, industrial hosts, cloning and expressing genes encoding precursor production pathways, and the creation of novel enzymes that catalyze unnatural reactions will be necessary to expand the range of products that can be produced from biological systems. When more of these tools are available, metabolic engineering should be just as powerful as synthetic chemistry, and together the two disciplines can greatly expand the number of products available from renewable resources.

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A11

NOVEL APPROACHES TO COMBAT BIOFILM DRUG TOLERANCE

*Kim Lewis*⁶⁰

The Nature of the Threat

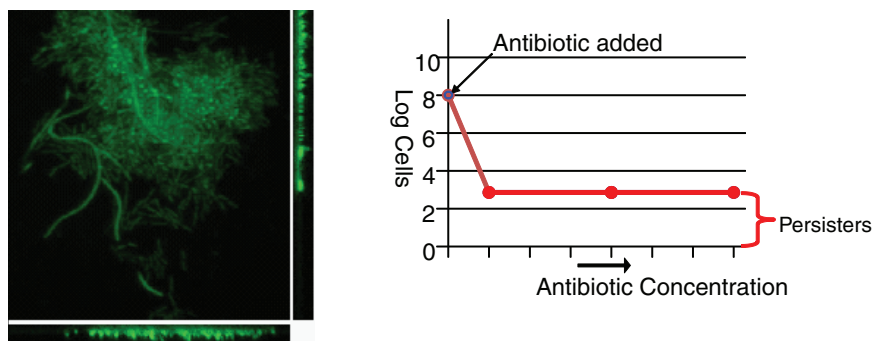
It is a given that new antibiotics are needed to combat drug-resistant pathogens. However, this is only part of the need—we actually never had antibiotics capable of eradicating an infection. Currently used antibiotics have been developed against rapidly growing bacteria, and most of them have no activity against stationary-state organisms, and none are effective against dormant persister cells. The relative effectiveness of antibiotics in treating disease is largely a result of a cooperation with the immune system, which mops up after antibiotics eliminate the bulk of a growing population. But the deficiency of existing antibiotics against supposedly drug-susceptible pathogens is becoming increasingly apparent with the rise of immunocompromised patients (HIV infected, undergoing chemotherapy) and the wide use of indwelling devices (catheters, prostheses, and heart valves), where the pathogen forms biofilms protecting cells from the components of the immune system. The ineffectiveness of the immune system leads to chronic diseases, which make up approximately half of all infectious disease cases in the developed world. The main culprit responsible for tolerance of pathogens to antibiotics are specialized survivors, persister cells (Lewis, 2007, 2010), which we examine in the following section.

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Persisters

Persisters represent a small subpopulation of cells that spontaneously go into a dormant, nondividing state. When a population is treated with a bactericidal antibiotic, regular cells die, while persisters survive (Figure A11-1). In order to kill, antibiotics require active targets, which explains the tolerance of persisters. Taking samples and plating them for colony counts over time from a culture treated with antibiotic produces a biphasic pattern, with a distinct plateau of surviving persisters. By contrast, resistance mechanisms prevent antibiotics from binding to their targets (Figure A11-2).

Infectious disease is often untreatable, even when caused by a pathogen that is not resistant to antibiotics. This is the essential paradox of chronic infections. In most cases, chronic infections are accompanied by the formation of biofilms, which seems to point to the source of the problem (Costerton et al., 1999; Del Pozo and Patel, 2007). Biofilms have been linked to dental disease, endocarditis, cystitis, urinary tract infection, deep-seated infections, indwelling device and catheter infections, and the incurable disease of cystic fibrosis. In the case of indwelling devices such as prostheses and heart valves, reoperation is the method of choice for treating the infection. Biofilms do not generally restrict penetration of antibiotics (Walters et al., 2003), but they do form a barrier for the larger com-



Child middle ear (*H. influenzae*)

Dental diseases (*Streptococcus*, *Actinomyces*)

Indwelling devices (catheters, prostheses) infection (*S. aureus*, *S. epidermidis*, other coagulase negative staph)

Endocarditis (*S. aureus*)

Urinary catheters, UTI (*E. coli*)

Cystic fibrosis (*P. aeruginosa*, *S. aureus*); Tuberculosis

FIGURE A11-1 Persisters and biofilms. Dose-dependent killing with a bactericidal antibiotic reveals a small subpopulation of tolerant cells, persisters, that are formed within a biofilm.

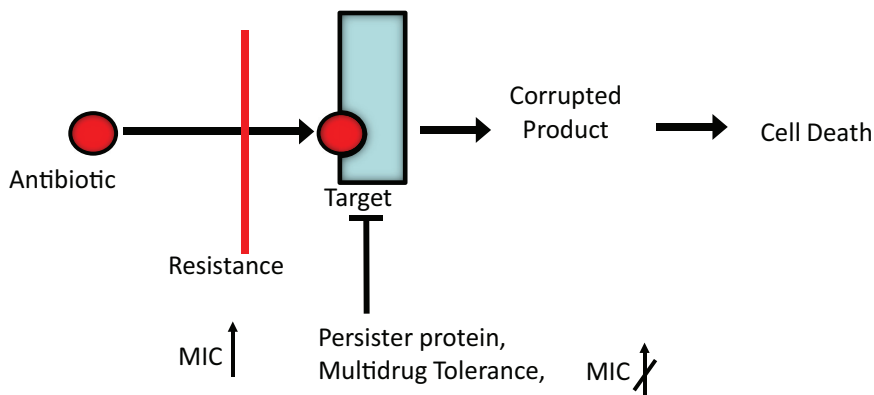


FIGURE A11-2 Resistance and tolerance. Bactericidal antibiotics kill cells by forcing the active target to produce corrupted products. Persister proteins act by blocking the target, so no corrupted product can be produced. By contrast, all resistance mechanisms prevent the antibiotic from binding to the target.

ponents of the immune system (Jesaitis et al., 2003; Leid et al., 2002; Vuong et al., 2004). The presence of biofilm-specific resistance mechanisms was suggested to account for the recalcitrance of infectious diseases (Stewart and Costerton, 2001). However, the bulk of cells in the biofilm are actually highly susceptible to killing by antibiotics; only a small fraction of persisters remains alive (Spoering and Lewis, 2001). Based on these findings, we proposed a simple model of a relapsing chronic infection: antibiotics kill the majority of cells, and the immune system eliminates both regular cells and persisters from the bloodstream (Lewis, 2001) (Figure A11-3). The only remaining live cells are then persisters in the biofilm. Once the level of antibiotic drops, persisters repopulate the biofilm, and the infection relapses. While this is a plausible model, it is not the only one. A simpler possibility is that antibiotics fail to effectively reach at least some cells in vivo, resulting in a relapsing infection.

Establishing potential causality between persisters and therapy failure is not trivial, since these cells form a small subpopulation with a temporary phenotype, which precludes introducing them into an animal model of infection. We reasoned that causality can be tested based on what we know about selection for high persister (*hip*) mutants in vitro. Periodic application of high doses of bactericidal antibiotics leads to the selection of strains that produce increased levels of persisters (Moyed and Bertrand, 1983; Wolfson et al., 1990). This is precisely what happens in the course of treating chronic infections: the patient is periodically exposed to high doses of antibiotics, which may select for *hip* mutants. But *hip* mutants would only gain advantage if the drugs effectively reach, and kill, the regular cells of the pathogen.

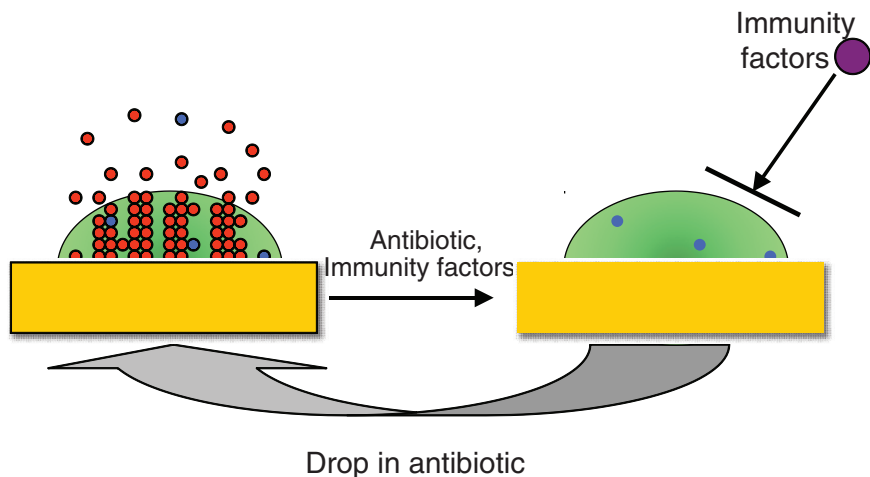


FIGURE A11-3 A model of a relapsing biofilm infection. Regular cells (red) and persisters (blue) form in the biofilm and are shed off into surrounding tissue and the bloodstream. Antibiotics kill regular cells, and the immune system eliminates escaping persisters. The matrix protects persisters from the immune system, and when the concentration of the antibiotic drops, they repopulate the biofilm, causing a relapse.

Patients with cystic fibrosis (CF) are treated for decades for an incurable *Pseudomonas aeruginosa* infection to which they eventually succumb (Gibson et al., 2003). The periodic application of high doses of antibiotics provides some relief by decreasing the pathogen burden, but it does not clear the infection. If *hip* strains of pathogens were selected in vivo, they would most likely be present in a CF patient. We took advantage of a set of longitudinal *P. aeruginosa* isolates from a single patient, collected over the course of many years (Smith et al., 2006). Testing persister levels by monitoring survival after challenge with a high dose of ofloxacin showed a dramatic, 100-fold increase in surviving cells in the last four isolates (Mulcahy et al., 2010). Testing paired strains from additional patients showed that, in most cases, there was a considerable increase in persister levels in the late isolate from a patient. Interestingly, most of the *hip* isolates had no increase in minimum inhibitory concentration (MIC) compared to their clonal parent strain to ofloxacin, carbenicillin, and tobramycin, suggesting that classical acquired resistance plays little or no role in the recalcitrance of CF infection. These experiments directly link persisters to the clinical manifestation of the disease and suggest that persisters are responsible for the therapy failure of chronic CF infection. But why have the *hip* mutants with their striking survival phenotype evaded detection for such a long time?

The main focus of research in antimicrobials has been on drug resistance, and the basic starting experiment is to test a clinical isolate for its ability to grow in

the presence of elevated levels of different antibiotics and to record any increases in the MIC. This is also the standard test employed by clinical microbiology laboratories. The *hip* mutants are of course missed by this test, which explains why they had remained undetected in spite of a major effort aimed at understanding pathogen survival to antimicrobial chemotherapy. Given that *hip* mutants are the likely main culprit responsible for morbidity and mortality of the CF infection, it makes sense to test for their presence. Testing for persister levels is not that much more difficult as compared to a MIC test.

Is selection for *hip* mutants a general feature of chronic infections? We recently examined patients with chronic oral thrush caused by *Candida albicans* (LaFleur et al., 2010). These were cancer patients undergoing chemotherapy, and suppression of the immune system caused the fungal infection. In patients where the disease did not resolve, the *C. albicans* isolates were almost invariably *hip* mutants, as compared to patients where the disease cleared within 3 weeks of treatment with chlorhexidine. The eukaryotic *C. albicans* forms persisters (Al-Dhaheer and Douglas, 2008; Harrison et al., 2007; LaFleur et al., 2006) through mechanisms that are probably analogous, rather than homologous, to that of their bacterial counterparts. Given the similar lifestyles of the unrelated *P. aeruginosa* and *C. albicans*, we may expect that the survival advantage of a *hip* mutation is universal. Just as multidrug resistance has become the prevalent danger in acute infections, multidrug tolerance of persisters and *hip* mutants may be the main, but largely overlooked, culprit of chronic infectious disease.

Biofilms apparently serve as a protective habitat for persisters (Harrison et al., 2005a,b, 2009; LaFleur et al., 2006; Spoering and Lewis, 2001), allowing them to evade the immune response. However, a more general paradigm is that persisters will be critical for pathogens to survive antimicrobial chemotherapy whenever the immune response is limited. Such cases would include disseminating infections in immunocompromised patients undergoing cancer chemotherapy or infected with HIV. Persisters are also likely to play an important role in immunocompetent individuals in cases where the pathogen is located at sites poorly accessible by components of the immune system. These include the central nervous system, where pathogens cause debilitating meningitis and brain abscesses (Honda and Warren, 2009), and the gastrointestinal tract, where hard-to-eradicate *Helicobacter pylori* causes gastroduodenal ulcers and gastric carcinoma (Peterson et al., 2000). Tuberculosis is perhaps the most prominent case of a chronic infection by a pathogen evading the immune system. The acute infection may resolve spontaneously or as a result of antimicrobial therapy, but the pathogen often remains in a “latent” form (Barry et al., 2009). It is estimated that one of every three people carry latent *Mycobacterium tuberculosis*, and 10 percent of carriers develop an acute infection at some stage in their lives. One simple possibility is that persisters are equivalent to the latent form of the pathogen. We recently isolated persisters from a growing culture of *M. tuberculosis* following lysis with D-cycloserine and found that these cells exhibit a general

shutdown of biosynthetic processes, a hallmark of dormancy (Keren et al., 2011). The *M. tuberculosis* persisters also had several toxin-antitoxin modules overexpressed. These findings will enable the use of the “persister signature” to establish the equivalence of these cells and the dormant form of the pathogen in vivo.

The above analysis underscores the significance of drug tolerance as a barrier to effective antimicrobial chemotherapy. Given its significance—roughly half of all cases of infection (Figure A11-4)—the number of studies dedicated to tolerance is tiny compared to the number of publications on resistance. The difficulty in pinpointing the mechanism of biofilm recalcitrance and the formidable barriers to studying persister cells account for the lack of parity between these two comparably significant fields. Hopefully a better balance will be achieved, and the following discussion summarizes recent advances in understanding the mechanism of tolerance.

Persisters were initially discovered in 1944, but the mechanism of their formation eluded us for a very long time. Only recently has the molecular mechanism of dormancy begun to emerge.

The most straightforward approach to finding an underlying mechanism of a complex function is by screening a library of transposon insertion mutants. This produces a set of candidate genes, and subsequent analysis leads to a pathway and a mechanism. This is indeed how the basic mechanisms of sporulation, flagellation, chemotaxis, virulence, and many other functions have been established.

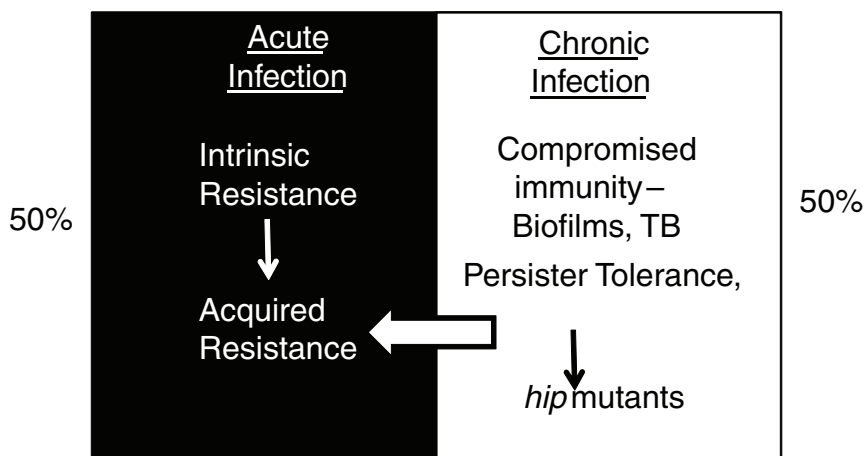


FIGURE A11-4 The two faces of recalcitrance. Drug resistance plays an important role in recalcitrance of acute infections, while drug tolerance is largely responsible for failures of chemotherapy in chronic infections. Tolerance allows the pathogen to survive for lengthy periods of time, and this relapsing infection increases the probability of producing classical resistant cells.

However, screening a Tn insertion library of *Escherichia coli* for an ability to tolerate high doses of antibiotics produced no mutants completely lacking persisters (Hu and Coates, 2005; Spoering, 2006). With the development of the complete, ordered *E. coli* gene knockout library by the Mori group (Baba et al., 2006) (the Keio collection), it seemed reasonable to revisit the screening approach. Indeed, there always remains a possibility that transposons missed a critical gene, or the library was not large enough. The use of the Keio collection largely resolves this uncertainty.

This advanced screen (Hansen et al., 2008), similarly to previous efforts, did not produce a single mutant lacking persisters, suggesting a high degree of redundancy. The screen did identify a number of interesting genes, with knockouts showing about a 10-fold decrease in persister formation. The majority of hits were in global regulators, DksA, DnaKJ, HupAB, and IhfAB. This is an independent indication of redundancy—a global regulator can affect expression of several persister genes simultaneously, resulting in a phenotype (Figure A11-5). The screen also produced two interesting candidate genes that may be more directly involved in persister formation: *YgfA*, which can inhibit nucleotide synthesis, and *YigB*, which may block metabolism by depleting the pool of flavin mononucleotide (FMN).

A similar screen of a *P. aeruginosa* mutant library was recently reported (De Groote et al., 2009). As in *E. coli*, no persisterless mutant was identified, pointing to the similar redundancy theme.

The main conclusion from the screens is that persister formation does not follow the main design theme of complex cellular functions—a single linear regulatory pathway controlling an execution mechanism. By contrast, persisters are apparently formed through a number of independent parallel mechanisms (Figure A11-5). There is a considerable adaptive advantage in this redundant design—no single compound will disable persister formation.

Screens for persister genes were useful in finding some possible candidate genes and pointing to redundancy of function. It seemed that a method better suited to uncover redundant elements would be transcriptome analysis. For this, persisters had to be isolated.

Persisters form a small and temporary population, making isolation challenging. The simplest approach is to lyse a population of growing cells with a β -lactam antibiotic and collect surviving persisters (Keren et al., 2004). This allows one to isolate enough *E. coli* cells to perform a transcriptome analysis. A more advanced method aimed at isolating native persisters was developed, based on a guess that these are dormant cells with diminished protein synthesis (Shah et al., 2006). If the strain expressed degradable green fluorescent protein (GFP), then cells that stochastically enter into dormancy will become dim. In a population of *E. coli* expressing degradable GFP under the control of a ribosomal promoter that is only active in dividing cells, a small number of cells indeed appeared to be dim. The difference in fluorescence allowed for the sorting of the

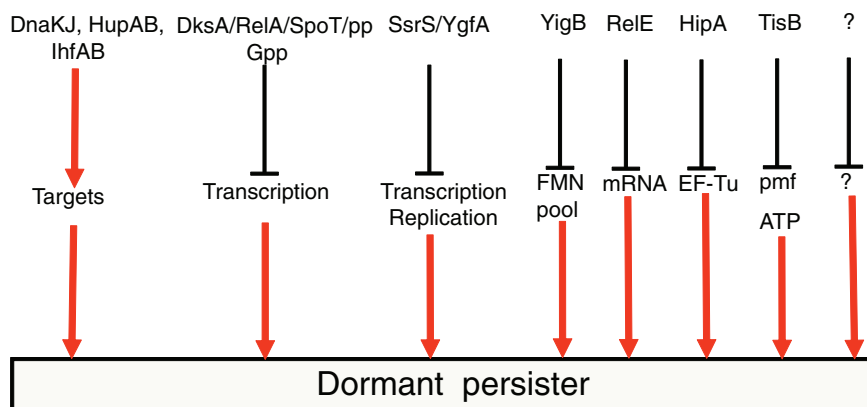


FIGURE A11-5 Candidate persister genes. Persisters are formed through parallel redundant pathways.

two subpopulations. The dim cells were tolerant to ofloxacin, confirming that they are persisters.

Transcriptomes obtained by both methods pointed to downregulation of biosynthesis genes and indicated increased expression of several toxin-antitoxin (TA) modules (RelBE, MazEF, DinJYafQ, YgiU). TA modules are found on plasmids, where they constitute a maintenance mechanism (Gerdes et al., 1986b; Hayes, 2003). Typically, the toxin is a protein that inhibits an important cellular function, such as translation or replication, and forms an inactive complex with the antitoxin. The toxin is stable, while the antitoxin is degradable. If a daughter cell does not receive a plasmid after segregation, the antitoxin level decreases due to proteolysis, leaving a toxin that either kills the cell or inhibits propagation. TA modules are also commonly found on bacterial chromosomes, but their role is largely unknown. In *E. coli*, MazF and an unrelated toxin RelE induce stasis by cleaving mRNA, which of course inhibits translation, a condition that can be reversed by expression of corresponding antitoxins (Christensen and Gerdes, 2003; Pedersen et al., 2002). This property of toxins makes them excellent candidates for persister genes.

Ectopic expression of RelE (Keren et al., 2004) or MazF (Vazquez-Laslop et al., 2006) strongly increased tolerance to antibiotics. The first gene linked to persisters, *hipA* (Moyed and Bertrand, 1983), is also a toxin, and its ectopic expression causes multidrug tolerance as well (Correia et al., 2006; Falla and Chopra, 1998; Korch and Hill, 2006; Vazquez-Laslop et al., 2006). Interestingly, a bioinformatics analysis indicates that HipA is a member of the TOR family of kinases, which have been extensively studied in eukaryotes (Schmelzle and Hall, 2000) but have not been previously identified in bacteria. HipA is indeed a kinase; it

autophosphorylates on ser150, and site-directed mutagenesis replaces it, or other conserved amino acids in the catalytic and Mg^{2+} -binding sites abolish its ability to stop cell growth and confer drug tolerance (Correia et al., 2006). The crystal structure of HipA in complex with its antitoxin HipB was recently resolved, and a pull-down experiment showed that the substrate of HipA is elongation factor EF-Tu (Schumacher et al., 2009). Phosphorylated EF-Tu is inactive, which leads to a block in translation and dormancy (Figure A11-6).

The deletion of potential candidates of persister genes noted above does not produce a discernible phenotype affecting persister production, possibly due to the high degree of redundancy of these elements. In *E. coli*, there are at least 15 TA modules (Alix and Blanc-Potard, 2009; Pandey and Gerdes, 2005; Pedersen and Gerdes, 1999) and there are more than 80 in *M. tuberculosis* (Ramage et al., 2009).

High redundancy of TA genes would explain the lack of a multidrug-tolerance phenotype in knockout mutants, and therefore it seemed useful to search

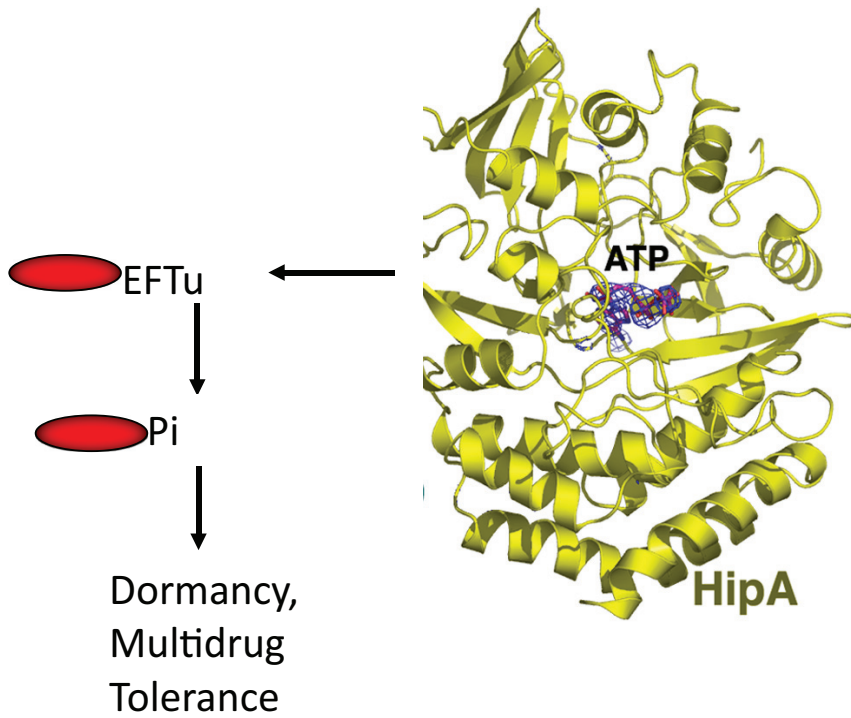


FIGURE A11-6 The HipA toxin causes dormancy in *E. coli* by phosphorylating elongation factor Tu, which inhibits protein synthesis.

for conditions where a particular toxin would be highly expressed in a wild-type strain and then examine a possible link to persister formation.

Several TA modules contain the Lex box and are induced by the SOS response. These are *symER*, *hokE*, *yafN/yafO*, and *tisAB/istr1* (Courcelle et al., 2001; Fernandez De Henestrosa et al., 2000; Kawano et al., 2007; McKenzie et al., 2003; Motiejunaite et al., 2007; Pedersen and Gerdes, 1999; Singletary et al., 2009; Vogel et al., 2004). Fluoroquinolones induce the SOS response (Phillips et al., 1987), and we tested the ability of ciprofloxacin to induce persister formation (Dorr et al., 2009).

Examination of deletion strains showed that the level of persisters dropped dramatically, 10- to 100-fold, in a Δ *tisAB* mutant. This suggests that TisB was responsible for the formation of the majority of persisters under conditions of SOS induction. The level of persisters was unaffected in strains deleted in the other Lex box containing TA modules. Persister levels observed in time-dependent killing experiments with ampicillin or streptomycin that do not cause DNA damage were unchanged in the Δ *tisAB* strain. TisB only had a phenotype in the presence of a functional RecA protein, confirming the dependence on the SOS pathway.

Ectopic overexpression of *tisB* sharply increased the level of persisters. The drop in persisters in a deletion strain and the increase upon overexpression gives reasonable confidence in functionality of a persister gene. The dependence of TisB-induced persisters on a particular regulatory pathway, the SOS response, further strengthens the case for TisB as a specialized persister protein (Figure A11-7). Incidentally, a *tisB* mutant is not present in the otherwise fairly complete Keio knockout library, and the small open reading frame might have been easily missed by Tn mutagenesis as well, evading detection by the generalized screens for persister genes.

The role of TisB in persister formation is unexpected based on what we know about this type of protein. TisB is a small, 29 amino acid hydrophobic peptide that binds to the membrane and disrupts the proton motive force (pmf), which leads to a drop in ATP levels (Unoson and Wagner, 2008). Bacteria, plants, and animals all produce antimicrobial membrane-acting peptides (Garcia-Olmedo et al., 1998; Sahl and Bierbaum, 1998; Zasloff, 2002). Toxins of many TA loci found on plasmids belong to this type as well. If a daughter cell does not inherit a plasmid, the concentration of a labile antitoxin decreases, and the toxin such as the membrane-acting *hok* kills the cell (Gerdes et al., 1986a). High-level artificial overexpression of TisB also causes cell death (Unoson and Wagner, 2008). It is remarkable from this perspective that the membrane-acting TisB under conditions of natural (mild) expression has the exact opposite effect of protecting the cell from antibiotics.

Fluoroquinolones such as ciprofloxacin are widely used broad-spectrum antibiotics, and their ability to induce multidrug-tolerant cells is unexpected and a cause of considerable concern. The induction of persister formation by fluoroquinolones may contribute to the ineffectiveness of antibiotics in eradicat-

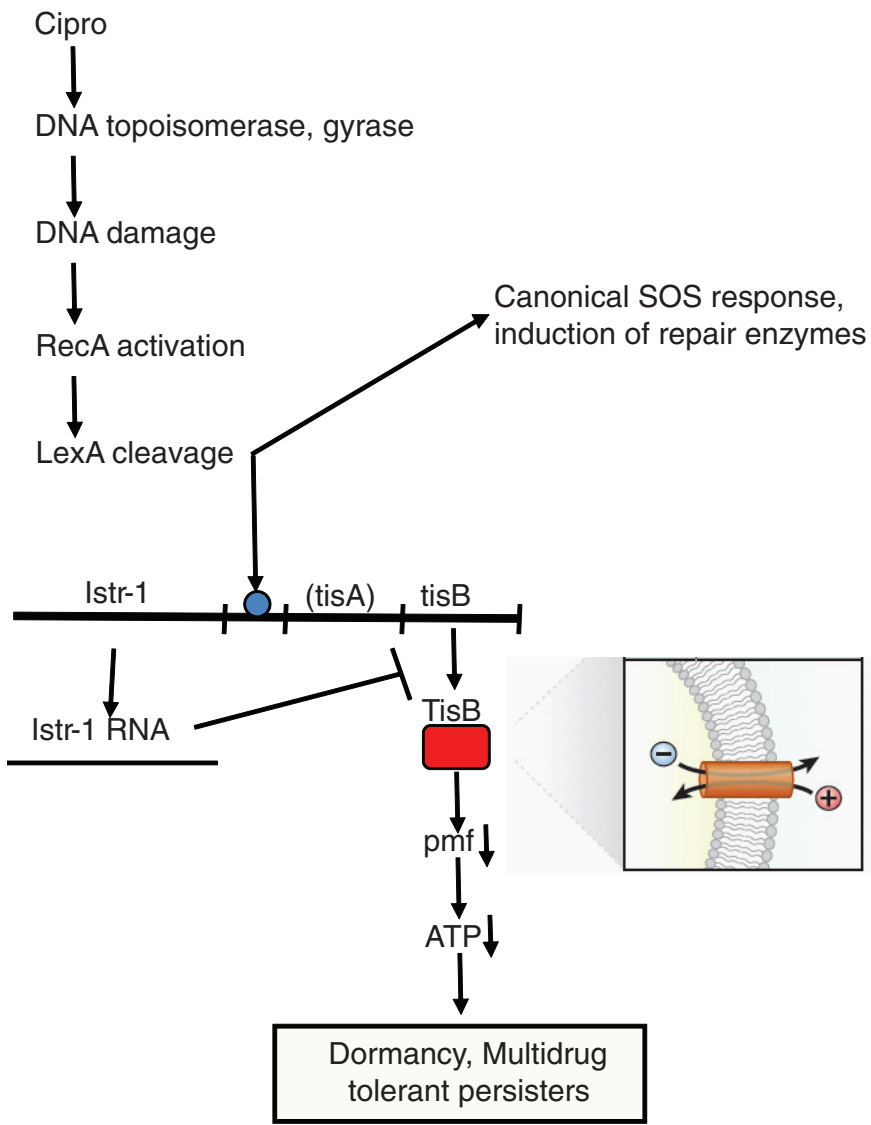


FIGURE A11-7 Persister induction by antibiotic. The common antibiotic ciprofloxacin causes DNA damage by converting its targets, DNA gyrase and topoisomerase, into endonucleases. This activates the canonical SOS response, leading to increased expression of DNA repair enzymes. At the same time, the LexA repressor that regulates expression of all SOS genes also controls transcription of the *TisAB* toxin-antitoxin module. The *TisB* toxin is an antimicrobial peptide, which binds to the membrane, causing an increase in pmf and ATP. This produces a systems shutdown, blocking antibiotic targets, which ensures multidrug tolerance.

ing infections. Indeed, preexposure with a low dose of ciprofloxacin drastically increased tolerance to subsequent exposure with a high dose, and TisB persisters are multidrug tolerant.

The finding of the role of TisB in tolerance opens an intriguing possibility of a wider link between other stress responses and persister formation. Pathogens are exposed to many stress factors in the host environment apart from DNA damaging agents: oxidants, high temperature, low pH, and membrane-acting agents. It is possible that all stress responses induce the formation of surviving persisters.

While resistance and tolerance are mechanistically distinct, there is sufficient reason to believe that tolerance may be a major cause for developing resistance. Indeed, the probability of resistance development is proportional to the size of the pathogen population, and a lingering chronic infection that cannot be eradicated due to tolerance will go on to produce resistant mutants and strains acquiring resistant determinants by transmission from other bacteria (Levin and Rozen, 2006). Combating tolerance then becomes a major component in preventing resistance.

The Discovery Challenge

Source Compounds

The discovery of penicillin was an isolated event, but the development of screening for antimicrobial activity from soil actinomycetes by Salman Waxman produced the first, and also the only known, effective platform technology for antibiotic discovery (Schatz et al., 1944). Cultivable actinomycetes, however, are a limited resource; ~99 percent of microbes do not readily grow in the lab and are known as “uncultured” (Lewis et al., 2010). Overmining of actinomycetes by the early 1960s replaced discovery of novel compounds with rediscovery of knowns.

In response to the dwindling returns in natural product antibiotic discovery, the industry responded by focusing on synthetics. Indeed, a number of antimicrobials are synthetic (metronidazole, trimethoprim, isoniazid, ethionamide, pyrazinamide, and ethambutol), and there is one highly effective class of synthetic broad-spectrum antibiotics, the fluoroquinolones. Encouraged by these examples, and by dramatic advances in synthetic and combinatorial chemistry, high-throughput robotics, genomics, and proteomics, a new discovery platform emerged (Figure A11-8). Combinatorial chemistry provided a large number of test compounds, which were screened in high-throughput format against isolated essential target proteins determined by genomics. This platform, however, failed to produce a new class of broad-spectrum antibiotics, leading to the closure of anti-infectives divisions in many of the Big Pharma companies. The main reasons for failure is well understood: high-throughput screening hits were literally running into the penetration barrier of Gram-negative bacteria, which is made of transenvelope multidrug resistance (MDR) pumps that extrude amphipathic

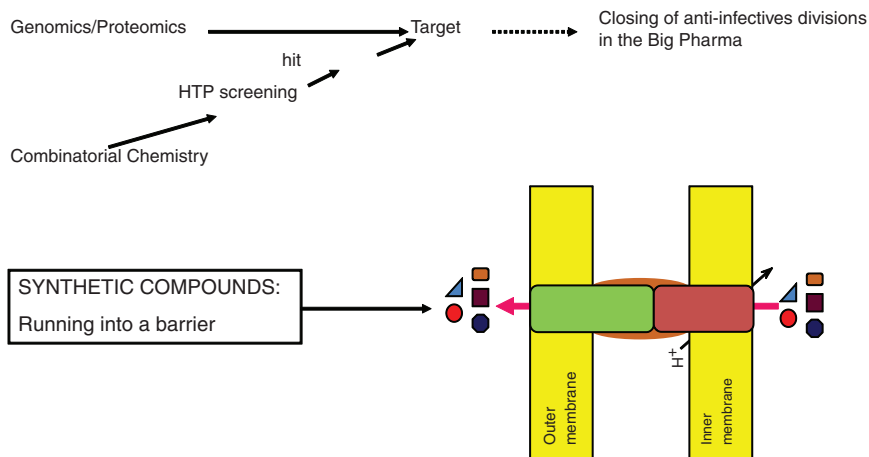


FIGURE A11-8 The high-tech platform. Early leads from screening compound libraries are extruded by transenvelope MDR pumps of Gram-negative species.

compounds across the outer membrane barrier (Lomovskaya et al., 2008). Drugs have to be amphipathic in order to penetrate across the hydrophobic inner membrane, but this is precisely the feature that the outer membrane restricts and the MDRs recognize. There are few compounds that pass this seemingly impenetrable barrier rather effectively: the broad-spectrum aminoglycosides, tetracyclines, fluoroquinolones, some β -lactams, chloramphenicol, and azithromycin. Fluoroquinolones are the only synthetics on this list, and they were discovered 50 years ago.

But what about less challenging narrow-spectrums, with good activity against at least Gram-positive species? Seventy high-throughput screens performed by GlaxoSmithKline, for example, against a large number of targets produced no viable leads (Payne et al., 2007). Glaxo scientists realized that penetration is a serious problem, and therefore also performed *in vivo* screens against *E. coli*, but only obtained “nuisance” hits, such as membrane-acting compounds. One obvious conclusion from this negative experience is that the libraries do not carry good starting compounds.

In part this is due to the fact that libraries are constructed based on Lipinski rules (Lipinski, 2003), which are good for predicting druglike properties for compounds acting against mammalian cell targets but do not work well for bacteria because of peculiarities of permeation (O’Shea and Moser, 2008; Silver, 2008). Another important consideration is the probability of resistance development. Pathogen populations produce 10^9 cells in an infected patient, which means that the probability of resistance development should be $<10^9$. This is readily achieved with most of the antibiotic classes currently in use, since they hit more than one

target (fluoroquinolones attack DNA gyrase and topoisomerase, β -lactams inhibit several penicillin-binding proteins, and ribosomal inhibitors bind to rRNA which is coded by multiple genes) (Silver, 2007). This requirement severely limits the number of realistic targets for antimicrobial drug discovery.

The above analysis presents an extremely bleak picture; if we cannot even discover compounds acting against rapidly growing Gram-positive bacteria, what are the prospects of finding broad-spectrum antimicrobials acting against non-growing stationary cells and persisters?

Opportunities

There are many steps in the drug discovery pipeline, but if there are no viable leads, there is no pipeline. Indeed, at the last Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) meeting (2009), there was not a single broad-spectrum lead presented. This means that the number of realistic broad-spectrum leads in the global antimicrobial drug discovery pipeline is zero. This is where the process needs to be restarted, and this is where allocation of resources will make a tangible impact.

A Fresh Look at Potential Sources of Compounds

Natural products There are two largely untapped and potentially enormous new sources of natural products: uncultured microorganisms and silent operons coding for secondary metabolites.

A recent resurgence in cultivation efforts aimed at gaining access to uncultured microorganisms has been sparked by the vast diversity of uncultured bacterial groups revealed by environmental surveys of 16S rRNA (Aoi et al., 2009; Bollmann et al., 2007; Bruns et al., 2002; Connon and Giovannoni, 2002; Davis et al., 2005; Ferrari et al., 2005; Gavrish et al., 2008; Kaerberlein et al., 2002; Nichols et al., 2008; Rappe et al., 2002; Stevenson et al., 2004; Zengler et al., 2002). While some novel bacterial species were successfully cultured by varying media and growth conditions (Joseph et al., 2003), significant departures from conventional techniques were clearly in order, and indeed the new technologies substantially diverged from traditional cultivation methods by adopting single-cell and high-throughput strategies (Connon and Giovannoni, 2002; Nichols et al., 2008; Rappe et al., 2002; Zengler et al., 2002), better mimicking the natural milieu (Aoi et al., 2009; Bruns et al., 2002; Ferrari et al., 2005; Stevenson et al., 2004), increasing the length of incubation, and lowering the concentration of nutrients (Davis et al., 2005). High-throughput extinction culturing is based on the dilution of natural communities of bacteria to 1 to 10 cells per well in low-nutrient, filtered marine water. This strategy resulted in the cultivation of the first member of the ubiquitous, previously uncultured clade, SAR11 (Rappe et al., 2002). Our research group contributed to the effort by developing three

cultivation methodologies (Gavrish et al., 2008; Kaeberlein et al., 2002; Nichols et al., 2008). All three strategies aim to provide microorganisms with their natural growth conditions by incubating them in simulated natural environments.

The diffusion chamber is designed to essentially “trick” cells into thinking they are growing in their natural environment by creating an incubation strategy that very closely mimics their natural habitat (Kaeberlein et al., 2002). The diffusion chamber consists of a stainless steel washer and 0.03- μm pore-size membranes (Figure A11-9). After gluing a membrane to one side of the washer, the inoculum (a mix of environmental cells and warm agar) is introduced, and the second membrane seals the chamber. Nutrients from the environment can diffuse into the chamber; therefore, it is not necessary to add them to the medium. Once inoculated and assembled, the chamber can be returned to the original location of sampling or in a simulated natural environment such as a block of sediment kept in an aquarium in lab. Microcolonies grow in the chamber during such incubation. A recovery rate of 22 percent on average was observed in the diffusion chambers. In this study and follow-up research (Bollmann et al., 2007; Nichols et al., 2008) we isolated numerous species that did not grow in Petri dishes inoculated with environmental samples but were successfully grown in the diffusion chambers.

Reinoculation of material both from marine and soil environments from chamber to chamber produces “domesticated” variants that grow on regular media on a Petri dish and can be exploited for secondary metabolite production (Bollmann et al., 2007; Nichols et al., 2008).

The diffusion chamber typically produces a mixed culture, which requires considerable time to isolate, purify, and reinoculate individual colonies. In order

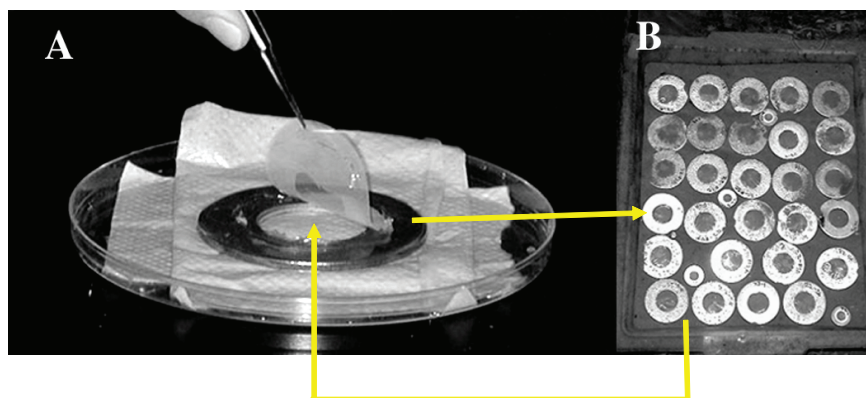


FIGURE A11-9 A diffusion chamber for growing bacteria in situ. A sample from marine sediment is diluted, mixed with agar, and sandwiched between the two semipermeable membranes of the diffusion chamber, which is returned to the environment.

to streamline this process into a high-throughput system, we developed a variant of the diffusion chamber for massively parallel microbial isolation. The Isolation Chip, or ichip for short (Nichols et al., 2010), consists of hundreds of miniature diffusion chambers that can be loaded with an average of one cell per chamber. The ichip enables microbial growth and isolation in a single step with hundreds of individual cultures incubating on a single chip.

Microorganisms that are particularly important for drug discovery, microscopic fungi and actinomycetes, grow by forming filaments capable of penetrating soft substrates. Since actinomycetes can pass through 0.2- μm pores, we reasoned this could be used to design a trap for the specific capture of these organisms (Gavriš et al., 2008). The trap is similar in design to the diffusion chamber, except the membranes have larger pores and the agar inside the trap is initially sterile when placed in the environment. Any growth observed afterward inside the trap is due to the movement of cells into the trap during incubation. The majority of organisms grown in the traps proved to be actinomycetes, some of which represented rare and unusual species from the genera *Dactylosporangium*, *Catellatospora*, *Catenulispora*, *Lentzea*, and *Streptacidiphilus*.

We noticed that some organisms forming colonies in the diffusion chamber can grow on a Petri dish, but only in the presence of other species from the same environment (Kaerberlein et al., 2002; Nichols et al., 2008) and suggested that uncultured bacteria only commit to division in a familiar environment, which they recognize by the presence of growth factors released by their neighbors. In order to assess the commonality of the growth dependence of uncultured organisms on neighboring species and pick good models for study, we chose an environment where bacteria live in a tightly packed community (D'Onofrio et al., 2010). This is a biofilm that envelopes sand particles of a tidal ocean beach (Figure A11-10). There were disproportionately more colonies appearing on densely inoculated plates compared with more dilute plates. This indicated that some of the cells that grew on the densely seeded plates were receiving growth factors from neighboring colonies. To test the possible growth dependence of microorganisms on neighboring species, pairs of colonies growing within a short distance of each other were restreaked in close proximity to each other. Potential uncultured isolates were identified by their diminishing growth with increasing distance from the cultivable “helper” strain on the cross-streak plates. Colonies of the culturable organism *Micrococcus luteus* KLE1011 (a marine sand sediment isolate 99.5 percent identical to *M. luteus* DSM 200030^T according to 16S rRNA gene sequence) grew larger as their distance from other colonies increased (Figure A11-10). Approximately 100 randomly picked pairs of colonies were re-streaked from the high-density plates, and 10 percent of these pairs showed this pattern of growth induction on cross-streaked plates.

In order to isolate growth factors, spent medium from the helper *M. luteus* KLE1011 was tested and shown to induce growth of the uncultured *M. polysiphoniae* KLE1104. An assay-guided fractionation led to isolation and structure

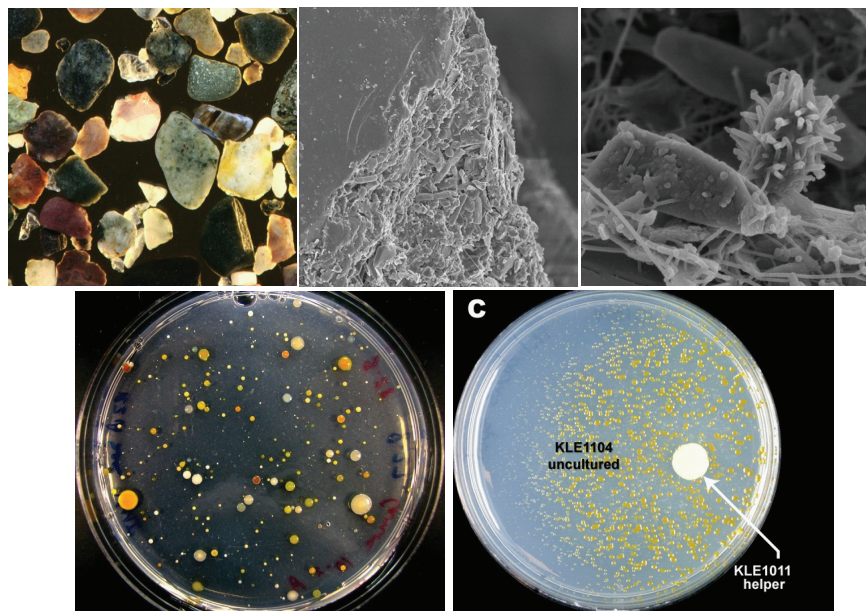


FIGURE A11-10 Understanding the mechanism of uncultivability. Marine sand particles are covered by a multispecies biofilm (top). Cells from the biofilm form colonies on a densely seeded plate, and pairing them together reveals that some of them are uncultured bacteria (evenly spread on the plate) that will only grow in the presence of a helper species spotted on the same plate (bottom).

determination of five different siderophores and each of them was able to induce growth of *M. polysiphoniae* KLE1104. This demonstrated that siderophores represent the growth factors responsible for the helping activity. The siderophores consisted of a central core with alternating *N*-hydroxycadaverine and succinic acid units and were of the desferrioxamine class (Challis, 2005). Both close relatives of known microorganisms and novel species were isolated by this approach. This study identified the first class of growth factors for uncultured bacteria and suggests that additional ones will come from analyzing organisms growing in co-culture.

Silent operons Whole-genome sequencing of several actinomycetes showed that there are many more potential biosynthetic pathways for production of secondary metabolites than there are known antibiotics made by these organisms (Ikeda et al., 2003). Ecopia used fermentation in 40 different media to entice production of additional compounds and discovered a novel type of enediyne with anticancer activity (Zazopoulos et al., 2003). No novel antimicrobials emerged from this

effort. However, in order to be effective, one needs to develop a high-throughput approach to induce production of such compounds. This is entirely doable.

Synthetics Are existing libraries, both commercially available and proprietary collections in Big Pharma, useless for antibiotic discovery? It does seem so, since they have obviously already been screened for actives, including nonbiased screens for growth inhibition of whole cells, and produced no viable leads. But does it not seem strange that a screen of a collection of 600 dyes by Domagk produced the first viable antibiotic, while a screen of the total global library of $\sim 10^7$ compounds produced nothing at all? As the libraries grew, a number of innovations were introduced aimed at improving the screening outcome; thus we have in vitro screening, targeted screens, Lipinski rules, and specificity validation. My feeling is that each time we tried to improve things, the result was to discard valuable compounds. I think that the existing libraries do harbor useful molecules; the question is how to identify them.

Good Compounds from Bad Libraries

Back to Domagk The first screen was also perfect: Domagk tested compounds against mice infected with streptococci. The result was the discovery of prontosil, a sulfa drug that has no activity in vitro. The compound is cleaved in the intestine by gut bacteria, releasing the active sulfonamide moiety, which inhibits dehydropteroate synthase in the folate pathway. An in vitro test would have missed prontosil. There are obvious advantages to testing compounds in situ—this automatically eliminates the significant burden of toxic molecules and demonstrates efficacy, again automatically eliminating substances with problems of action in an animal, such as serum binding, instability, or poor tissue distribution. In addition, different types of compounds may be uniquely uncovered, such as those requiring activation in situ and those hitting targets that are only important in an infection but not in vitro. While this would theoretically be the perfect way to go, testing in 10^7 mice is not an option for a variety of reasons, including ethical considerations and the large amounts of required test compound. We therefore considered a useful intermediate between in vitro and a mammal—an animal that, unlike mice, can be dispersed in microtiter wells. *Caenorhabditis elegans* can be infected with human pathogens by simply ingesting them, and we found that the worm can be cured by common antibiotics such as tetracycline and vancomycin, and at concentrations typically achieved in human plasma (Moy et al., 2006). Worms infected with a pathogen such as *Enterococcus faecalis* die, stop moving, their shape changes from curved to straight, and they can be detected by typical eukaryotic vital dyes (Figure A11-11). Using these parameters, an automated approach was developed, and a large pilot screen of compound libraries uncovered hits, some of which had no activity in vitro (Moy et al., 2006, 2009). This ap-

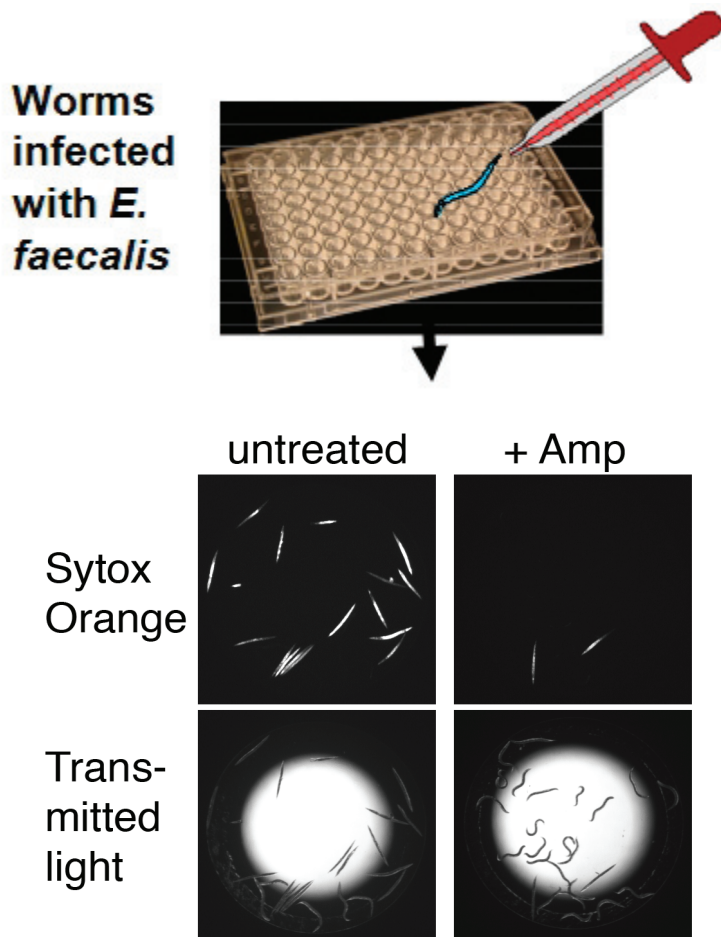


FIGURE A11-11 A high-throughput screen for antimicrobials in an animal model. *C. elegans* are infected with *E. faecalis*, and cured with ampicillin. This provides for an assay of compounds that cure the worm in situ.

proach shows that *C. elegans* points us in the right direction—back to Domagk, but with larger libraries.

Better libraries and rules of penetration Of course it would be great to have a better library, constructed based not on Lipinski rules but on “rules of penetration.” We have a small number of broad-spectrum compounds that are able to largely bypass the MDRs and get across the impermeable barrier of Gram-negative membranes—tetracycline, chloramphenicol, aminoglycosides, trimethoprim,

β -lactams (these only need to traverse the outer membrane), fluoroquinolones, and metronidazole. This set is too small to enable us to discern rules for penetration. But testing a large number of unbiased compounds from a library for their ability to enter into the cytoplasm of Gram-negative bacteria should allow us to deduce general rules that favor penetration. Once these are available, this would drive the synthesis and combinatorial chemistry of new compound libraries specifically geared toward antimicrobial discovery.

Prodrugs It is useful to consider the theoretically perfect antibiotic from first principles and then decide whether it is realistic. Approaches we discussed so far do not address the daunting challenge of killing persister cells while at the same time showing broad-spectrum activity. It is useful to start with the end result: a highly reactive compound will kill all cells, including persisters. In order to spare the host, the compound must be delivered as a prodrug, and then a bacteria-specific enzyme will activate it into a generally reactive molecule which will covalently bind to unrelated targets. Importantly, this mechanism creates an irreversible sink, largely resolving the issue of MDR efflux, so the antimicrobial is automatically broad spectrum. Is this realistic? Several existing antimicrobials closely match the properties of this idealized prodrug antibiotic. These are isoniazid, pyrazinamide, ethionamide, and metronidazole. The first three are anti-Mtb drugs, while metronidazole is a broad-spectrum compound acting against anaerobic bacteria. All four compounds convert into active antiseptic-type molecules inside the cell that covalently bind to their targets. It seems to be no accident that prodrug antibiotics make up the core of the anti-Mtb drug arsenal, since an ability to kill the pathogen is critical for treating the disease. Preferred targets have been identified for isoniazid and ethionamide (Vilcheze et al., 2005), suggesting a relatively limited reactivity of these compounds. The existence of preferred targets indicates that the prodrug products are not that reactive, and there is considerable room for developing better sterilizing antibiotics based on the same principle.

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A12

NEXT-GENERATION SYNTHETIC GENE NETWORKS⁶¹

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Synthetic biology is focused on the rational construction of biological systems based on engineering principles. During the field's first decade of development, significant progress has been made in designing biological parts and assembling them into genetic circuits to achieve basic functionalities. These circuits have been used to construct proof-of-principle systems with promising results in industrial and medical applications. However, advances in synthetic biology have been limited by a lack of interoperable parts, techniques for dynamically probing biological systems and frameworks for the reliable construction and operation of complex, higher-order networks. As these challenges are addressed, synthetic biologists will be able to construct useful next-generation synthetic gene networks with real-world applications in medicine, biotechnology, bioremediation and bioenergy.

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Ten years since the introduction of the field's inaugural devices—the genetic toggle switch (J.J.C. and colleagues) (Gardner *et al.*, 2000) and repressilator (Elowitz and Leibler, 2000)—synthetic biologists have successfully engineered a wide range of functionality into artificial gene circuits, creating switches (Gardner *et al.*, 2000; Kramer *et al.*, 2004; Isaacs *et al.*, 2003; Ham *et al.*, 2006, 2008; Deans *et al.*, 2007; Ajo-Franklin *et al.*, 2007; Friedland *et al.*, 2009), oscillators (Elowitz and Leibler, 2000; Fung *et al.*, 2005; Stricker *et al.*, 2008; Tiggles *et al.*, 2009), digital logic evaluators (Rinaudo *et al.*, 2007; Win and Smolke, 2008), filters (Basu *et al.*, 2005; Hooshangi *et al.*, 2005; Sohka *et al.*, 2009), sensors (Bayer and Smolke, 2005; Kobayashi *et al.*, 2004; Win and Smolke, 2007) and cell-cell communicators (Basu *et al.*, 2005; Kobayashi *et al.*, 2004). Some of these engineered gene networks have been applied to perform useful tasks such as population control (You *et al.*, 2004), decision making for whole-cell biosensors (Kobayashi *et al.*, 2004), genetic timing for fermentation processes (J.J.C. and colleagues) (Ellis *et al.*, 2009) and image processing (Levskaya *et al.*, 2005, 2009; Tabor *et al.*, 2009). Synthetic biologists have even begun to address important medical and industrial problems with engineered organisms, such as bacteria that invade cancer cells (Anderson *et al.*, 2006), bacteriophages with enhanced abilities to treat infectious diseases (Lu and Collins, 2007, 2009), and yeast with synthetic microbial pathways that enable the production of antimalarial drug precursors (Ro *et al.*, 2006). However, in most application-driven cases, engineered organisms contain only simple gene circuits that do not fully exploit the potential of synthetic biology. There remains a fundamental disconnect between low-level genetic circuitry and the promise of assembling these circuits into more complex gene networks that exhibit robust, predictable behaviors.

Thus, despite all of its successes, many more challenges remain in advancing synthetic biology to the realm of higher-order networks with programmable functionality and real-world applicability. Here, instead of reviewing the progress that has been made in synthetic biology, we present challenges and goals for next-generation synthetic gene networks, and describe some of the more compelling circuits to be developed and application areas to be considered.

Synthetic Gene Networks: What Have We Learned and What Do We Need?

The engineering of mechanical, electrical and chemical systems is enabled by well-established frameworks for handling complexity, reliable means of probing and manipulating system states and the use of testing platforms—tools that are largely lacking in the engineering of biology. Developing properly functioning biological circuits can involve complicated protocols for DNA construction, rudimentary model-guided and rational design, and repeated rounds of trial and error followed by fine-tuning. Limitations in characterizing kinetic processes and interactions between synthetic components and other unknown constituents *in vivo* make troubleshooting and modeling frustrating and prohibitively time

consuming. As a result, the design cycle for engineering synthetic gene networks remains slow and error prone.

Fortunately, advances are being made in streamlining the physical construction of artificial biological systems, in the form of resources and methods for building larger engineered DNA systems from smaller defined parts (Ellis *et al.*, 2009; Czar *et al.*, 2009; Guido *et al.*, 2006; Shetty *et al.*, 2008). Additionally, large-scale DNA sequencing and synthesis technologies are gradually enabling researchers to directly program whole genes, genetic circuits and even genomes, as well as to re-encode DNA sequences with optimal codons and minimal restriction sites (see review, Carr and Church [2009]).

Despite these advances in molecular construction, the task of building synthetic gene networks that function as desired remains extremely challenging. Accelerated, large-scale diversification (Wang *et al.*, 2009) and the use of characterized component libraries in conjunction with *in silico* models for a priori design (Ellis *et al.*, 2009) are proving useful in helping to fine-tune network performance toward desired outputs. Even so, in general, synthetic biologists are often fundamentally limited by a dearth of interoperable and modular biological parts, predictive computational modeling capabilities, reliable means of characterizing information flow through engineered gene networks and test platforms for rapidly designing and constructing synthetic circuits.

In the following subsections, we discuss four important research efforts that will improve and accelerate the design cycle for next-generation synthetic gene networks: first, advancing and expanding the toolkit of available parts and modules; second, modeling and fine-tuning the behavior of synthetic circuits; third, developing probes for reliably quantifying state values for synthetic (and natural) biomolecular systems; and fourth, creating test platforms for characterizing component interactions within engineered gene networks, designing gene circuits with increasing complexity and developing complex circuits for use in higher organisms. These advances will allow synthetic biologists to realize higher-order networks with desired functionalities for satisfying real-world applications.

Interoperable parts and modules for synthetic gene networks

Although there has been no shortage of novel circuit topologies to construct, limitations in the number of interoperable and well-characterized parts have constrained the development of more complex biological systems (Ellis *et al.*, 2009; Guido *et al.*, 2006; Purnick and Weiss, 2009; Lucks *et al.*, 2008). The situation is complicated by the fact that many potential interactions between biological parts, which are derived from a variety of sources within different cellular backgrounds, are not well understood or characterized. As a result, the majority of synthetic circuits are still constructed *ad hoc* from a small number of commonly used components (e.g., LacI, TetR and lambda repressor proteins and regulated promoters) with a significant amount of trial and error. There is a pressing need

to expand the synthetic biology toolkit of available parts and modules. Because physical interconnections cannot be made in biological systems to the same extent as electrical and mechanical systems, interoperability must be derived from chemical specificity between parts and their desired targets. This limits our ability to construct truly modular parts and highlights the need for rigorous characterization of component interactions so that detrimental interactions can be minimized and factored into computational models.

Engineered zinc fingers constitute a flexible system for targeting specific DNA sequences, one which could significantly expand the available synthetic biology toolkit for performing targeted recombination, controlling transcriptional activity and making circuit interconnections. Zinc-finger technology has primarily been used to design zinc-finger nucleases that generate targeted double-strand breaks for genomic modifications (Maeder *et al.*, 2008). These engineered nucleases may be used to enhance recombination in large-scale genome engineering techniques (Wang *et al.*, 2009). A second and potentially very promising use of engineered zinc fingers is as a source of interoperable transcription factors, which would greatly expand the current and limited repertoire of useful activators and repressors. In fact, zinc fingers have already been harnessed to create artificial transcription factors by fusing zinc-finger proteins with activation or repression domains (Beerli *et al.*, 2000; Park *et al.*, 2003). Libraries of externally controllable transcriptional activators or repressors could be created by engineering protein or RNA ligand-responsive regulators, which control the transcription or translation of zinc finger–based artificial transcription factors themselves (Bayer and Smolke, 2005). These libraries would enable the construction of basic circuits, such as genetic switches (Gardner *et al.*, 2000), as well as more complex gene networks. In fact, several of the higher-order networks we describe below rely on having multiple reliable and interoperable transcriptional activators and repressors for proper functioning.

Even so, these engineered transcription factors have not yet been fully characterized, and if they are to be used as building blocks for complex gene networks, then knowledge of their *in vivo* kinetics and input-output transfer functions would be beneficial. In addition, much of the rich dynamics associated with small, synthetic gene networks is attributable to the cooperative binding or multimerization of transcription factors, and it is not yet clear what further engineering is required to endow zinc-finger transcription factors with such features.

Nucleic acid–based parts, such as RNAs, are also promising candidates for libraries of interoperable parts because they can be rationally programmed based on sequence specificity (Deans *et al.*, 2007; Isaacs *et al.*, 2004; Win *et al.*, 2009). Novel circuit interconnections could be established using small interfering RNAs (siRNAs) to control the expression of specific components. Recombinases, which target specific DNA recombinase-recognition sites, also represent a fruitful, underutilized source of interoperable parts. Recombinases have been used in the context of synthetic biology to create memory elements and genetic

counters (Friedland *et al.*, 2009). However, more than 100 natural recombinases are known, and these can be engineered by mutagenesis and directed evolution for greater diversity and sequence specificity (Buchholz *et al.*, 1998; Kilby *et al.*, 1993; Santoro and Schultz, 2002; Groth and Calos, 2004).

Libraries of well-characterized, interoperable parts, such as transcription factors and recombinases, would vastly enhance the ability of synthetic biologists to build more complex gene networks with greater reliability and real-world applicability. In addition to libraries of individual parts, it would be of great value to have well-characterized and interoperable modules (e.g., switches, oscillators and interfaces) that could be used in a plug-and-play fashion to create higher-order networks and programmable cells. As the number of parts and modules expands, high-throughput, combinatorial efforts for quantifying the levels of interference and cross-talk between multiple components within cells will be increasingly important as guides for choosing the most appropriate components for network assembly.

Modeling and fine-tuning synthetic gene networks

Integrated efforts for modeling and fine-tuning synthetic gene circuits are useful for ensuring that assembled networks operate as intended. Such approaches will be increasingly important as more complex circuits are constructed along with the expanded development of interoperable parts. Although studies have shown that in some cases, component properties alone are sufficient for predicting network behavior (Ellis *et al.*, 2009; Guido *et al.*, 2006; Kaplan *et al.*, 2008), others have demonstrated the need for modeling and fine-tuning networks after their basic topologies have been established (Gardner *et al.*, 2000; Ellis *et al.*, 2009). A multi-step design cycle that involves creating diverse component libraries, constructing, characterizing and modeling representative network topologies, and assembling and fine-tuning desired circuits, followed by subsequent refinement cycles (Ellis *et al.*, 2009), will be crucial for the successful design and construction of next-generation synthetic gene networks.

The fine-tuning of biomolecular parts and networks can be achieved by developing diverse component libraries through mutagenesis followed by in-depth characterization and modeling (Ellis *et al.*, 2009; Alper *et al.*, 2005; Cox *et al.*, 2007; Hammer *et al.*, 2006; Jensen and Hammer, 1998; Murphy *et al.*, 2007). Significant progress has been made in tuning gene expression by altering transcriptional, translational and degradation activities. For example, promoter libraries with a range of transcriptional activities can be created and characterized, plugged into *in silico* models and then used to develop synthetic gene networks with defined outputs, without significant post-hoc adjustments (Ellis *et al.*, 2009; Alper *et al.*, 2005; Cox *et al.*, 2007; Hammer *et al.*, 2006; Jensen and Hammer, 1998; Murphy *et al.*, 2007). Alternatively, synthetic ribosome binding site (RBS) sequences can be used to optimize protein expression levels. Recently, Salis *et*

al. (2009) have developed a thermodynamic model for predicting the relative translational initiation rates for a protein with different upstream RBS sequences, a model that can also be used to rationally forward-engineer RBS sequences to give desired protein expression. In addition, protein degradation can be controlled by tagging proteins with degradation-targeting peptides that impart different degradation dynamics (Andersen *et al.*, 1998).

By automating the construction and characterization of biomolecular components, extensive libraries could be created for the rapid design and construction of complex gene networks. These efforts, coupled with *in silico* modeling, would serve to fast-track synthetic biology (more detailed discussions of modeling techniques for synthetic biology are found in Ellis *et al.*, 2009; Guido *et al.*, 2006; Nevozhay *et al.*, 2009; Chandran *et al.*, 2009; Kaznessis, 2009; Hasty *et al.*, 2001). However, to build reliable models of biomolecular parts and networks, new methods for probing and acquiring detailed *in vitro* and *in vivo* measurements are needed, which we discuss below.

Probes for characterizing synthetic gene networks

Significant advances have been made in the development of new technologies for manipulating biological systems and probing their internal states. At the single-molecule level, for instance, optical tweezers and atomic force microscopes provide new, direct ways to probe the biophysical states of single DNA, RNA and protein molecules as they undergo conformational changes and other dynamical processes (Khalil *et al.*, 2007, 2008; Svoboda and Block, 1994; Bustamante *et al.*, 2003; Neuman and Nagy, 2008). However, we lack similar tools for tracking the *in vivo* operation of synthetic gene circuits in a high-throughput fashion. Ideally, making dynamical measurements of biological networks would involve placing sensors at multiple internal nodes, akin to how current and voltage are measured in electrical systems. Furthermore, external manipulation of synthetic biomolecular systems is typically accomplished by the addition of chemical inducers, which can suffer from cross-talk (Lee *et al.*, 2007), be difficult to remove and be consumed over time. As a result, inputs are often troublesome to control dynamically.

Microfluidic devices have been coupled to single-cell microscopy and image processing techniques to enable increasingly precise manipulation and measurement of cells, especially since inputs can be modulated over time (Gulati *et al.*, 2009; Bennett and Hasty, 2009). These systems allow the rapid addition and removal of chemical inducers, enabling more sophisticated, time-dependent inputs than conventional step functions, while also enabling researchers to track and quantify single cells for long periods of time. These developments make possible the wider use of well-established engineering approaches for analyzing circuits and other systems in synthetic biology. For example, frequency-domain analysis, a technique used commonly in electrical engineering (Simpson *et al.*, 2003;

Mettetal *et al.*, 2008), can be employed with microfluidics to characterize the transfer functions and noise behaviors of synthetic biological circuits (Simpson *et al.*, 2003; Mettetal *et al.*, 2008; Bennett *et al.*, 2008). Additionally, small-signal linearization of nonlinear gene circuits can be achieved by applying oscillatory perturbations with microfluidics and measuring responses at the single-cell level (Mettetal *et al.*, 2008; Bennett *et al.*, 2008).

Indeed, microfluidics provides a useful platform for perturbing synthetic gene circuits with well-controlled inputs and observing the outputs in high-resolution fashion. Without the proper ‘sensors’ (that is, for quantitatively and simultaneously probing all the internal nodes of a given gene circuit), however, this technology alone is not sufficient to bring full, engineering-like characterization to synthetic gene networks.

Thus far, probes enabling quantitative measurements of synthetic gene circuits have primarily focused on the use of fluorescent proteins for *in vivo* quantification of promoter activity or protein expression. With the advent of novel mass spectrometry-based methods that provide global, absolute protein concentrations in cells (Malmstrom *et al.*, 2009), quantitative transcriptome data can now be merged with proteome data, improving our ability to characterize and model the dynamics of synthetic gene networks. Global proteomic data may also assist synthetic biologists in understanding the metabolic burden that artificial circuits place on host cells. Further efforts to devise fluorescent-based and other types of reporters for the simultaneous monitoring of transcriptome and proteome dynamics *in vivo* are needed to close the loop on full-circuit accounting. Some promising tools under development include tracking protein function by incorporating unnatural amino acids that exhibit fluorescence (Summerer *et al.*, 2006; Wang *et al.*, 2006), quantum dots (Michalet *et al.*, 2005) and radiofrequency-controlled nanoparticles (Hamad-Schifferli *et al.*, 2002).

As the field awaits entire-circuit probes, there are, in the meantime, several potentially accessible technologies for increasing the throughput and pace of piecewise gene-circuit characterization. Recent advances in engineering light-inducible biological parts and systems (Levskaya *et al.*, 2005, 2009; Boyden *et al.*, 2005) have unlocked the potential for optical-based circuit characterization, expanding the number and type of tunable knobs available to synthetic biologists. For instance, by coupling a synthetic gene network of interest to a biological light/dark sensor as well as to fluorescent protein outputs, one could potentially measure the network’s input/output transfer function in a high-throughput fashion using spectrophotometric microplate readers, without having to add varying concentrations of chemical inducers. In essence, both control and monitoring of biomolecular systems would be accomplished using reliable and high-speed optics that are typically associated with fluorescence readouts and microscopy. This is an exciting prospect, particularly in the context of microfluidic devices, which would facilitate the focusing of optical inputs and readouts to single cells.

Using electrical signals, in lieu of chemical or optical signals, for control

and monitoring of biological systems would also present high-speed advantages. Recently, advances have been made in integrating silicon electronics with lipid bilayers containing transmembrane pores to perform electronic signal conduction (Misra *et al.*, 2009). This technology may eventually allow direct communication and control between engineered cells and electronic circuits by means of ionic flow. The incorporation of these and other technologies to perturb and monitor the *in vivo* performance of synthetic gene networks will enable us to achieve desired functionality faster and more reliably.

Test platforms for engineering complex gene circuits

Increasing complexity—whether assembling larger synthetic gene networks from smaller ones or engineering circuits into higher organisms—dramatically increases the number of potential failure modes. In the former case, combining multiple individually functioning genetic circuits into a single cellular background can lead to unintended interactions among the synthetic components or with host factors, and these various failure modes are often difficult to pinpoint and isolate from one another. In the latter case, engineering synthetic networks for mammalian systems poses additional challenges beyond engineering circuits for bacterial and yeast strains, which have comparatively well-characterized genomes, transcriptomes, proteomes and metabolomes. Mammalian systems are much more complex and possess substantially less well-characterized components for engineering (Weber and Fussenegger, 2009), but for these and other reasons, constitute fertile ground for new applications and genetic parts.

The development of test platforms where engineered gene circuits can be designed and validated before being deployed in other or more complex cellular backgrounds would mitigate failure-prone jumps in complexity. These platforms could be used to verify or debug circuit topology and basic functionality in well-controlled environments. For example, cells optimized for testing may be engineered to have minimal genomes to decrease the risk of pleiotropic or uncharacterized interactions between the host and the synthetic networks (Gibson *et al.*, 2008; Glass *et al.*, 2006; Lartigue *et al.*, 2007, 2009; Carrera *et al.*, 2009). The use of orthogonal parts that are decoupled from host cells may enable the dedication of defined cellular resources to engineered functions, which can simplify the construction and troubleshooting of gene circuits. For example, nucleic acid-based parts can be designed to function orthogonally to the wild-type cellular machinery (Rackham and Chin, 2005; Wang *et al.*, 2007; An *et al.*, 2009). Artificial codons and unnatural amino acids, which have enabled new methods for studying existing proteins and the realization of proteins with novel functions, could also be used to produce synthetic circuits that function orthogonally to host cells (Wang *et al.*, 2009). Simplifying backgrounds would additionally enable more accurate computational modeling of complex circuits before they are deployed into their ultimate environments. Furthermore, minimal cells could

themselves contain synthetic circuits that provide useful testing functionalities, such as multiplexed transcriptional and translational controls and output probes.

Lower organisms can also be useful for the construction and characterization of synthetic gene networks before such systems are extended and deployed into higher organisms. In fact, several synthetic circuits, such as clocks and switches, were initially developed in bacteria and later translated into mammalian counterparts using analogous design principles (Kramer *et al.*, 2004; Deans *et al.*, 2007; Tigges *et al.*, 2009). Additionally, lower-organism test platforms could be endowed with certain features of interest from desired higher-organism hosts. For example, RNA interference–based circuits could be built first in *Saccharomyces cerevisiae* before being used in mammalian cells (Drinnenberg *et al.*, 2009). In one case, mitochondrial DNA was engineered into *Escherichia coli* before retransplantation into mammalian hosts (Yoon and Koob, 2003). Other biomolecular systems and components that are ripe for engineering in lower organisms include chromatin, ubiquitins and proteosomes.

The introduction of synthetic gene networks into higher organisms also runs the risk of compromising natural networks, which have evolved to maintain cellular robustness. Accordingly, methods for simplifying organisms for designing and testing synthetic circuits could be extended to engineer final deployment hosts, making them more conducive to synthetic gene circuits. Ultimately, *in vivo* directed evolutionary methods, based on repeated rounds of mutagenesis and selection within final cellular backgrounds, could be used to identify the optimal performance conditions of synthetic gene networks after their basic functionalities have been validated in earlier test platforms (Wang *et al.*, 2009).

Next-Generation Gene Networks

Advancing synthetic gene circuits into the realm of higher-order networks with programmable functionality is one of the ultimate goals of synthetic biology. Useful next-generation gene networks should attempt to satisfy at least one of the following criteria: first, yield insights into the principles that guide the operation of natural biological systems; second, highlight design principles and/or provide modules that can be applied to the construction of other useful synthetic circuits; third, advance the tools available for novel scientific experiments; and fourth, enable real-world applications in medicine, industry and/or agriculture. Below, we describe several next-generation gene circuits and discuss their potential utility in the context of the above criteria.

Tunable filters and noise generators

Fine-tuning the performance of a synthetic gene network typically means reengineering its components, be it by replacing or mutating its parts. Networks whose responses can be tuned without the reengineering of its parts, such as the

biological version of a tunable electronic filter, would enable more sophisticated cellular-based signal processing. Synthetic transcriptional cascades can exhibit low-pass filter characteristics (Hooshangi *et al.*, 2005), and artificial gene circuits with negative autoregulation are capable of pushing the noise spectra of their outputs to higher frequencies, where it can be filtered by the low-pass characteristics of a downstream gene cascade (Austin *et al.*, 2006). Tunable genetic filters with respect to time could be implemented by tuning RNA and/or protein degradation in autoregulated negative-feedback circuits (Simpson *et al.*, 2003; Mar *et al.*, 1999; McGinness *et al.*, 2006; Banaszynski *et al.*, 2006) (Fig. A12-1). Such circuits would be useful in studying and shaping noise spectra to optimize the performance of artificial gene networks.

Recently, an externally tunable, bacterial bandpass-filter has been described (Sohka *et al.*, 2009) that uses low-pass and high-pass filters in series to derive bandpass activity with respect to enzymes and inducer molecules. These types of filters, when coupled to quorum-sensing modules, can be used for spatial patterning applications (Basu *et al.*, 2005; Sohka *et al.*, 2009). They could also be

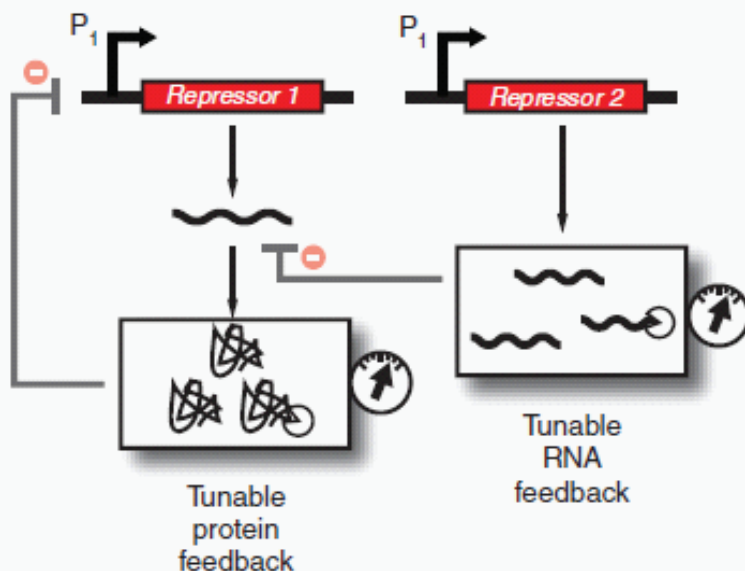


FIGURE A12-1 Tunable genetic filter. Filter characteristics can be adjusted by tuning the degradation of RNA and protein effectors in negative-feedback loops. Examples of RNA effectors include siRNAs, riboregulators and ribozymes. Examples of protein effectors include transcriptional activators and repressors. In this example, the P_1 promoter is suppressed by transcriptional repressor proteins expressed from the *Repressor 1* gene.

readily extended to complex multicellular pattern formation by engineering a suite of different cells, each carrying filters that respond to different inputs. Synthetic gene circuits based on tunable filters may also make useful platforms for studying cellular differentiation and development, as artificial pattern generation is a model for how natural systems form complex structures (Basu *et al.*, 2005; Sohka *et al.*, 2009).

Along similar lines, recent developments in stem cell biology have unlocked important potential roles for synthetic gene networks (Takahashi *et al.*, 2007). For example, it has been shown that stochastic fluctuations in protein expression in embryonic stem cells are important for determining differentiation fates (Macarthur *et al.*, 2009). Indeed, stochasticity might be harnessed in differentiation to force population-wide heterogeneity and provide system robustness, though it may also be detrimental if it causes uncontrollable differentiation.

The effects of stochasticity in stem cell differentiation could be studied with synthetic gene circuits that act as tunable noise generators. Lu *et al.*, for instance, considered two such designs for modulating the noise profile of an output protein (Lu *et al.*, 2008). This showed that the mean value and variance of the output can be effectively tuned with two external signals, one for regulating transcription and the other for regulating translation, and to a greater extent with three external signals, the third for regulating DNA copy number (Lu *et al.*, 2008). By varying noise levels while keeping mean expression levels constant, the thresholds at which gene expression noise yields beneficial versus detrimental effects on stem cell differentiation can be elucidated (J.J.C. and colleagues) (Blake *et al.*, 2006).

Furthermore, the discovery of induced pluripotent stem cells (iPSCs), based on the controlled expression of four transcription factors (OCT4, SOX2, KLF2 and MYC) in adult fibroblasts, has created a source of patient-specific progenitor cells for engineering (Takahashi *et al.*, 2007). Genetic noise generators and basic control circuits could be used to dissect the mechanism for inducing pluripotency in differentiated adult cells by controlling the expression levels of the four iPSC-dependent transcription factors. Ultimately, these efforts could lead to the development of timing circuits (Ellis *et al.*, 2009) for higher-efficiency stem cell reprogramming.

Lineage commitment to trophoderm, ectoderm, mesoderm and endoderm pathways are controlled by distinct sets of genes (Macarthur *et al.*, 2009), and many interacting factors, including growth factors, extracellular matrices and mechanical forces, play important roles in cellular differentiation (Discher *et al.*, 2009). As differentiation pathways become better understood, synthetic gene cascades may be used to program cellular commitment with increased fidelity for applications in biotechnology and regenerative medicine.

Analog-to-digital and digital-to-analog converters

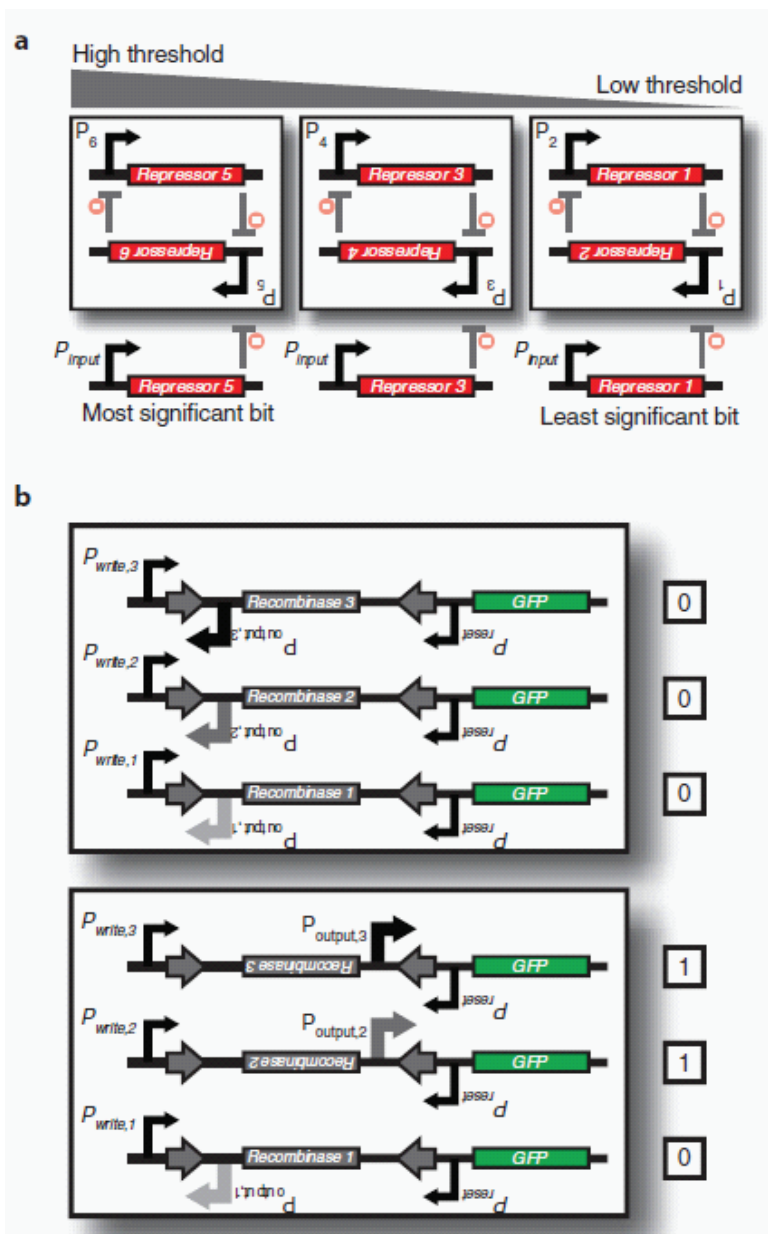
Electrical engineers have used digital processing to achieve reliability and flexibility, even though the world in which digital circuits operate is inherently

analog. Although synthetic biological circuits are unlikely to match the computing power of digital electronics, simple circuits inspired by digital and analog electronics may significantly increase the reliability and programmability of biological behaviors.

For example, biological analog-to-digital converters could translate external analog inputs, such as inducer concentrations or exposure times, into internal digital representations for biological processing. Consider, for instance, a bank of genetic switches with adjustable thresholds (Fig. A12-2a). These switches could be made out of libraries of artificial transcription factors, as described above. This design would perform discretization of analog inputs into levels of digital output. Depending on the level of analog inputs, different genetic pathways could be activated. Cells possessing analog-to-digital converters would be useful as biosensors in medical and environmental settings. For example, whole-cell biosensors (Kobayashi *et al.*, 2004), resident in the gut, may be engineered to generate different reporter molecules that could be measured in stool depending on the detected level of gastrointestinal bleeding. Expressing different reporter molecules rather than a continuous gradient of a single reporter molecule would yield more reliable and easily interpretable outputs. Digital-to-analog converters, on the other hand, would translate digital representations back into analog outputs (Fig. A12-2b); such systems could be used to reliably set internal system states. For example, instead of fine-tuning transcriptional activity with varying amounts of chemical inducers, a digital-to-analog converter, composed of a bank of genetic switches, each of which is sensitive to a different inducer, might provide better control. If each activated switch enabled transcription from promoters of varying strengths ($P_{\text{output},3} > P_{\text{output},2} > P_{\text{output},1}$), then digital combinations of inducers could be used to program defined levels of transcriptional activities (Fig. A12-2b). Such a circuit might be useful in biotechnology applications, where reliable expression of different pathways is needed for programming different modes of operation in engineered cells. In addition, digital-to-analog converters may be useful in providing a multiplexed method for probing synthetic circuits. For example, because each analog level is associated with a distinct digital state, a single analog output can allow one to infer the internal digital state of a synthetic gene network (Fig. A12-2b).

Adaptive learning networks

Synthetic gene networks that can learn or adapt to exogenous conditions could provide insight into natural networks and be useful for applications where adaptation to external stimuli may be advantageous, such as autonomous whole-cell biosensors (Fernando *et al.*, 2009; Fritz *et al.*, 2007). Endogenous biomolecular networks in bacteria can exhibit anticipatory behavior for related perturbations in environmental stimuli (Tagkopoulos *et al.*, 2008; Mitchell *et al.*, 2009). This type of behavior and the associated underlying design principles could, in principle, be harnessed to endow transcriptional networks with the ability to learn



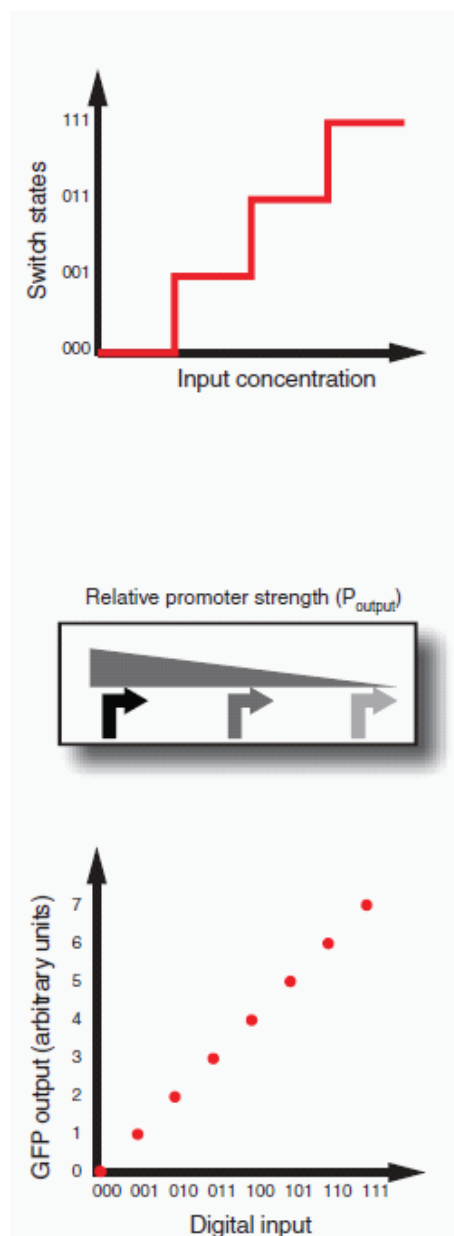


FIGURE A12-2 Genetic signal converters. **(a)** Analog-to-digital converter circuit that enables the discretization of analog inputs. The circuit is composed of a bank of toggle switches that have increasing response thresholds so that sequential toggling is achieved as input levels increase. The design could enable different natural or synthetic pathways to be activated depending on distinct input ranges, which may be useful in cell-based biosensing applications. Inputs into promoters and logic operations are shown explicitly except when the promoter (P) name is italicized, which represents an inducible promoter. **(b)** Digital-to-analog converter circuit that enables the programming of defined promoter activity based on combinatorial inputs. The circuit is composed of a bank of recombinase-based switches, known as single-invertase memory modules (SIMMs) (Friedland et al., 2009). Each SIMM is composed of an inverted promoter and a recombinase gene located between its cognate recognition sites, indicated by the arrows. Upon the combinatorial addition of inducers that activate specific P_{write} promoters, different SIMMs will be flipped, enabling promoters of varying strength to drive green fluorescent protein (GFP) expression. This allows combinatorial programming of different levels of promoter activity.

(Fernando *et al.*, 2009), much like synaptic interconnections between neurons. A basic design that would enable this functionality involves two transcriptional activators (Activator A and Activator B), each of which is expressed in the presence of a different stimulus (Fig. A12-3a). Suppose that both transcriptional activators drive the expression of effector proteins (Effector A and Effector B), which control distinct genetic pathways. When both transcriptional factors are active, indicating the simultaneous presence of the two stimuli, a toggle switch is flipped ON. This creates an associative memory. Subsequently, if either of the transcription factors is activated, AND logic between the ON toggle switch and one transcriptional activator produces the effector protein that controls the pathways of the other activators. On the basis of this design, cells could be programmed to associate simultaneous inputs and exhibit anticipatory behavior by activating the pathways of associated stimuli, even in the presence of only one of the stimuli. In another example of a learning network, one could design bacteria that could be taught ‘winner-take-all’ behavior in detecting stimuli, similar to cortical neural processing (Lee *et al.*, 1999). In this example, bacteria could be exposed to different types of chemical stimuli (Inducers A–C; Fig. A12-3b). An exogenously added inducer (Inducer ‘Learn’) acts as a trigger for learning and serves as one input into multiple, independent transcriptional AND gates, which possess secondary inputs for detecting the presence of each of the different chemical stimuli. Each gate drives an individual toggle switch that, when flipped, suppresses the flipping of the other switches. This creates a winner-take-all system in which the presence of the most abundant chemical stimuli is recorded. Furthermore, the toggle switch outputs could be fed as inputs into transcriptional AND gates, which once again possess secondary inputs for detecting the presence of the different stimuli. If these gates drive different fluorescent reporters when activated, then the overall system will associate only a single type of stimuli with the learning trigger and respond with an output only in the presence of the single type of stimuli in the future. This system could potentially be adapted to create chemotactic bacteria that ‘remember’ a particular location or landmark and only respond to the gradient of one chemoattractant.

In more complicated instances of learning networks, it is conceivable that synthetic gene circuits could be designed to adapt on their own, that is, without external mutagenesis or exogenous nucleic acids. For example, transcription-based interconnections could be dynamically reconfigured based on the expression of DNA recombinases (Friedland *et al.*, 2009). Another design could involve error-prone RNA polymerases, which create mutant RNAs that could be reverse-transcribed and joined back into the genome based on double-stranded breaks created by zinc-finger nucleases. Specificity for where the mutations would occur could be achieved by using promoters that are uniquely read by the error-prone RNA polymerases, such as T7 promoters with a T7 error-prone RNA polymerase, and zinc-finger nucleases that define where homologous recombination can occur (Brakmann and Grzeszik, 2001). In this design, enhanced mutagenesis frequencies could be targeted to specific regions of the genome.

Protein-based computational circuits

Beyond DNA- and RNA-based circuits, protein-based synthetic systems have the potential to enable flexible and fast computation through post-translational mechanisms (Yeh *et al.*, 2007; Dueber *et al.*, 2007; Bashor *et al.*, 2008). Protein-based circuits are advantageous in that they can be designed to target synthetic activities to subcellular locations (Levskaia *et al.*, 2009). In this way, different sites within the same cell could have different protein circuit states rather than relying solely on shared cellular promoter states, thereby enabling researchers to explore the functional dynamics and consequences of cellular localization. Protein-based designs can also operate on much shorter time scales than genetic circuits because their operation is independent of the transcription and translation machinery (Goldberg *et al.*, 2009). Accordingly, it would be exciting to develop protein-based circuits that can act as rapidly responding logic gates, smart sensors or memory elements.

With regards to this last application, synthetic amyloids could serve as novel components for epigenetic memory circuits. By fusing a yeast prion determinant from Sup35 to the rat glucocorticoid receptor, a transcription factor regulated by steroid hormone, Li and Lindquist (2000) demonstrated that the state of transcriptional activity from the fused protein could be affected and inherited stably in an epigenetic fashion. Given the increasing number of identified prionogenic proteins (Alberti *et al.*, 2009), there is an opportunity to create amyloid-based memory systems that transmit functionality from one generation to the next (Fig. A12-4). In these systems, aggregation could be induced by the transient expression of the prionogenic domain (PD), whereas disaggregation could be achieved by expressing protein remodeling factors, such as chaperones (heat shock protein 104). Though this system relies on the transcription and translation of prionogenic and disaggregating factors, it may enable the control of protein effectors that can operate on shorter time scales. For example, enzymes fused to a prionogenic domain may exhibit different activity levels depending on whether they are attached to an amyloid core.

Because genetic circuits and proteins function on different time scales, it would also be worthwhile to develop synthetic networks that couple both modalities. For example, the output of protein-based computation could be stored in recombinase-based memory elements (Ham *et al.*, 2006, 2008; Friedland *et al.*, 2009). It would also be conceivable to couple the two types of networks to harness their varied filtering capabilities. For example, the mitogen-activated protein kinase cascade contains both positive-feedback and negative-feedback loops that enable rapid activation followed by deactivation (Bhalla *et al.*, 2002), thus acting like a high-pass filter. On the other hand, transcription- and translation-based gene networks operate on longer time scales rendering them effective low-pass filters. Thus, synthetic kinase/phosphatase circuits that in turn drive gene-based networks could be used to create bandstop filters, which could be coupled with other bandpass filters and used for complex patterning applications.

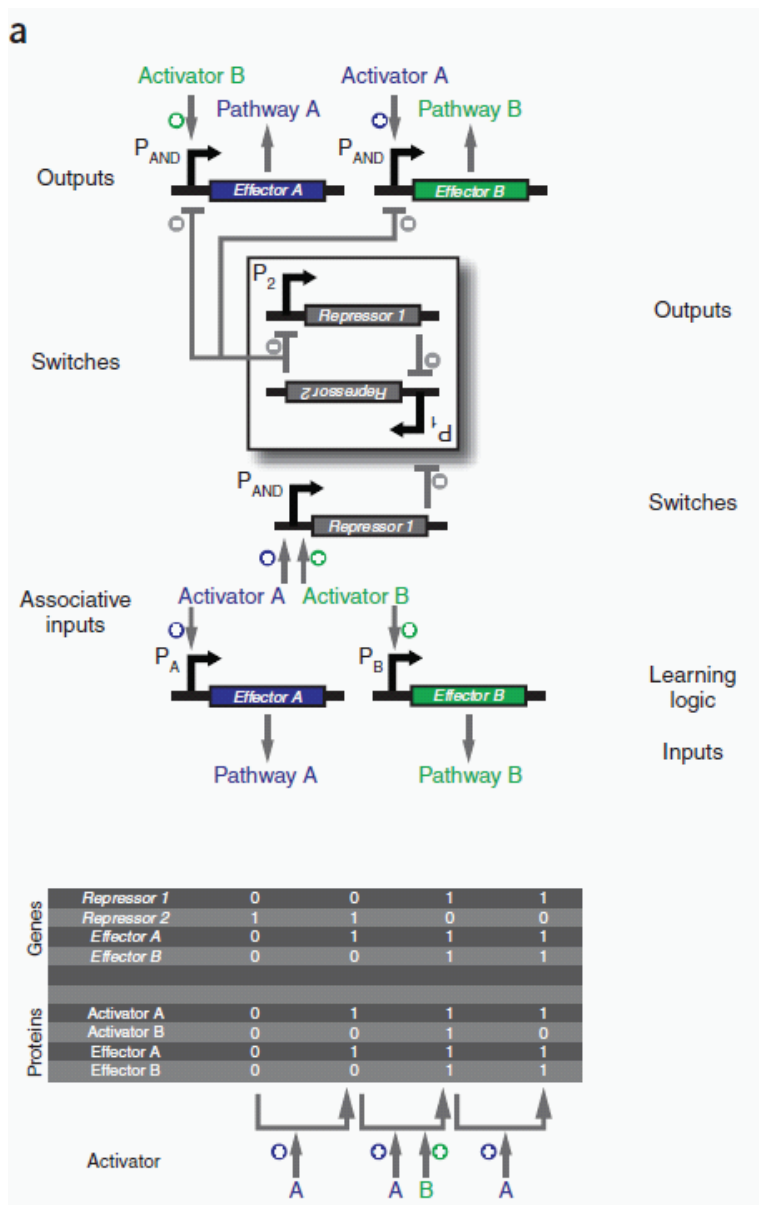
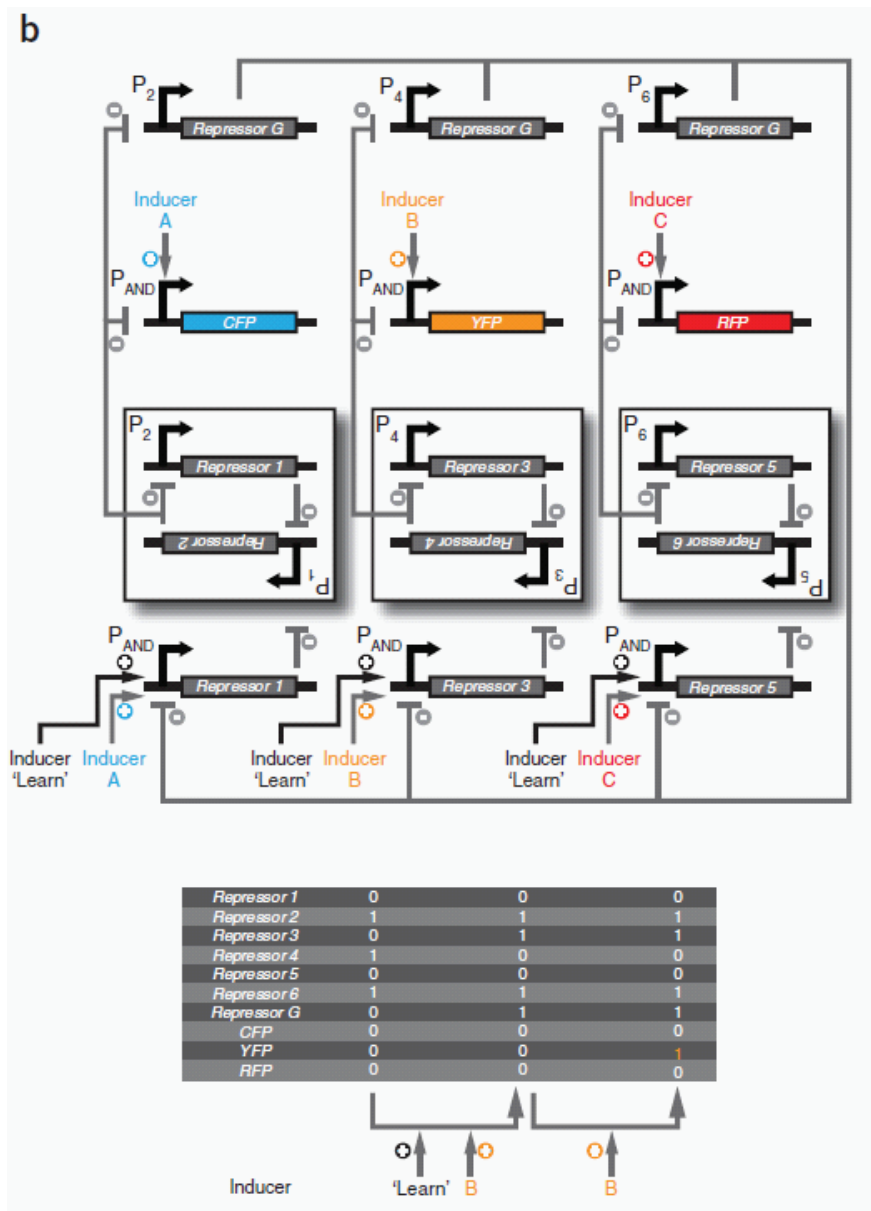


FIGURE A12-3 Adaptive learning networks. (a) Associative memory circuit enables association between two simultaneous inputs ('Activator A' and 'Activator B') so that the subsequent presence of only a single input can drive its own pathway and the pathway of the other input. Associations between inputs are recorded by a promoter ' P_{AND} ' that is activated in the presence of Activator A and Activator B to toggle the memory switch. Inputs



into promoters and logic operations are shown explicitly except when the promoter name is italicized, which represents an inducible promoter. **(b)** Winner-take-all circuit allows only one input out of many to be recorded. This effect is achieved by a global repressor protein that gates all inputs and prevents them from being recorded if there has already been an input recorded in memory.

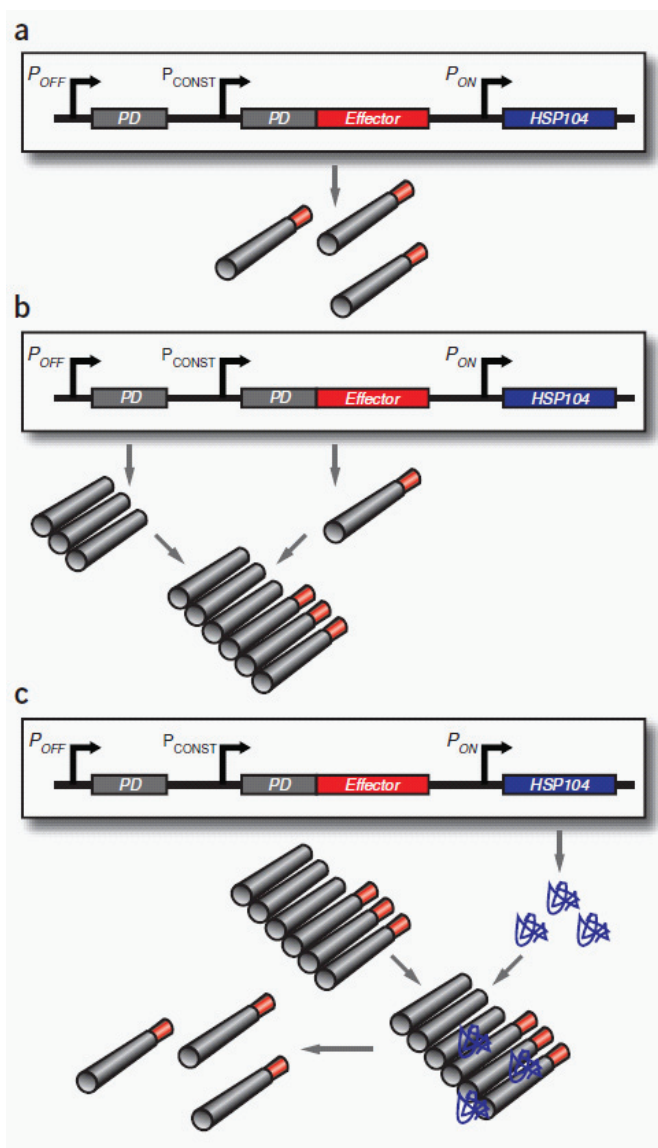


FIGURE A12-4 Amyloid-based memory. (a) Amyloid-based memory can be implemented by fusing a prionogenic domain (*PD*) to an effector gene, such as a transcriptional activator. (b) Overexpressing the prion-determining region via promoter ' P_{OFF} ' causes aggregation of the fusion protein, rendering the effector inactive. (c) Subsequent overexpression of chaperone proteins (e.g., *HSP104*), which act to disaggregate amyloids, via promoter ' P_{ON} ' releases the effector from the amyloid state and enables it to fulfill its function. Inputs into promoters and logic operations are shown explicitly except when the promoter name is italicized, which represents an inducible promoter.

Intercell signaling circuits and pulse-based processing for genetic oscillators

Robust genetic oscillators with tunable periods have been developed through a combination of experimental and computational efforts (Stricker *et al.*, 2008; Tiggas *et al.*, 2009; Tsai *et al.*, 2008). In addition to shedding light on the design principles guiding the evolution of naturally occurring biological clocks and circadian rhythms, these synthetic oscillators may also have significant utility in biotechnology applications, such as in the synthesis and delivery of biologic drugs. Glucocorticoid secretion, for instance, has a circadian and ultradian pattern of release, resulting in transcriptional pulsing in cells that contain glucocorticoid receptors (Stavreva *et al.*, 2009). Therefore, pulsatile administration of hormones may have therapeutic benefit compared with synthetic hormones applied in a non-ultradian schedule.

An alternative to device-based periodic drug delivery systems could be engineered bacteria that reside in the human gut and synthesize an active drug at fixed time intervals. To realize such an application, one would need to develop and implement intercell signaling circuits for synchronizing and entraining synthetic genetic oscillators (McMillen *et al.*, 2002; Garcia Ojalvo *et al.*, 2004). Such circuits could be based, for example, on modular components from bacterial quorum sensing systems. Along similar lines, one could engineer light-sensitive (Levskaya *et al.*, 2005, 2009) entrainment circuits for synchronizing mammalian synthetic genetic oscillators. This may help in the construction of oscillators that can faithfully follow circadian rhythms. Spike- or pulse-based processing is present in neurons and has been adapted for use in hybrid computation in electrical systems, where interspike times are viewed as analog parameters and spike counts are viewed as digital parameters (Sarpeshkar and O'Halloran, 2002). In synthetic gene circuits, pulse-based processing may open up exciting new methods for encoding information in engineered cells. For example, instead of transmitting information between cells by means of absolute levels of quorum-sensing molecules, the frequency of a robust genetic oscillator could be modulated. This might be useful in delivering information over longer distances, as frequency information may be less susceptible to decay over distance than absolute molecule levels. Representing signals in this fashion is analogous to frequency modulation encoding in electrical engineering.

Engineered circuits for biological containment

Biological containment, which refers to efforts for ensuring that genetically modified organisms do not spread throughout the natural environment, can be achieved by passive or active techniques. In passive containment, cells are engineered to be dependent on exogenous supplementation to compensate for gene defects, whereas in active containment, cells are engineered to directly express toxic compounds when located outside their target environments (Molin *et al.*, 1993). Synthetic genetic counters or timers for programmed cell death could be

used as an active containment tool. Counting circuits could, for example, be designed to trigger cell suicide after a defined number of cell cycles or a sequence of events. Recently, we have developed two designs for synthetic counters—a recombinase-based cascade of memory units and a riboregulated transcriptional cascade—that could be adapted for this purpose (Friedland *et al.*, 2009). In each case, one could incorporate into the counters promoters that are cell cycle-dependent and replace the output reporter proteins with toxic proteins (Fig. A12-5). Circuits of this sort would enable cells to be programmed to have limited, prescribed lifetimes.

Redundant circuits that implement digital logic allowing for the conditional survival of engineered cells only within their desired environments would also potentially reduce the failure rate of biological containment. If a broad set of interoperable parts were developed, multiple layers of control circuits could be built for increased reliability. As in electrical and mechanical engineering, quantitative analysis of failure rates in biological systems would enable improved systems-level design and robustness of synthetic gene networks. This could be accomplished, for example, by subjecting synthetic containment circuits to a variety of stressful conditions that would lead to increased mutation rates and thus improper functioning. Rational and directed evolutionary methods to engineer cells with decreased mutation rates or the application of redundant circuits could then be employed to minimize failure rates.

Whole-cell biosensors and response systems

Programmable cells that act as whole-cell biosensors have been created by interfacing engineered gene networks with the cell's natural regulatory circuitry (Kobayashi *et al.*, 2004) or with other biological components, such as light-responsive elements (Levskaya *et al.*, 2005, 2009). The development of novel or reengineered sensory modalities and components would expand the range of applications that programmable cells could address. This could involve engineering proteins or RNAs to detect a range of small molecules (Looger *et al.*, 2003; Win *et al.*, 2006), or designing protein-based synthetic signaling cascades by rationally rewiring the protein-protein interactions and output responses of prokaryotic two-component signal transduction systems (Skerker *et al.*, 2008).

The detection of electrical signals or production of biological energy (e.g., mimicking the operation of electrical electrocytes [Xu *et al.*, 2008]) could also be enabled by incorporating natural or synthetic ion channels into engineered cells. In addition, magnetoresponsive bacteria could play useful roles in environmental and medical applications (Jogler and Schuler, 2009). Synthetic bacteria, designed to form magnetosomes and seek out cancer cells, could be used to enhance imaging, and magnetic bacteria could be engineered to interact with nanoparticles to enhance the targeting of cancer cells. Moreover, the introduction of mechanosensitive ion channels (e.g., MscL from *Mycobacterium tuberculosis* and MscS from

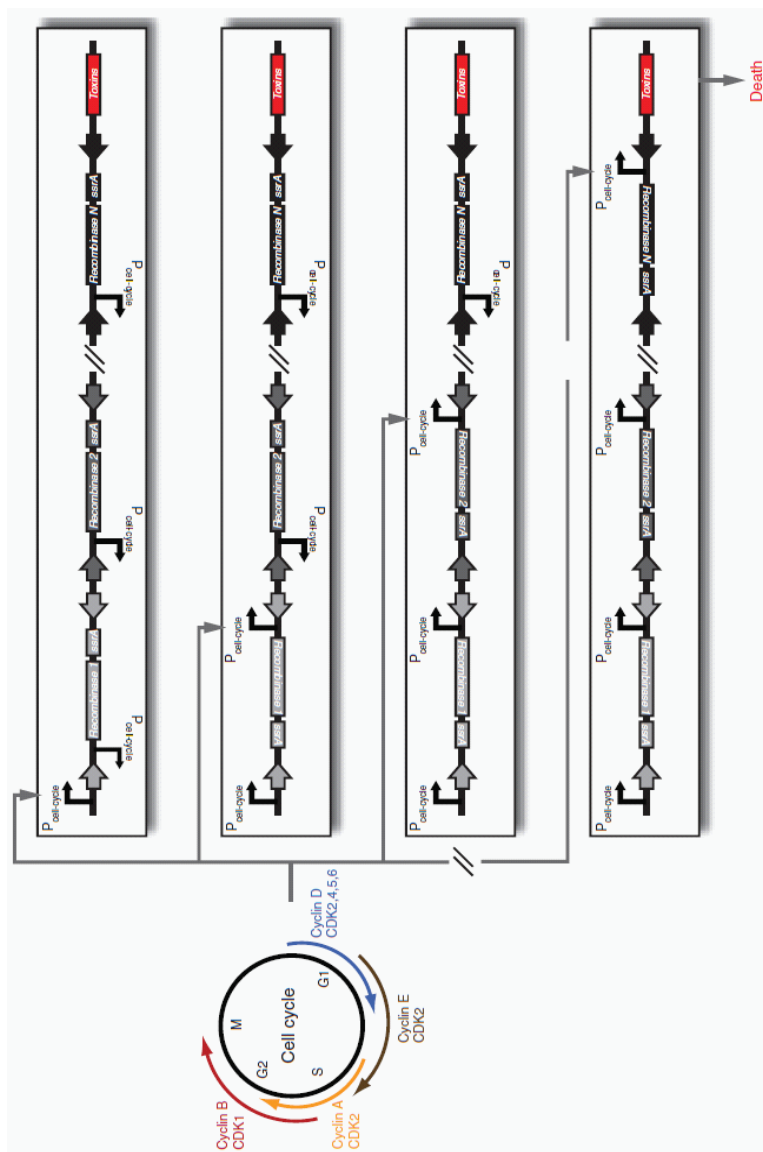


FIGURE A12-5 Cell-cycle counter for biological containment. Cell-cycle counting is accomplished with a cascade of single recombinease-based memory units (e.g., SIMMs [Friedland et al., 2009]), each of which is driven by a cell cycle-dependent promoter. After N cell-cycle events are counted, the gene circuit unlocks the expression of a toxic protein triggering cell death. Protein degradation tags (*ssrA*) are fused to the recombinease genes to ensure stability of the circuit.

E. coli) could endow designer cells with the ability to detect mechanical forces (Booth *et al.*, 2007). Such cells may be useful *in vivo* sensors for studying cellular differentiation signals or the effects of external stresses on the body.

Ultimately, programmable cells possessing novel sensory modules could be integrated with mechanical, electrical and chemical systems to detect, process and respond to external stimuli, and exploited for a variety of environmental and biomedical applications. For example, bacteria could be engineered to seek out hazardous chemicals or heavy metals in the environment, perform cleanup and return to their origin to report on the number of hazardous sites encountered via analysis by microfluidic devices. To eventually achieve such complex tasks, an intermediate goal might involve programming chemotactic bacteria to swim from waypoint to waypoint. A dish containing gradients of several chemoattractants would constitute the navigational course (Fig. A12-6a).

At the core of this design could be a synthetic gene network made up of a series of sequential toggle switches that control the expression of receptors needed for bacterial chemotaxis toward chemoattractants (Falke *et al.*, 1997) (Fig. A12-6b). The programmable cells would initially express only a single chemoattractant receptor, and therefore would migrate up only one of the chemoattractant gradients (Falke *et al.*, 1997). To determine that a waypoint has been achieved, a threshold-based toggle switch would be turned ON upon reaching a sufficiently high concentration of the chemoattractant. When the first toggle switch is ON, production of the first chemoattractant receptor would be suppressed and production of a second receptor allowed, resulting in cells swimming up the second chemical gradient. The ON switch would additionally prime the next toggle switch in the series to be switched ON when the second waypoint is reached. When that second toggle flips ON, the previous switch would be flipped OFF to ensure that only one chemoattractant is being followed at a time. The final chemoattractant would lead the bacteria back to its origin so that the engineered cells would complete a multi-stop round trip.

Designer circuits and systems for microbiome engineering

The human microbiome is fertile ground for the application of engineered organisms as scientific tools and therapeutic agents. There are unique bacterial populations residing in distinct locations in the human body that are perturbed in disease states (Gao *et al.*, 2008; Grice *et al.*, 2009). Each represents an exciting opportunity for reengineering the human microbiome and designing targeted therapeutics for a range of conditions, including dermatologic, genitourinary, gastrointestinal, metabolic and immunologic diseases (Ley *et al.*, 2008; Turnbaugh *et al.*, 2006, 2009).

Recently, bacteria have been engineered to infiltrate cellular communities for the purposes of delivering probes, gene circuits or chemicals (Steidler *et al.*, 2000; Braat *et al.*, 2006). In a similar fashion, bacteriophages carrying synthetic

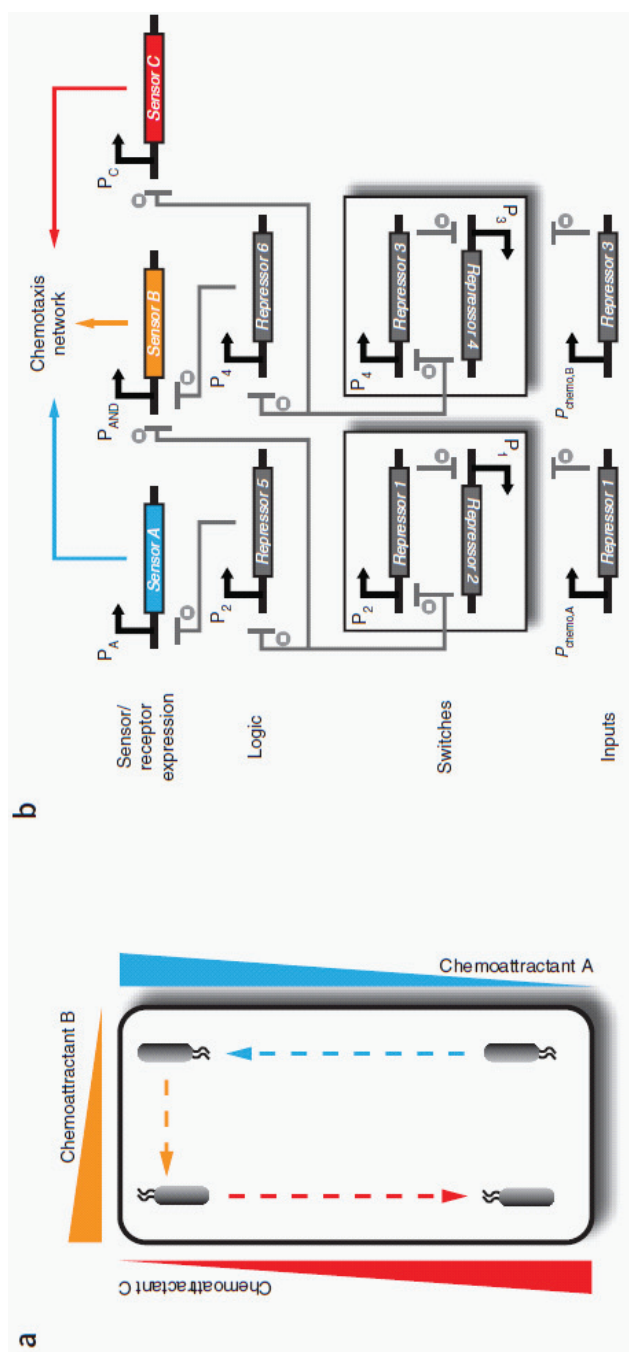
gene circuits could transform existing microbiome bacteria with new functionalities. For instance, given that anaerobic bacteria are known to migrate to hypoxic and necrotic regions of solid tumors (Wei *et al.*, 2008), bacteriophages could be designed to infect cancer-targeting bacteria. These bacteriophages could encode conditional expression of chemotherapeutic agents using synthetic logic gates or switches that are coupled to environmental sensors.

Bhatia and colleagues (von Maltzahn *et al.*, 2007) recently have developed nanoparticles that perform Boolean logic based on proteolytic activity. Viruses that infect tumor cells or bacteria could carry synthetic gene circuits that regulate in a programmable fashion the expression of enzymes that trigger nanoparticle activity. In these ways, one could develop targeted therapies against cancer or infectious diseases that exploit the human microbiome and synthetic gene networks.

Switchboard for dynamically controlling the expression of multiple genes

Engineered cells have long been used to produce recombinant proteins and chemicals for the biotechnology industry, and one of the major applications of synthetic biology to date has been in enhancing microbial production of biofuels (Stephanopoulos, 2007) and biomaterials (Teule *et al.*, 2009; Slotta *et al.*, 2007; Rammensee *et al.*, 2008; Widmaier *et al.*, 2009). Improving production from cells involves numerous engineering decisions related to the entire organism, including codon optimization, choosing whether or not to export recombinant proteins (Choi and Lee, 2004), rational or evolutionary methods for improving metabolic yields (Klein-Marcuschamer and Stephanopoulos, 2008; Dueber *et al.*, 2009), and optimization of growth conditions. Often some or all of the genes required for production are non-optimal for bacterial expression and contain repetitive sequences that are unstable in bacterial hosts. Whole-gene synthesis techniques are increasingly being used to optimize coding sequences for recombinant production (Widmaier *et al.*, 2009).

These innovative approaches, as well as more traditional knockout techniques, introduce hard-wired changes into the genomes of interest. However, for many industrial and bioprocess applications, there is a need to dynamically modulate and control the expression of multiple genes, depending upon the state of the bioreactor. These situations would benefit from the development of a synthetic switchboard, one that could tune the expression of many different genes simultaneously and independently. Such a switchboard could be made up of a series of adjustable threshold genetic switches, riboregulators or riboswitches, and designed to respond to different environmental and intracellular variables, such as pH, light intensity and the metabolic state of the cell. The switchboard design, which would integrate novel sensory modalities with tunable, interoperable genetic circuits, would have broad functionality. It could be programmed, for example, to shift carbon flux between different pathways depending upon cellular



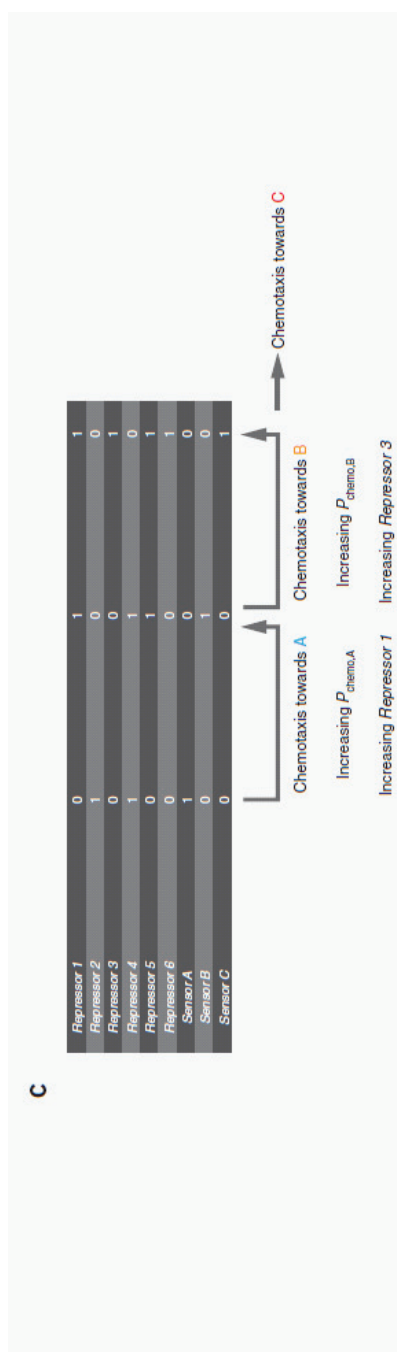


FIGURE A12-6 Autonomous chemotaxis. **(a)** Chemotactic environment made up of three chemoattractant gradients (A, B, C). **(b)** The synthetic gene network, whereby toggle switches control the sequential expression of three chemotaxis sensor receptors, for autonomously navigating bacteria down three chemoattractant gradients. Inputs into promoters and logic operations are shown explicitly, except when the promoter name is italicized, which represents an inducible promoter. **(c)** Boolean ON/OFF values for the network genes illustrate the sequential order of operations.

conditions, thereby optimizing the production of biofuels, specialty chemicals and other materials.

Conclusions

The past decade has witnessed the power of intelligently applying engineering principles to biology in the development of many exciting, artificial gene circuits and biomolecular systems. We are convinced that next-generation synthetic gene networks will advance understanding of natural systems, provide new biological modules and create new tools that will enable the construction of even more complex systems. Most importantly, if the current pace of progress in synthetic biology continues, real-world applications in fields such as medicine, biotechnology, bioremediation and bioenergy will be realized.

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Competing Interests Statement

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturebiotechnology/>.

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A13

ENGINEERING SCALABLE BIOLOGICAL SYSTEMS⁶⁶*Timothy K. Lu*⁶⁷

Synthetic biology is focused on engineering biological organisms to study natural systems and to provide new solutions for pressing medical, industrial and environmental problems. At the core of engineered organisms are synthetic biological circuits that execute the tasks of sensing inputs, processing logic and performing output functions. In the last decade, significant progress has been made in developing basic designs for a wide range of biological circuits in bacteria, yeast and mammalian systems. However, significant challenges in the construction, probing, modulation and debugging of synthetic biological systems must be addressed in order to achieve scalable higher-complexity biological circuits. Furthermore, concomitant efforts to evaluate the safety and biocontainment of engineered organisms and address public and regulatory concerns will be necessary to ensure that technological advances are translated into real-world solutions.

In the last century, scientists have made giant strides in identifying and studying biological parts such as proteins and nucleic acids (Watson and Crick, 1953; Lander et al., 2001; Venter et al., 2001; Fiers et al., 1976; Fleischmann et al., 1995), understanding regulatory networks (Johnson et al., 1981), and constructing engineered organisms using the ever-advancing tools of genetic engineering (Itakura et al., 1977). In the last decade, synthetic biologists have

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Key words: synthetic biology, biological circuits, engineered organisms, modelling, high-throughput design, regulatory issues, biological probes, biological modulators

Abbreviations: PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Mbp, mega-basepairs; Kbp, kilo-basepairs; qRT-PCR, quantitative reverse-transcriptase PCR; FRET, fluorescence resonance energy transfer; IPTG, isopropyl β -D-1-thiogalactopyranoside; SELEX, systematic evolution of ligands by exponential enrichment; RNAi, RNA interference; GMOs, genetically modified organisms

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leveraged the power of modern molecular biology using frameworks translated from traditional disciplines such as electrical engineering, computer science, mechanical engineering and chemical engineering to create a wide range of synthetic biological circuits, including switches (Gardner et al., 2000; Kramer et al., 2004; Isaacs et al., 2003; Ham et al., 2006, 2008; Deans et al., 2007; Ajo-Franklin et al., 2007; Dueber et al., 2007), oscillators (Elowitz and Leibler, 2000; Stricker et al., 2008; McMillen et al., 2002), digital logic gates (Rinaudo et al., 2007; Win and Smolke, 2008; Anderson et al., 2007; Dueber et al., 2003; Yeh et al., 2007), filters (Hooshangi et al., 2005; Basu et al., 2005; Sohka et al., 2009), modular and interoperable memory devices (Friedland et al., 2009), counters (Friedland et al., 2009), sensors (Win and Smolke, 2007; Bayer and Smolke, 2005), and protein scaffolds (Bashor et al., 2008). Using these circuits, biological engineers have created synthetic organisms that can be used for bioremediation, biosensing, computation, bioenergy and medical therapeutics (reviewed in Lu et al., 2009; Lim, 2010; Khalil and Collins, 2010). Despite these advances, the realization of synthetic-biology-based applications will require future breakthroughs in our ability to create sufficiently complex and reliable biological systems. Here, I will discuss current limitations and potential solutions for the construction, probing, modulation and debugging of scalable biological systems as well as hurdles for the deployment of engineered organisms from bacteria to mammalian cells which adds to the discussion of next-generation synthetic gene networks in Lu et al. (2009) (**Fig. A13-1**).

Physical Construction of Scalable Biological Systems

Construction of early synthetic circuits largely relied on restriction enzymes and polymerase chain reaction (PCR)-based techniques to assemble existing genetic components. These methods do not scale well with increasing complexity due to a lack of sufficient unique restriction sites and the need to have physical DNA templates from which to amplify genetic parts. Standards for library construction and the assembly of parts libraries (Smolke, 2009) have been integral in circumventing this dependency on templates and restriction sites. However, since these parts must be devoid of restriction sites used in the defined standards and should ideally be optimized for use in one's organism of choice (Okano et al., 2008), the use of whole-gene DNA synthesis is on the rise (Carlson, 2009). Using direct chemical synthesis, circuits can be designed *in silico* and implemented in DNA with significantly less effort from researchers. As DNA synthesis becomes increasingly economical and efficient, it will become possible to construct complex systems with less reliance on restriction enzymes. For example, DNA synthesis productivity has exceeded 1 Mbp per person per day while Venter and colleagues recently succeeded in synthesizing a 1.08 Mbp genome (Gibson et al., 2010). However, most synthetic gene circuits to date have not exceeded the 50 Kbp level, indicating that there is a large gap between our ability to read and

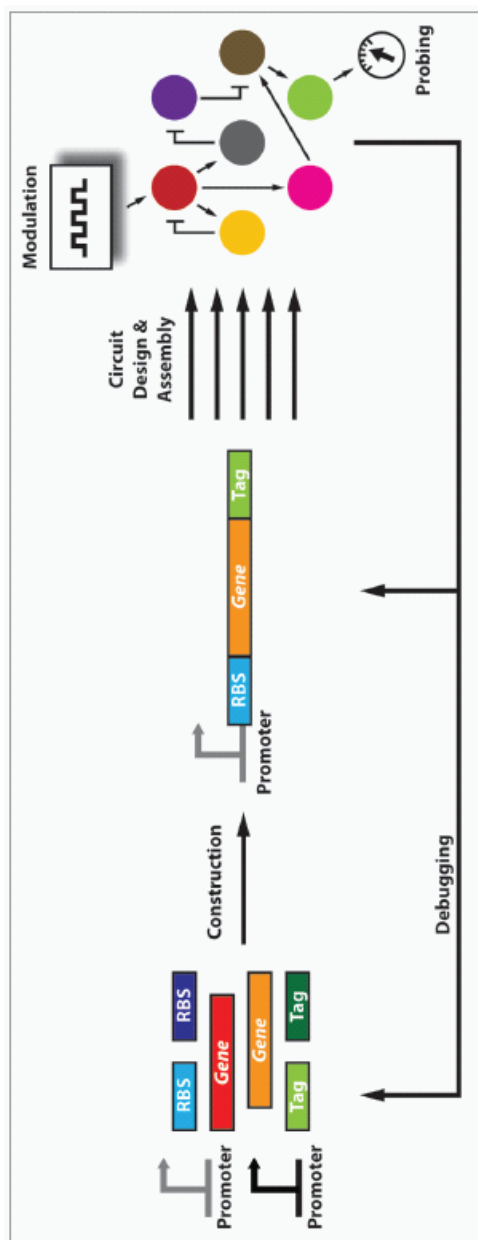


FIGURE A13-1 A basic design cycle for synthetic biology includes creating well-characterized parts (e.g., regulatory elements, genes, proteins, RNAs), constructing synthetic devices and modules and designing and assembling higher-order networks. All steps of this cycle are aided by modelling, probes and modulators to analyze circuit performance. Debugging is an iterative process based on parts optimization, fine-tuning regulatory components, modelling and changing circuit architecture.

write DNA and knowing what DNA to write (**Fig. A13-2**). Just as the decoding of the human genome sequence did not immediately reveal the functions of all human genes, the utility of high-throughput DNA synthesis technology will only gradually become evident as synthetic biologists learn how to create complex systems. For example, future synthesized circuits should be designed with ease of probing, modulating and debugging in mind. These features could be implemented by including validated RNA “handles” that can be easily measured with standard probe sets to determine internal RNA concentrations, gene circuits that allow inducers to modulate synthetic circuit protein levels, and properly situated restriction sites for the rapid cloning of components that need systematic optimization, such as ribosome binding sequences.

Significant advances in well-characterized, interoperable devices are necessary for the construction of higher-order modules that will enable scalable biological systems (Andrianantoandro et al., 2006). The majority of biological

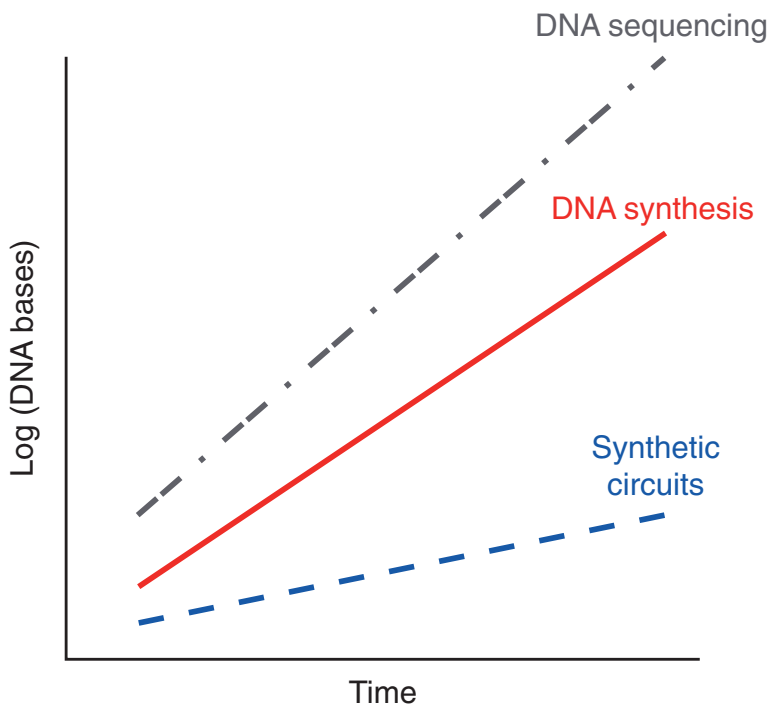


FIGURE A13-2 DNA sequencing and synthesis technologies are advancing at exponential rates, outpacing the ability of synthetic biologists to construct useful and scalable biological circuits (Carlson, 2009). These trends are similar to Moore’s law for integrated circuits (Moore, 1965) and suggest that there is substantial room for growth in the field of synthetic circuits.

circuits have been constructed using a handful of synthetic parts (Lu et al., 2009). Furthermore, it is often the case that when new designs for biological parts are developed, only a few instantiations are created and tested, usually in single cellular backgrounds. As a result, there is a need for the systematic development and characterization of compatible biological parts. Specificity in biological systems largely relies on spatial distribution and chemical interactions. This is in stark contrast to electrical engineering, where specificity is achieved through direct electrical wiring. Thus, strategies for achieving inter-part compatibility include targeting circuits to isolated compartments (Parsons et al., 2010; Tanaka et al., 2010), mutagenesis and directed evolution of existing parts, and using comparative genomics to identify, synthesize, and test homologous proteins or nucleic acids. These efforts may be complicated by unknown global factors (e.g., growth rates, endogenous transcription factors with off-target effects on synthetic circuits, protein-protein interactions, small RNAs) that can confound device testing and render it difficult to use pre-defined parts in a wide range of organisms and environmental conditions without additional alterations and characterization (Klumpp et al., 2009). Therefore, combinatorial methods to test single-component performance, multi-component interactions and biological crosstalk (e.g., cross-activation or cross-repression of transcription, non-specific enzymatic activity, inappropriate triggering of signalling pathways) will be important for parts libraries (**Fig. A13-3**). These results should be incorporated into mathematical models to aid future model-based design. Indeed, institutions such as BIOFAB are attempting to systematically assemble and characterize libraries of synthetic devices. However, development efforts for certain platforms that are promising for library construction, such as zinc finger proteins and RNA interference, may be slowed by the presence of existing intellectual property (Scott, 2005).

As an example of combinatorial characterization (**Fig. A13-3**), suppose one would like to construct multiple interoperable NOR (NOT-OR) gates to constitute a universal logic system. NOR functionality can be built by placing pairwise combinations of unique operator sites for transcriptional repressors within synthetic promoters. To identify orthogonal repressors, one can encode individual transcription factors under inducible control on one set of plasmids and individual cognate operator sites driving expression of a reporter gene on another set of plasmids. Then, all possible combinations of transcription factor plasmids and reporter plasmids can be co-transformed into cells and tested for single-component performance (e.g., when a transcription factor is co-transformed with its cognate operator site) and potential crosstalk interactions (e.g., when a transcription factor is co-transformed with non-cognate operator sites). Standard induction curves can be derived by varying the concentration of transcription factors using the inducible promoters and measuring the resulting output (Kelly et al., 2009). Based on these results, an optimal set of non-interacting transcription factors and cognate operators can be selected. To create the NOR gates, all possible pairwise combinations of operators can be constructed in synthetic promoters

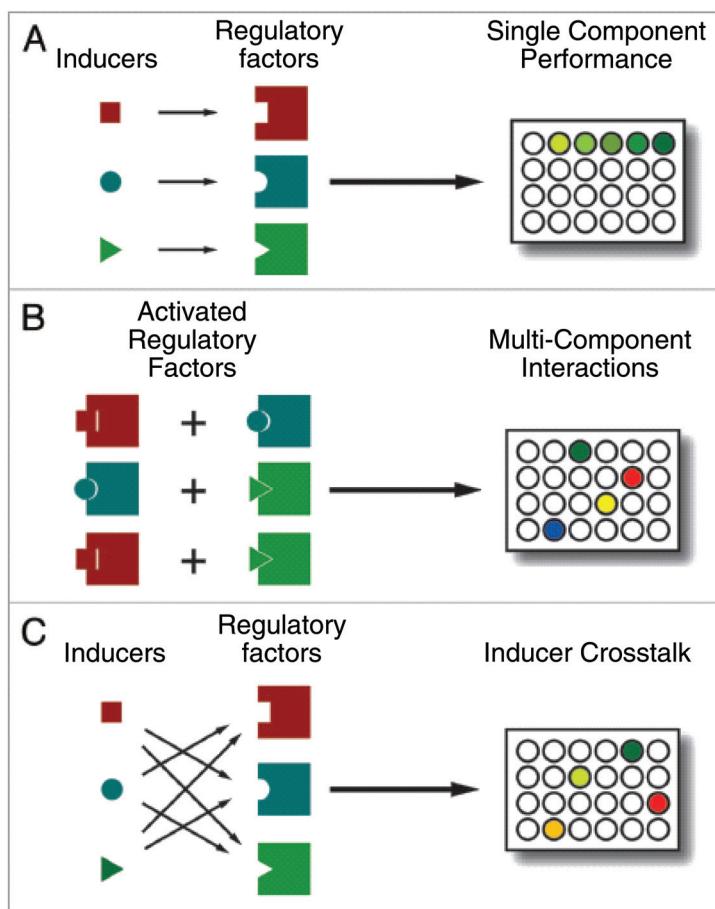


FIGURE A13-3 Combinatorial high-throughput methods will be useful in the assembly of well-characterized libraries of synthetic parts and devices. For example, transcriptional regulators and their cognate inducers can be analyzed for (A) single-component performance, (B) interactions between multiple components and (C) inducer crosstalk (e.g., cross-activation and/or cross-inhibition).

and co-transformed into cells with all pairwise combinations of transcription factors under independent inducible control. Proper NOR gate functionality and crosstalk can then be determined in a high-throughput fashion for all potential gates by varying inducer levels and measuring reporter gene output. In addition to enabling interoperable gate selection, large-scale experiments such as these should yield substantial data for models that can predict the orthogonality of transcription factors and operators for future circuits (e.g., using heuristic or

thermodynamically guided algorithms). Moreover, matrices of cross-repression interactions can be constructed and incorporated into transcriptional models when cross-interacting transcription factors must be used in other systems.

Model-guided design is crucial for the construction of complicated electrical and mechanical systems. Time-based simulations for electrical and mechanical systems are possible since mathematical models are established and parameters are well known. In contrast, most parameters in biological circuits are unknown and the computational resources required to accurately simulate noise and multiple component interactions are significant. Recent advances in modeling chemical networks, transcription, translation and biological noise using the inherent physics of solid-state electronic devices should enable the construction of large-scale real-time electronic models of synthetic biological systems (Mandal and Sarpeshkar, 2009). Other techniques from control theory such as small-signal linearization and modularization enable tractable modelling and simulations prior to implementation. Biological systems exhibit nonlinearity (e.g., cooperativity) which can be linearized in different regions of operation (**Fig. A13-4A**). Frequency-domain analysis in linearized systems allows for block modelling and deeper understanding of system dynamics, such as noise, stability, time constants and performance (**Fig. A13-4B**) (Cox et al., 2006; Simpson et al., 2003). Small-signal linearization and frequency-domain modelling have not been extensively used for studying and designing synthetic biological circuits even though advances in microfluidics and time-lapse microscopy can now achieve frequency modulation of inputs and long time-scale data collection necessary for frequency-domain analysis (Cox et al., 2006; Mettetal et al., 2008). Furthermore, microfluidics devices can be coupled with electronic controllers to stabilize and alter the dynamics of synthetic biological circuits similar to electronic controllers that are used to control mechanical systems.

The insights that can be gained from linearized block models of complex systems can complement the accuracy of time-domain state-space representation, time-based mathematical simulations and non-linear control theory. To enable successful time-based and frequency-based modelling of biological systems, accurate parameters will need to be derived by high-throughput *in vitro* and *in vivo* probes and microarrays, as described below.

Molecular Probes and Modulators for Scalable Biological Systems

High-throughput methods for probing multiple nodes, such as protein and RNA levels, in complex biological circuits are necessary to achieve reliable and scalable performance (Tyagi, 2009). Ideal probe features include high signal-to-noise ratios, specificity, low cost, multiplexability, non-invasiveness and the ability to reveal real-time dynamics. Complementary techniques for the reliable and multiplexed modulation of synthetic circuits are also needed to generate

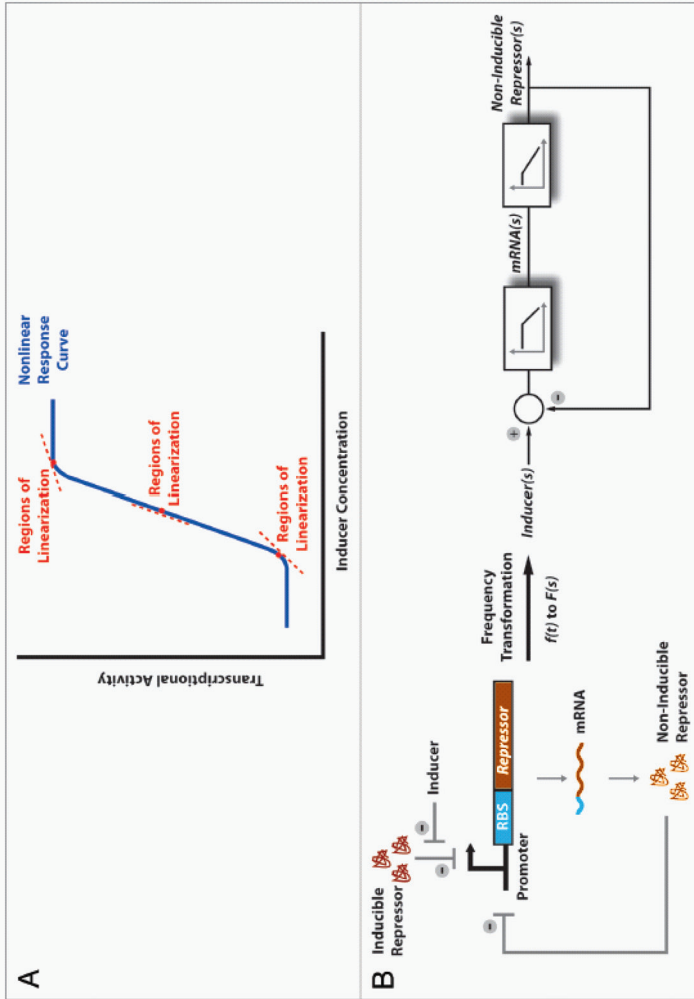


FIGURE A13-4 Control theory techniques for modelling synthetic biological circuits. (A) Small-signal linearization of biological components in different regions of operation enables the development of linear models. (B) Linearization can enable frequency-domain analysis, systems modelling using block diagrams and deeper insights into system dynamics. For example, transcription and translation can be understood as low-pass filters and block diagrams can be drawn for simple negative-feedback loops to yield understanding into system interconnections and responses to different input types (Cox et al., 2006; Simpson et al., 2003). In the block diagram shown, s refers to $j\omega$ where j is $\sqrt{-1}$ and ω is angular frequency.

perturbations to obtain system parameters, drive accurate models and monitor system performance.

Analyses of most synthetic circuits rely on fluorescent proteins, which are well-characterized, easy to detect and can be multiplexed, as reporters of combined transcriptional and translational activity. However, fluorescent proteins are large, require folding and maturation and can be too stable to track rapid dynamics without additional modifications (Andersen et al., 1998). To address these issues, methods have recently been developed to create fast-folding fluorescent proteins, degradation tags and *in vivo* ligation of fluorophores to proteins (Uttamapinant et al., 2010). Alternative methods for monitoring protein levels in synthetic circuits include luminescence or colorimetric assays although these methods can be more difficult to multiplex. Advances in high-throughput proteomic characterization may eventually enable global monitoring of protein levels across time without the need for multiplexed reporter proteins (Malmstrom et al., 2009). Nucleic-acid aptamer beacons may also play a role in protein detection but have not been extensively applied to *in vivo* settings and require selection protocols since there are no algorithms that can reliably predict the affinity of binding between aptamers and proteins of interest (Hamaguchi et al., 2001). Thus, new techniques for multiplexed and global protein detection are needed for scalable biological system design.

Transcriptional activity can be monitored by quantifying RNA in engineered cells, which is easier to do in a multiplexed and global fashion than quantifying protein levels. Multiplexed RNA levels can be assayed with qRT-PCR or microarrays but these techniques require nucleic acid extraction and cellular destruction. Fusing RNAs of interest to aptamers that bind fluorescent proteins or fluorescent small molecules can be used to monitor *in vivo* RNA levels but requires modifying RNAs of interest (Tyagi, 2009). Side-by-side fluorescence resonance energy transfer (FRET) probes, quenched autoligation probes and molecular beacons are sequence-specific tools for detecting RNA levels *in vivo* with multiplexing capabilities but face challenges such as intracellular delivery, compartmentalization and degradation (Tyagi, 2009). Nonetheless, nucleic-acid-based probes are likely to be early enablers for high-throughput *in vivo* monitoring of transcription in synthetic biological circuits given that they are relatively easy to design for different targets. Thus, methods for achieving efficient delivery of nucleic-acid-based probes are crucially needed to make them widely useful to synthetic biologists for monitoring *in vivo* RNA levels in real time.

The majority of modulators used in synthetic circuits today are small molecules that induce or repress the activity of existing transcriptional regulators. As increasing number of transcriptional regulators are identified or created, it will be important to identify corresponding inducers that can control *in vivo* function, characterize crosstalk between different inducers and design interoperable modulators. For example, directed evolution of the AraC transcriptional regulator was necessary to increase compatibility between arabinose-controlled

and IPTG-controlled systems (Lee et al., 2007). Other modulators for synthetic circuits include aptamers that respond to small molecules and trigger translational activities (Bayer and Smolke, 2005). These aptamers can be discovered using techniques such as Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Light-inducible proteins provide rapid and well-controlled signal transduction that could potentially be enhanced by developing variants which preferentially respond to different wavelengths of light (Levskaia et al., 2009; Boyden et al., 2005). An underutilized technology in synthetic biology is RNA interference (RNAi), which allows straightforward design of sequence-specific modulators. However, RNAi is not available in many organisms of interest and must be coupled with efficient delivery mechanisms for nucleic acids (Rinaudo et al., 2007). Fortunately, RNAi has been reconstituted in well-studied organisms such as *Saccharomyces cerevisiae* and significant advances are being made in RNAi-delivery technology (Whitehead et al., 2009).

Debugging Malfunctioning Biological Systems

The harsh reality of engineering synthetic biological circuits is that most designs fail to perform as expected. Debugging of synthetic circuits is a painstakingly iterative process involving the detailed characterization of all available internal nodes, trial-and-error modification of constituent components and modelling. However, debugging will be greatly facilitated by improved techniques for sequencing, probing and modulating synthetic circuits, as described above. For example, an initial debugging step is usually to fully sequence all circuit components to ensure that the intended genetic program has been constructed. Subsequent debugging can include dynamically probing all possible RNA and protein nodes and interactions using techniques such as qRT-PCR, fluorescent protein fusions and circuit-specific methods (e.g., in vitro gel shift assays, protein phosphorylation assays, single-cell microscopy, and so forth). Based on these results, it can often be determined whether system failure is due to inherent circuit topology or due to poor component performance leading to circuit operation in non-functional parameter regions. In the former case, redesigns are needed and can benefit from detailed modelling using insights and measurements from the failed attempts. In the latter case, systematic and randomized mutagenesis and screening techniques are helpful to optimize system performance. For example, synthetic circuits often do not work properly because of mismatched expression levels resulting in outputs that do not fit into the dynamic range of other inputs. Mismatches in dynamic range can usually be corrected by systematically altering the strengths of promoters and ribosome binding sequences using mutagenesis (Ellis et al., 2009; Salis et al., 2009).

In addition, optimization of synthetic genes can help resolve problems associated with the expression of heterologous genes in foreign hosts including DNA instability and poor translational efficiency (Widmaier et al., 2009). Techniques

for the high-throughput alteration of genetic circuits should also help speed up debugging cycles by enabling testing across larger parameter spaces (Wang et al., 2009). Modelling can generate insights into potential failure points in synthetic circuits but is inadequate at identifying problems with components or parameters that are not included in the models themselves. For example, global effects of host factors can change the performance of synthetic circuits (Klumpp et al., 2009). To model these effects, high-throughput data for global transcriptional responses to defined perturbations should be obtained from large promoter libraries and incorporated into whole-cell simulations (Zaslaver et al., 2006). Moreover, test platforms based on well-characterized host organisms or minimal organisms may be ideal backgrounds for validating system designs prior to deployment in final vehicles (Lu et al., 2009; Forster and Church, 2006). Finally, given the significant time and effort that is spent on fixing biological systems and the wealth of information that can be gleaned from malfunctioning designs, it would be beneficial to the general synthetic biology community to establish repositories where results from debugging cycles and system failures can be disseminated.

Public and Regulatory Considerations for Engineered Biological Systems

In addition to technological advances, efforts to address public and regulatory concerns over synthetic biology are necessary to ensure the successful translation of scalable biological systems to real-world applications. The synthetic biology community has recognized that safety, security and ethical issues must be addressed in an open and earnest fashion (Parens et al., 2008; Bugl et al., 2007). Although there is little data to suggest that genetic engineering or synthetic biology have produced harmful constructs over the last few decades, concerns from the public and the media are often voiced when significant technological milestones in synthetic biology are achieved. Some of these concerns may be rooted in inadequate scientific understanding or communication of the current state of synthetic biology. Thus, enhanced outreach and education efforts between researchers and the public are necessary to ensure the field's continued progress. Furthermore, global variations in cultures and attitudes towards engineered life should be recognized and addressed by researchers and advocates. For example, differences in opinion between the United States and the European Union regarding the use of Genetically Modified Organisms (GMOs) as food products may translate to synthetic organisms. Thus, it is imperative that synthetic biologists act and communicate their roles as critics of the field to ensure that safety, reliability and the realization of broad social benefits are maintained as the utmost research priorities.

The regulatory issues associated with deploying synthetic biological organisms vary greatly depending on application. The areas associated with the lowest regulatory hurdles include metabolic engineering and bioenergy where biocontainment is straightforward to achieve, direct contact with humans is limited and

ultimate products are chemical compounds produced by engineered cells. For environmental deployment of synthetic organisms in the United States, the Environmental Protection Agency and the Food and Drug Administration can become involved. Regulatory issues for environmental applications are similar to those involved with GMOs, such as the use of recombinant DNA, biocontainment and impact on natural ecosystems. Standardized methods and technologies must be developed with input from regulatory agencies to evaluate these concerns. The most difficult regulatory hurdles pertain to the use of synthetic biological systems for human therapeutics such as adoptive immunotherapy (Varela-Rohena et al., 2008), cancer-seeking bacteria (Anderson et al., 2006), engineered phage targeting bacterial biofilms (Lu and Collins, 2007) and antibiotic-resistant organisms (Lu and Collins, 2009). These hurdles include immunogenicity, biocontainment, recombinant DNA and manufacturing purity. Stem-cell-based therapies face similar hurdles and therefore it will be informative to follow the path of stem cells into the clinic (Kiskinis and Eggan, 2010). Reliable methods to eliminate engineered organisms as needed (Molin et al., 1993), stringent techniques to evaluate safety in animals and humans, techniques for encapsulating and isolating engineered cells (Lim et al., 2010), assays for mutation rates in deployed organisms, utilization of probiotic strains as substrates for engineering microbe-based therapeutics and close communication with regulatory agencies will be necessary to translate synthetic biological organisms into the important frontier of human treatments. Furthermore, synthetic biologists interested in human therapeutics should focus their efforts on areas of significant unmet need to optimize risk-benefit tradeoffs and should be open to proof-of-concept applications in areas with lower regulatory hurdles such as environmental or veterinary use.

Conclusions

The past decade has delivered significant advances in the design and construction of basic synthetic circuits. In the upcoming decade, novel technologies for composing, probing, modulating and debugging scalable biological circuits will enable the robust performance of useful tasks by engineered organisms. Scientific advancements must be accompanied by concomitant efforts to address societal and regulatory concerns over synthetic biology. These endeavors should yield exciting new solutions for real-world problems in critical areas of medical, industrial, environmental and energy applications.

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A14

METABOLIC SYSTEMS BIOLOGY⁶⁸*Bernhard Palsson⁶⁹***Abstract**

The first full genome sequences were established in the mid-1990s. Shortly thereafter, genome-scale metabolic network reconstructions appeared. Since that time, we have witnessed an exponential growth in their number and uses. Here I discuss, from a personal point of view, four topics: (1) the placement of metabolic systems biology in the context of broader scientific developments, (2) its foundational concepts, (3) some of its current uses, and (4) some of the expected future developments in the field.

Metabolic Systems Biology in the Grand Scheme of Things

Ever since Gregor Mendel discovered discrete quanta of information passed from one generation to the next that determined form and function of an organism, the genotype–phenotype relationship has been of fundamental importance in the life sciences. For monogenic traits, the genotype–phenotype relationship can be readily understood. However, most phenotypic traits involve multiple gene products. This makes the genotype–phenotype relationship a challenge to reconstruct and understand, given the complex interactions that can form among the gene products.

With the publication of the first full genome sequences in the mid-1990s (Fleischmann et al., 1995) it became possible, in principle, to identify all the gene products involved in complex biological processes in a single organism. The well-studied biochemistry of metabolic transformations made it possible to reconstruct, on a genome-scale, metabolic networks for a target organism in a biochemically detailed fashion (Edwards and Palsson, 1999, 2000). Such

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metabolic network reconstructions can be converted into a mathematical format yielding mechanistic genotype–phenotype relationships for microbial metabolism (Palsson, 2006). The mathematical format of the underlying biochemical, genetic, and genomic (BiGG) knowledge allows the formulation of genome-scale models (GEMs). GEMs enable the computation of phenotypic traits based on the genetic composition of the target organism (Palsson, 2006; Price et al., 2004).

Since the establishment of the first metabolic genome-scale reconstruction in 1999 and *in silico* models thereof, many more have followed (Fig. A14-1); perhaps most notably for human metabolism in 2007. The scope and content of network reconstructions continues to grow, for instance to include the entire transcription/translation apparatus of a cell (Thiele et al., 2009) and the structural information about the metabolic enzymes (Zhang et al., *in press*).

Foundations of Metabolic Systems Biology

The Basic Paradigm

We can now enumerate various cellular components, describe their interactions chemically, formulate a mathematical description of the totality of such interactions, identify the constraints that the resulting network operates under, and apply optimality principles to evaluate likely physiological functions in a

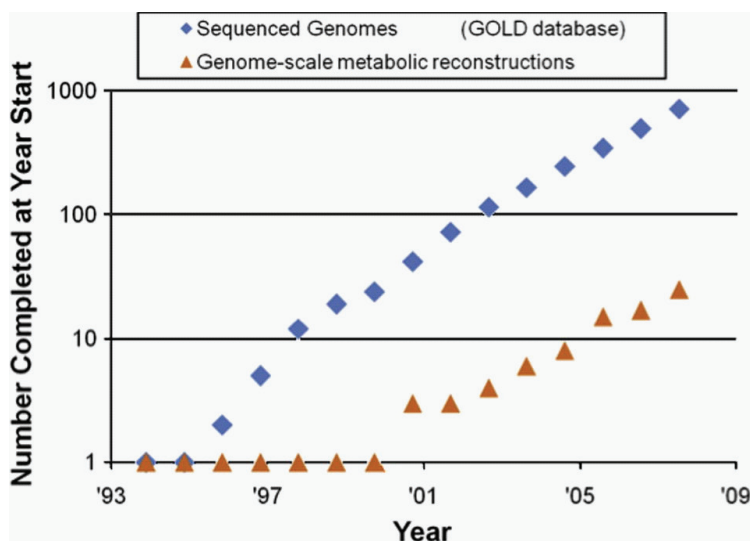


FIGURE A14-1 Growth of genome sequences and genome-scale metabolic reconstructions. The number of network reconstruction has grown exponentially, at a similar pace as genome sequences have appeared (prepared by Adam Feist and Ines Thiele).

given environment. These capabilities provide a consistent framework on which a mechanistic basis for the microbial metabolic genotype–phenotype relationship can be formulated. The underlying process is based on an emerging paradigm to relate the genotype to the phenotype through reconstruction and in silico model building (Fig. A14-2) is comprised of four steps:

1. generation of ‘omics’ and collection of literature data on the target organism;
2. network reconstruction and the formulation of a BiGG knowledge-base;
3. conversion of the reconstruction into a mathematical format and the implementation of in silico query tools; and
4. enablement of a variety of basic and applied uses.

This fundamental paradigm allows for the first time the genome-scale computation of phenotypic functions of an organism. The establishment of a mechanistic formulation of the most fundamental relationship in biology – the genotype–phenotype relationship – for a limited number of phenotypic functions, differs from that developed for the basic physical laws about a century ago. This mechanistic description must account for both proximal and distal causation. Proximal (or proximate) causation occurs against a fixed genetic background (i.e., an individual organism), while distal (or ultimate) causation results from (genomic) changes that occur from generation to generation, i.e., evolution.

Some Basic Principles

This basic paradigm has been implemented for a number of organisms and a variety of biological results have been obtained (Feist and Palsson, 2008; Papin et al., accepted for publication). As a result of this successful reduction to practice, one is tempted to try to determine and state the underlying reasons for this success. Below, I attempt to start this process.

Axiom #1: All cellular functions are based on chemistry. A simple but consequential statement, as it implies the fundamental events in a cell can be described by chemical equations. These equations, in turn, come with chemical information and physico-chemical principles.

Axiom #2: Annotated genome sequences along with experimental data enable the reconstruction of genome-scale metabolic networks. The reconstruction process is a grand-scale systematic assembly of information in a quality-controlled/quality assured (QC/QA) setting (Thiele and Palsson, in press) that leads to a BiGG knowledge-base, which is a collection of established biochemical, genetic, and genomic data represented by a network reconstruction. The reconstruction process has been reviewed elsewhere (Feist et al., 2009; Reed et al., 2006b), and a growing number of reconstructions are available (Fig. A14-1).

Axiom #3: Cells function in a context-specific manner. When a cell is placed

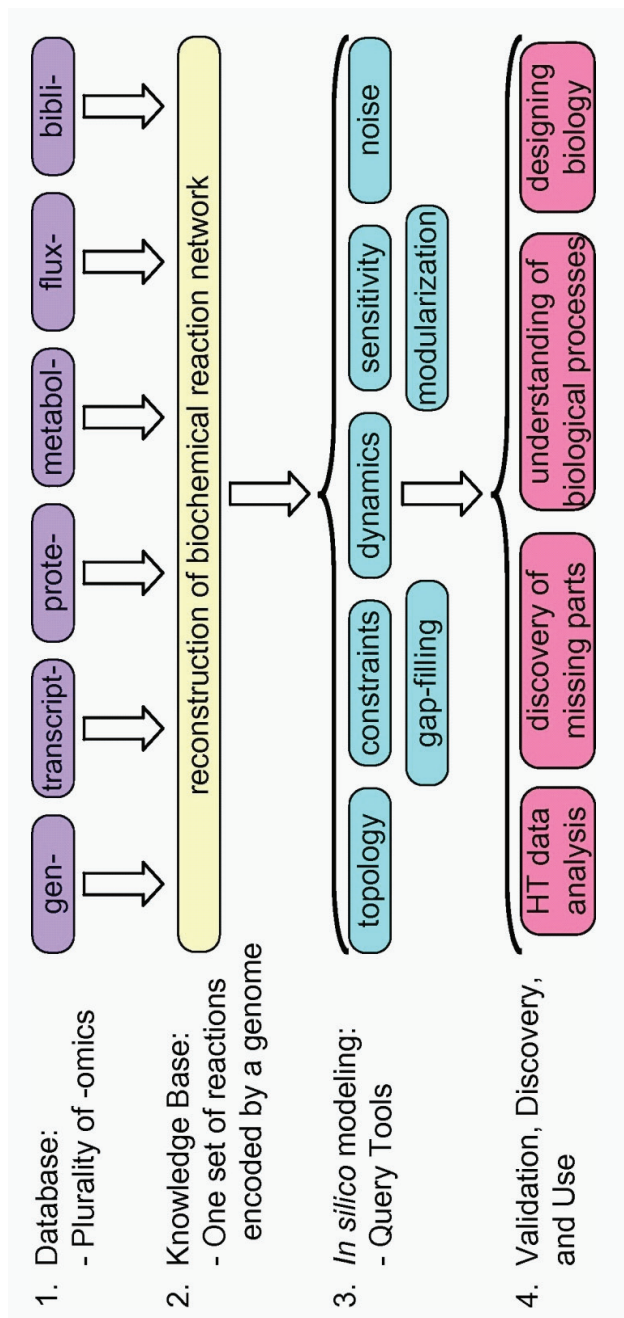


FIGURE A14-2 The four-step paradigm for metabolic systems biology. Adapted from Palsson (2006). Prepared by Adam Feist.

in a particular environment, it expresses a subset of its genes in response to environmental cues. The abundance of cellular components can be profiled using ‘omic’ methods (i.e., transcriptomics, proteomics, metabolomics). Such omic data can be mapped onto a network reconstruction to tailor it to the particular condition being considered.

Axiom #4: Cells operate under a series of constraints. Factors constraining cellular functions fall into four principal categories (Palsson, 2006): physico-chemical (see Axiom #5), topological (molecular crowding effects and steric hindrance), environmental (Axiom #3), and regulatory (basically self-imposed constraints, or restraints). These constraints cannot be violated allowing the estimation of all functional (i.e., physiological) states that a genome-scale reconstruction can achieve. Mathematically, such statements are translated into fundamental subspaces associated with the stoichiometric matrix (S), whose properties can be characterized (Palsson, 2006). In this so-called S matrix, where S stands for stoichiometric, the rows correspond to the network metabolites and the columns to the network reactions. The coefficients of the substrates and products of each reaction are entered in the corresponding cell of the matrix.

Axiom #5: Mass (and energy) is conserved. This statement is one of the basic physical laws. Since all proper chemical equations can be described by stoichiometric coefficients, and since a set of chemical equations can be described by the stoichiometric matrix, S , this means that all steady states (normally close to the homeostatic states of interest) of a network can be described by a simple linear equation, $S \cdot \mathbf{v} = 0$, where \mathbf{v} is a vector of fluxes through chemical reactions (Palsson, 2006). Thus, the computation of functional states of a network is enabled based on the known underlying chemistry.

Axiom #6: Cells evolve under a selection pressure in a given environment. This Darwinian statement has implicit optimality principles built into it. Consequently, if we know the selection pressure, we can state a so-called objective function and determine optimal states given a network reconstruction and governing constraints.

Each one of these statements by themselves is almost trivial and accepted in various scientific disciplines as being fundamental. Taken together, though, they combine to form the conceptual basis for constraint-based reconstruction and analysis (COBRA), and enable the development of the mechanistic genotype–phenotype relationship for metabolism. The recent emergence of genome-scale reconstructions (Axiom #2) has proven key to this formulation.

Practicing COBRA

The COBRA approach (Palsson, 2006; Price et al., 2004) has been widely used to analyze network reconstructions. It uses stoichiometric information about biochemical transformations taking place in a target organism to construct the model. While a metabolic reconstruction is unique to the target organism, one can derive many different condition-specific models from a single reconstruc-

tion. This conversion of a metabolic reconstruction of an organism into models requires the imposition of physico-chemical and environmental constraints to define systems boundaries. The conversion also includes the transformation of the reaction list into a computable, mathematical matrix format (e.g., using the COBRA toolbox [Becker et al., 2007]).

Formulation of BiGG knowledge-bases: The four-step procedure to reconstruct genome-scale metabolic networks has been detailed elsewhere (Feist et al., 2009; Reed et al., 2006b; Durot et al., 2009). Briefly, all metabolic functions encoded by an organism's genome are systematically retrieved, curated, and translated into a list of biochemical reactions that comprise the network. The association between the biochemical reactions and the catalyzing gene products is achieved using Boolean logic through the gene-protein-reaction (GPR) associations (Reed et al., 2006b). Further metabolic functions supported by experimental data can be included without gene association. Extensive QA/QC procedures ensure that the BiGG knowledge-base is self-consistent, comprehensive, and exhibits similar physiological properties as the target organism (Thiele and Palsson, in press). A BiGG knowledge-base effectively represents a two-dimensional annotation of a genome (Palsson, 2004) and represents the implementation of Axioms #1 and #2.

From a knowledge-base to a GEM: BiGG knowledge-bases can be converted into GEMs (genome-scale models) by implementing Axioms #3, #4, and #5. While BiGG accounts for genome-scale information, a GEM can represent the capacities of a cell in a particular environmental and genetic state. Subsequently, there are different possible GEMs that can be derived from a given organism's reconstruction. In this conversion, the BiGG knowledge-base is represented in a mathematical format, S .

System boundaries are defined around the entire reaction network. Exchange reactions are added to all transportable extracellular metabolites and can be constrained in simulations to represent different environmental conditions. Demand reactions are added, including the biomass reaction, that details all precursors and their fractional contributions to a cell's macromolecular composition, as well as any maintenance energy requirements (Feist et al., 2007).

Computational tools: A broad spectrum of methods has been developed under this umbrella (Palsson, 2006; Price et al., 2004; Durot et al., 2009), collectively called COBRA methods. Constraint-based metabolic models can be imported into Matlab in SBML format. COBRA methods can then be applied (Becker et al., 2007). With a growing number of metabolic reconstructions available (see http://www.systemsbiology.ucsd.edu/In_Silico_Organisms/Other_Organisms) and the accessibility of COBRA tools, the number of practitioners in this field is growing.

In a recent review (Feist and Palsson, 2008), the uses of the *Escherichia coli* GEM were classified into five categories: (1) metabolic engineering (i.e., genome-scale synthetic biology), (2) gap-filling (i.e., systematic generation of hypothesis), (3) phenotypic screens (data analysis), (4) determining network

properties (in silico systems biology), and (5) evolutionary studies (i.e., COBRA methods can analyze both proximal and distal causation). Similarly, a review of applications to other organisms, (Papin et al., accepted for publication), classifies them as: (1) contextualization of high-throughput data, (2) guidance of metabolic engineering, (3) directing hypothesis-driven discovery, (4) interrogation of multi-species relationships, and (5) network property discovery. Thus, the basic and applied uses of reconstructions and associated GEMs are growing.

Biological Science in the Era of Systems Biology

Genome-scale reconstructions enable biological science to proceed in fundamentally new ways. Here I discuss four new possibilities.

Integration of High-throughput Data

Omics data can be analyzed using a reconstruction as a scaffold. As stated above, a reconstruction is a BiGG knowledge-base, and if ‘omics’ data are mapped onto the reconstruction, it enables the analysis of the ‘omics’ data against the curated knowledge about the target organism as a context.

Several examples of this use of reconstructions have been demonstrated. Tissue-specific expression-profiling data has been mapped against the reconstruction of the global human metabolic map (Shlomi et al., 2008) to yield draft reconstructions of tissue-specific metabolic networks in humans. Proteomic data from the human cardiac mitochondria has been used to form tissue-specific organelle models (Vo et al., 2004) and used for the analysis of SNPs through the use of cosets (Jamshidi and Palsson, 2006). The use of reconstructions to analyze expression-profiling data from *E. coli* in many studies has been reviewed recently (Lewis et al., 2009). In a similar fashion, metabolomic and fluxomic data can be analyzed, e.g., see Jamshidi and Palsson (2008).

Thus, curated genome-scale reconstructions provide a new way to analyze ‘omics’ data. This ability is likely to help with revealing the information content in an ‘omics’ data set, as purely statistical approaches have proven to be somewhat limited.

Gap-filling

BiGG knowledge-bases are not complete; they have ‘gaps’ in them. These gaps come in at least two fundamental varieties. First, there can be a missing reaction or a path between two metabolites in the reconstruction, and secondly, a metabolite can be detected that has no connections to the network – representing an ‘island’ on the metabolic map. The former can be filled with a gap-filling process (Reed et al., 2006a). Missing links have been discovered this way (Fuhrer et al., 2007) and the methods developed to date for gap-filling have been described

(Breitling et al., 2008); the latter calls for the discovery of a new pathway. Computational tools for suggesting missing pathways have been recently described (Hatzimanikatis et al., 2005; Kumar and Maranas, 2009).

These methods may be viewed as representing interesting computational and algorithmic challenges. However, these developments are more profound and fundamental. They represent the computational generation of hypotheses using genome-scale BiGG knowledge-bases. The reconstruction is used as a context for analyzing data and determining a candidate explanation for discrepancies between experimental data and computational predictions. In fact, mixed-integer linear programming (MILP) algorithms represent the generation of the 'most parsimonious' hypothesis as they can be used to grade the complexity of the candidate explanations. In the initial study of this kind, the simplest explanation was always found to be the correct one (Reed et al., 2006a).

Understanding Complex Biological Phenomena

Phenotypic functions rely on the coordinated and simultaneous action of multiple gene products. This makes complex biological processes hard to comprehend. In addition, with changes over generations, such comprehension may be even more challenging. COBRA tools enable the computation of both proximal and distal causation at a genome-scale, and thus possesses the potential to provide a framework for a deep understanding of complex biological phenomena.

This expectation is perhaps being realized through work on bacterial adaptation. The ability of GEMs to predict the outcome of bacterial adaptation to new nutritional environments (Ibarra et al., 2002), even in the face of gene deletions (Fong and Palsson, 2004), opens up new and fascinating avenues to study fundamental biological phenomena. The network level predictions are made using GEMs and phenotypic functions. Fortunately, the third generation sequencing methods enable the full resequencing of microbial genomes following adaptation (Shendure et al., 2005; Herring et al., 2006). With available allelic replacement methods the causality of the mutations can be assessed (Herring et al., 2006; Conrad and Palsson, in press).

The convergence of these developments, COBRA, GEMs, cheap resequencing, and allelic replacement, allows the study of evolution in a laboratory setting. This fascinating prospect is likely to help us understand the plasticity and functions of bacterial genomes better than before. For instance, the RNA polymerase has been shown to be a highly mutable enzyme (Conrad and Palsson, in press) and we are now beginning to determine the objective functions that wild type strains seem to adhere to (Fischer and Sauer, 2005). Taken together, this means that teleology could be studied in an experimental setting.

Thus, metabolic systems biology is enabling a totally new scientific pursuit. Although the application of COBRA methods has been to bacterial adaptation, one might expect similar applications to pathogenic processes. For instance,

carcinogenesis has been conceptualized as the alleviation of a series of constraints (Hanahan and Weinberg, 2000) and metabolism is now seen as an integral part of this process (Vander Heiden et al., 2009). Thus, the analysis of the adjustment of metabolic processes to enable this pathologic state is likely to be possible using COBRA methods.

Metabolic Engineering

Microbial metabolism has been and is being modified to achieve practical ends through willful genetic manipulation of a wild type organism to generate a production strain. This field is called metabolic engineering; with a foundational paper by Bailey appearing in 1991 (Bailey, 1991). A recent review describes three phases in the history of metabolic engineering (Park and Lee, 2008): first is the use of random mutagenesis and screening; second, the use of targeted genetic manipulations to achieve ‘local’ results in the function of a network; and third, the use of GEMs to perform ‘global,’ or genome-scale analysis of genetic manipulations. Algorithms have been developed to perform the computation of genetic changes to achieve such global results (Burgard et al., 2003; Patil et al., 2005; Pharkya et al., 2004).

The engineering dictum, “*there is nothing more practical than reliable theory,*” is at the foundation of engineering design and practice. Good computational models accelerate design processes, and minimize prototyping, testing, and experimentation. The fact that the third phase in metabolic engineering has been ushered in through the use of GEMs signifies that we are now beginning to contemplate and practice synthetic biology at the genome-scale. Many commercial enterprises that are developing sustainable technologies benefit from this capability.

Beyond Metabolism in Microbes

With the successes of metabolic systems biology in microbes and the statement of some of its underlying axioms, it is natural to wonder what will happen next in this field. The foray into human metabolism seems like an obvious extension with the clear challenges of greater organismic complexity and function. i.e., what are the objectives of various metabolic functions in man?

We have already shown that similar bottom-up reconstructions of the transcription–translation machinery can be achieved (Thiele et al., 2009). Given the chemically detailed representation of this process on a genome-scale, COBRA approaches could be used for the analysis of systems properties. Several chemically detailed reconstructions of signaling systems have appeared (Thiele et al., 2009; Papin and Palsson, 2004) that could be analyzed using COBRA tools. New genome-scale data types (transcription start sites, tiled arrays for expression profiling, ChIP-chip, and proteomic methods) are now yielding the data that is needed to reconstruct transcriptional regulatory networks at a genome-scale (Cho

et al., accepted for publication). Other data types, such as phosphoproteomics (Macek et al., 2009; Soufi et al., 2008) and rapid metabolic regulatory mechanisms (Ralser et al., 2009) will expand the scope of reconstruction of regulatory phenomena.

Closing

The emergences of mechanistic genotype–phenotype relationships that can account for dual causality are likely to have a broad impact on the life sciences. A number of metabolic networks have already been reconstructed for target organisms. New biological science is being performed with these reconstructions. The new avenues that have been opened up by COBRA tools and a growing number of GEMs are probably just at the early stages of their exploration.

This piece is written as a personal account of the development of the field of genome-scale systems biology of metabolism. It is not meant to be a comprehensive view of the field but a discussion of the four topics stated in the summary. Although most of the references are to the author's own work, many of them are reviews citing the major developments in the field.

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A15

SYSTEMS VACCINOLOGY⁷⁰

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Vaccination is one of the greatest triumphs of modern medicine, yet we remain largely ignorant of the mechanisms by which successful vaccines stimulate protective immunity. Two recent advances are beginning to illuminate such mechanisms: realization of the pivotal role of the innate immune system in sensing microbes and stimulating adaptive immunity, and advances in systems biology. Recent studies have used systems biology approaches to obtain a global picture of the immune responses to vaccination

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in humans. This has enabled the identification of early innate signatures that predict the immunogenicity of vaccines, and identification of potentially novel mechanisms of immune regulation. Here, we review these advances and critically examine the potential opportunities and challenges posed by systems biology in vaccine development.

“We are drowning in a sea of data and thirsting for knowledge. Most biology today is low input, high throughput, no output biology.”

—Sydney Brenner

“We must make this the decade of vaccines.”

—Bill Gates

Introduction

In the epic saga of the evolutionary struggle between microbes and humans, the invention of vaccination is a defining moment, one that represents the victory of our wits over their genes. Ironically, however, despite the common origins of vaccinology and immunology in the pioneering work of giants such as Pasteur and Jenner, the two disciplines have evolved such different trajectories that immunologists remain largely ignorant about the mechanisms of action of successful vaccines (empirically made), and vaccinologists have, until recently, displayed little interest in the intricacies of immune regulation. Understanding the immunological mechanisms of vaccination, however, is of paramount importance in the rational design of future vaccines against pandemics such as HIV, malaria, and tuberculosis and against emerging infections. Recent advances in our understanding of the innate immune system and the use of systems biological approaches are beginning to reveal the fundamental mechanisms by which the innate immune system orchestrates protective immune responses to vaccination (Pulendran and Ahmed, 2006; Steinman, 2008). The innate immune system is capable of sensing viruses, bacteria, parasites, and fungi through the expression of so-called pattern recognition receptors (PRRs), which are expressed by dendritic cells (DCs) and other cells of the innate immune system (Reviewed by Coffman et al. [2010], this issue of *Immunity*). Toll-like receptors (TLRs) represent the most studied family of PRRs (Iwasaki and Medzhitov, 2010; Kawai and Akira, 2010). However, other non-TLR families of innate receptors, such as C type lectin-like receptors (Geijtenbeek and Gringhuis, 2009), nucleotide-binding oligomerization domain-like receptors (Ting et al., 2010), and retinoic acid-inducible gene I (RIG-I)-like receptors (Wilkins and Gale, 2010), also play critical roles in innate sensing of pathogens and induction of inflammatory responses. Emerging evidence suggests that the nature of the DC subtype, as well as the particular PRR triggered, plays a critical role in modulating the strength, quality, and persistence of adaptive immune responses (Pulendran and Ahmed, 2006; Steinman, 2008). Such insights

about the molecular basis of immune regulation have accrued largely through the traditional scientific method of hypothesis creation, and experimental validation, particularly through the reductionist approaches of molecular biology. However, as powerful as such approaches are, they offer a very limited view of complex biological systems. Thus, there are estimated to be more than 26,000 genes in our genomes, and entry of a vaccine or a pathogen into the body perturbs the expression of a substantial fraction of them. Systems biological tools offer us a solution to this problem. In vaccinology, recent studies have highlighted the use of such approaches in offering a global picture of the biological response to a vaccine. Here we highlight these advances and discuss their potential importance. This review is divided into four parts. In the first part (Biology of the 21st Century), we provide a broad overview of systems biology, its goals and challenges, and highlight the features that distinguish it from reductionistic biology. Next, (in Systems Biology in Vaccinology) we review recent studies that have applied systems biological approaches to vaccinology and suggest key areas where such approaches may impinge on vaccine development. These include identification of potentially novel correlates of immunity, predicting the efficacy of vaccines, accelerating the clinical trial platform of vaccines, and learning new biological insights about immune regulation. In part three (Low-Input, High-Throughput, No Output Biology), we critically examine the challenges and potential pitfalls of systems biological approaches. Finally (in A Framework for Systems Vaccinology), we conclude by offering a conceptual framework of how systems approaches can guide vaccine design and development.

Biology of the 21st Century

Two of the greatest scientific achievements of the 20th century were the discovery of the structure of DNA and the sequencing of the human genome. The grand challenge for biology and medicine at the turn of the 21st century is to understand the biological complexity that emerges from interactions between our genomes and the environment. We are uniquely poised to tackle this challenge of biological complexity by the convergence of a new intellectual framework (a systems rather than a reductionistic view) and new technologies (for measuring and visualizing the behavior of genes, molecules, cells, organs, and organisms), coupled with the innovation of computational and mathematical tools for dealing with complex data sets. The convergence of these disparate threads offers us an unprecedented opportunity to understand the fundamental features of life—from a holistic rather than solely reductionistic; from a predictive rather than descriptive; in short, from a systems biological viewpoint.

Systems biology is an interdisciplinary approach that systematically describes the complex interactions between all the parts in a biological system, with a view to elucidating new biological rules capable of predicting the behavior of the biological system (Kitano, 2002). Although reductionist molecular biology

works by isolating and characterizing each component of the system (e.g., a gene, a protein, or a cell type), systems biology focuses on studying the structure and dynamics of the whole system (Kitano, 2002). Under different types of perturbation, data are collected from all the components of a biological system, analyzed, and integrated in order to generate a mathematical model that describes or predicts the response of the system to individual perturbations (Ideker et al., 2001). A key goal of systems biology is to understand the nature of biological networks, which access, integrate, and communicate information from the genome to the environment, and back (Ideker et al., 2001; Kitano, 2002). These networks represent, in a sense, the lowest functional units of life processes, such as development, disease, immunity, and aging. Therefore, understanding these life processes requires understanding the nature and behavior of these networks, both their robustness and plasticity, in the face of a dynamic environment. What is needed to delineate these networks is the acquisition of high-throughput data on the genes, mRNAs, microRNAs, and proteins that constitute the networks. Systems biology capitalizes on several so-called “omic” technologies that are used to define and monitor all the components of the systems. DNA microarrays and high-throughput sequencing can be applied to identify global differences on gene expression (transcriptomics), genomic rearrangements, and genetic polymorphisms (genomics) as well as to provide a high-resolution global map of protein-DNA interactions (chromatin immunoprecipitation followed by DNA sequencing or hybridization to the array). Other enabling technologies include modern mass spectrometry (powering proteomics, lipidomics, and metabolomics), yeast two-hybrid system (mapping protein interactions), and genome-wide RNA interference screening (identifying genes required for a process). In addition, systems biology features the integration and modeling the huge amount of data generated by high-throughput techniques. An array of computational methods has been developed in the context of systems biology, and data integration and network inference are of special interest (Bansal et al., 2007; Hyduke and Palsson, 2010). Such methods can be closely coupled with experimental studies to generate testable hypotheses and improve the understanding of molecular mechanisms.

Systems biological approaches have changed prognosis and therapy response prediction in oncology (Alizadeh et al., 2000; Sørlie et al., 2001) and are beginning to be applied to understanding mechanisms of innate and adaptive immunity (Aderem and Hood, 2001; Germain, 2001; Gilchrist et al., 2006; Haining et al., 2008; Haining and Wherry, 2010; Kaech et al., 2002; Wherry et al., 2007; Zak and Aderem, 2009), in identifying diagnostic biomarkers of different infections (Chaussabel et al., 2008; Lee et al., 2008; Otaegui et al., 2009; Ramilo et al., 2007), and autoimmunity (Pascual et al., 2010). Systems biological approaches also offer unprecedented opportunities to study immune responses in humans (Aderem and Hood, 2001; Germain, 2001). However, only recently have they started to be applied to vaccinology. There are two broad applications of systems approaches in vaccinology: prediction of immunogenicity and efficacy of vac-

cines and scientific discovery. These two areas use distinct methodologies and have different rationales and output, and they are discussed below.

Systems Biology in Vaccinology

One potential application of systems biology in vaccinology is in predicting vaccine efficacy. The identification of molecular signatures (e.g., patterns of gene expression induced after vaccination), induced rapidly in the blood after vaccination that correlate with and predict the later development of protective immune responses, represents a strategy to prospectively determine vaccine efficacy. In the field of cancer genomics, predictions of cancer outcome have been based on gene expression profiles of the cancer cells themselves (see Box A15-1).

However, in the human immune system, there is no analogous single tissue from which to sample cells for dissecting biology and creating predictors. The immune system spans multiple lineages, is anatomically distributed, and is highly interregulated. Sampling all these cellular components and assaying their gene expression profiles is obviously not feasible. However, two critical features of the

BOX A15-1 Prediction and Classification Based on Gene Expression Signatures

In cancer genomics, gene expression signatures have been used to predict the patient's clinical outcome and response to therapies. In vaccinology, patterns of gene expression induced after vaccination (i.e., signatures) could be used to predict immunogenicity or efficacy. For example, a particular gene expression signature induced early after vaccination may be able to accurately classify whether the antibody titers are above or below the threshold necessary to confer protective immunity. For many vaccines, such correlates of protection have been established (e.g., Table A15-1). Such a signature can then be used to predict, in an independent trial, whether, for example, a vaccinee would generate an antibody response above the threshold necessary for protection. Toward this end, we have used a machine-learning approach to identify signatures that were capable of predicting the immunogenicity of individuals vaccinated with the yellow fever vaccine 17D (Querec et al., 2009) or the inactivated influenza vaccine.

The identification of signatures that predict the immunogenicity of vaccines could have broad public health utility in several situations: (1) identification of individuals who respond suboptimally to vaccination (e.g., the elderly, infants, the immunocompromised); (2) rapid screening of first responders during emergency outbreaks to identify vaccinees who respond suboptimally; (3) identification of nonresponders in partially effective vaccines (e.g., RTS, S malaria vaccine); (4) accelerated assessment of vaccine immunogenicity and of efficacy (e.g., new meningococcal vaccine); (5) identification of novel correlates of immunity and/or protection.

TABLE A15-1 Methods to Measure Antibody Correlates of Protection.

Vaccine (Pathogen)	Test	Correlate of Protection
Diphtheria (<i>C. diphtheriae</i>)	Toxin neutralization	0.01–0.1 IU/ml
Hepatitis A	ELISA	10 mIU/ml
Hepatitis B	ELISA	10 mIU/ml
Hib polysaccharide (<i>Hib</i>)	ELISA	1 µg/ml
Hib conjugate (<i>Hib</i>)	ELISA	0.15 µg/ml
Influenza	HAI	1/40 dilution
Lyme disease	ELISA	1100 EIA U/ml
Measles	Microneutralization	120 mIU/ml
Pneumococcus (<i>S. pneumoniae</i>)	ELISA; opsonophagocytosis	0.2–0.35 mg/ml (for children); 1/8 dilution
Polio	Neutralization	1/4–1/8 dilution
Rabies	Neutralization	0.5 IU/ml
Rubella	Immunoprecipitation	10–15 mIU/ml
Tetanus	Toxin neutralization	0.1 IU/ml
Chickenpox (VZV)	FAMA; gpELISA	≥1/64 dilution; ≥5 IU/ml

Historically, correlates of protection have relied on the measurement of the magnitude of the antigen-specific antibody response stimulated by vaccination. Such measurements typically include the concentration of the binding antibody titers (ELISA) or some measure of the activity of the antibody, such neutralization titers or opsonophagocytic titers. When a given threshold of such a measurement is achieved or exceeded, vaccination is assumed to have reached a signature of protective immunization. These tests have become well standardized and relatively straightforward to perform. The name of the pathogen is included in parenthesis, where its name is different from the commonly used name for the vaccine. The following abbreviations are used: *C. diphtheriae*, *Corynebacterium diphtheriae*; *Hib*, *Haemophilus influenzae* type B; *S. pneumoniae*, *Streptococcus pneumoniae*; HAI, hemagglutination inhibition; EIA, enzyme immunoassay; FAMA, fluorescent antibody to membrane antigens; gpELISA, glycoprotein antibody ELISA; VZV, varicella zoster virus. Adapted from Plotkin (2008).

immune response provide the rationale for applying genomic approaches to study the response to vaccines. First, cells of the immune system are easily accessible in peripheral blood samples. Each blood sample provides a snapshot of many lineages and dozens of differentiation states within the immune system. Moreover, because migration and trafficking is a central and ongoing feature of the immune response, peripheral blood leukocytes represent recent emigrants of peripheral tissues, including vaccine sites. Second, cells of the immune system are uniquely sensitive to perturbation. As discussed below and as we (Querec et al., 2009) and others (Gaucher et al., 2008) have demonstrated, individuals who have been vaccinated manifest marked and characteristic changes in the gene expression profiles of their peripheral blood leukocytes. Thus, the population of immune cells in the peripheral blood provides a sensitive bellwether of localized or systemic immunologic events.

The first examples of studies using systems biological tools to understand vaccine-induced immune responses came from two independent studies that

identified early molecular signatures induced in humans vaccinated with the yellow fever vaccine YF-17D (Gaucher et al., 2008; Querec et al., 2009). YF-17D is a live attenuated vaccine, which was generated after serial passage of a corresponding pathogenic strain (Asibi strain) of the yellow fever virus (Theiler and Smith, 1937) and is one of the most successful vaccines ever developed because it confers protection in nearly 90% of vaccinees. Over 600 million people have received this vaccine and a single immunization results in a broad spectrum of immune responses (neutralizing antibodies, cytotoxic T cells, and T helper 1 (Th1) and Th2 cells) and neutralizing antibody responses that persist for nearly 4 decades. The goal of our study (Querec et al., 2009) was to use YF-17D as a model to determine the feasibility of applying systems biological approaches (1) to identify molecular signatures induced early after vaccination, which could predict the later immunogenicity of the vaccine (i.e., to identify biomarkers of vaccine efficacy) and (2) to obtain biological insights about the mechanism of action of YF-17D. Fifteen individuals who had previously not been vaccinated with YF-17D or infected with yellow fever (and were thus immunologically naive to the vaccine or pathogen) were vaccinated, and blood samples were isolated at baseline and at various time points after vaccination and analyzed with respect to several immunological parameters. There was a striking variation in the magnitude of the antigen-specific CD8⁺ T cell responses, and the neutralizing antibody titers measured at day 15 or 60, between different individuals (Querec et al., 2009). We then measured cytokine induction in the plasma using a multiplex cytokine assay, and the frequencies and activation status of innate immune cells such as DC and monocyte subsets at days 1, 3, or 7 after vaccination, but these measurements did not correlate with the later T cell or antibody responses. Microarray analyses using the Affymetrix Human Genome U133 Plus 2.0 array of total peripheral blood mononuclear cells (PBMCs) revealed a molecular signature comprised of genes involved in innate sensing of viruses and antiviral immunity in most of the vaccinees. Thus, in addition to enhanced expression of endosomal TLRs, the gene expression of members of the 2',5'-oligoadenylate synthetase family (e.g., OAS 1,2,3 and L, which are essential proteins involved in the innate immune response to viral infection), *DDX58* (RIG-I), and *IFIH1* (MDA-5) were all upregulated (Figure A15-1). Two key transcription factors that mediate type I interferon responses, *IRF7* and *STAT1*, were also upregulated. Members of the ISGylation pathway, which preserve essential proteins from being degraded during the IFN-induced cellular antiviral state, were increased, including *ISG15*, *HERC5*, and *UBE2L6*. Another PRR group where both positive and negative regulation is induced by YF-17D is in the complement cascade. The complement signature of YF-17D included the upregulation of genes for C1q and its feedback inhibitor C1IN and the increased expression of the gene-encoding C3a receptor 1 with corresponding increase in the C3a protein in plasma (Figure A15-1). Thus YF-17D activates multiple pathogen surveillance mechanisms in several cellular compartments: extracellular, cell membrane, cytoplasmic, and vesicular

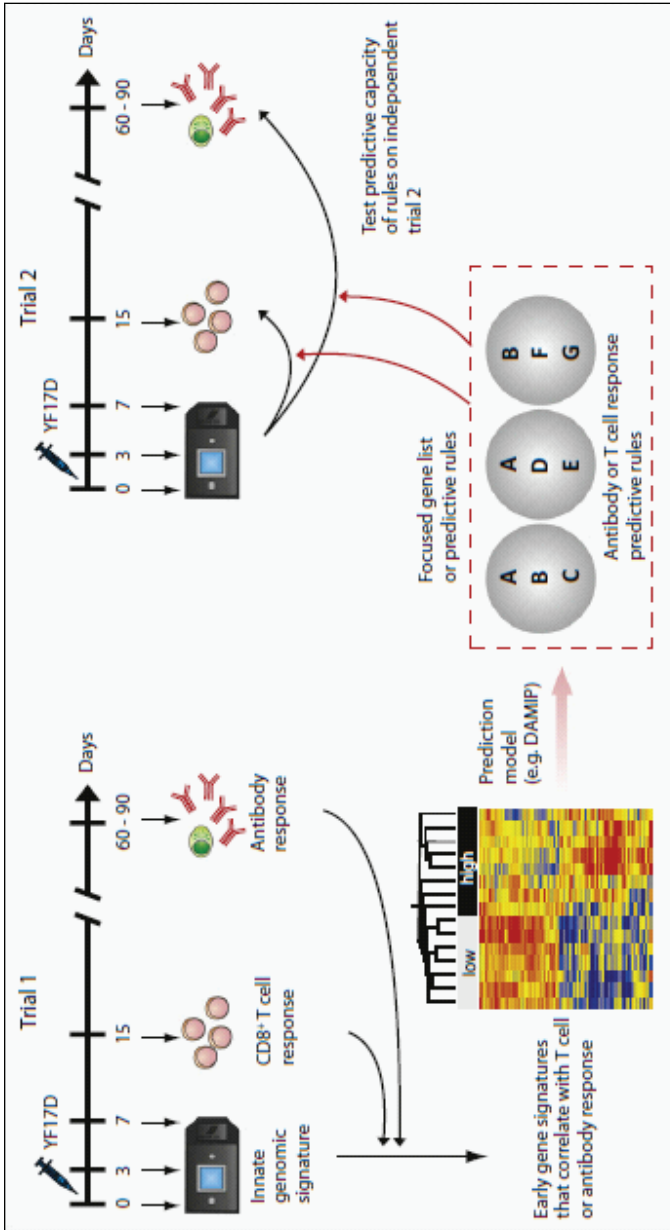


FIGURE A15-1 Using systems biology to predict the immunogenicity of the YF-17D vaccine. Schematic representation of the systems biology approach used to predict the T and B responses of YF-17D vaccinees (Querec et al., 2009). Healthy humans vaccinated with YF-17D are bled at the indicated time points and the innate and adaptive responses studied. Innate signatures obtained with microarrays are found to correlate with the later adaptive immune responses. The predictive power of such signatures is tested in an independent trial (trial 2).

(Figure A15-1). However, these signatures did not correlate with the magnitude of the antigen-specific CD8⁺ T cell or antibody responses.

We then used additional bioinformatics approaches to identify gene signatures that did correlate with the magnitude of antigen-specific CD8⁺ T cell responses and antibody titers and that were capable of predicting the magnitude of these responses in an independent trial of YF-17D vaccination in humans. We observed that signatures for CD8⁺ T cell responses from the first trial were predictive with up to 90% accuracy in the second trial and vice versa. Of the genes present in these predictive signatures, *EIF2AK4* is known to be a critical player in the integrated stress response (Wek et al., 2006) and regulates protein synthesis in response to changes in amino acid levels by phosphorylating the elongation initiation factor 2 (eIF2a) (Figure A15-1). This results in a global shutdown of translation of constitutively active proteins by redirection of their mRNAs from polysomes to discrete cytoplasmic foci known as stress granules (SGs), where they are transiently stored (Kedersha and Anderson, 2007). Consistent with this, YF-17D induced the phosphorylation of eIF2a and formation of stress granules (Querec et al., 2009). Moreover, several other genes involved in the stress response pathway, like calreticulin, protein disulfide isomerase, the glucocorticoid receptor, and c-Jun, were observed to correlate with the CD8⁺ T cell response (Figure A15-1). These observations stimulate the hypothesis that the induction of the integrated stress response in the innate immune system might play a key role in shaping the CD8⁺ T cell response to YF-17D. Experiments to test the hypothesis are currently underway. In the case of antibody responses, *TNFRSF17* (BCMA), a receptor for the B cell growth factor BLyS or BAFF (known to play a key role in B cell differentiation) (Avery et al., 2003), was a key gene in the predictive signatures. Thus, taken together, these studies provide a global description of the innate and adaptive immune responses that are induced after YF-17D vaccination and stimulate the generation of testable hypotheses about the biological mechanisms that regulate the magnitude and nature of the immune response to YF-17D (Figure A15-1).

The utility of such an approach in predicting the immunogenicity and protective efficacy of other vaccines needs to be determined. The question of whether the signatures that predict the T and B cell responses to YF-17D can also predict such responses to other vaccines remains to be determined. In one scenario, it could be envisioned that all vaccines that stimulate antibody responses would induce a common archetypal signature, capable of predicting the magnitude of the antibody response to any vaccine. Similarly, there could be an archetypal signature that predicts the antigen-specific CD8⁺ T cell responses to any vaccine. However, B and T cell responses come in different flavors, and different vaccines induce different types of B and T cell responses. It seems unlikely, therefore, that a common archetypal signature would be capable of predicting all the different types of B or T cell responses induced by different vaccines. A second scenario is that each vaccine could have a very unique signature, which was capable of predicting the particular type of T or B cell responses only to that vaccine. How-

ever, many vaccines induce similar types of immune responses (e.g., neutralizing antibodies or polyfunctional CD8⁺ T cells), so it is reasonable to suggest that vaccines that stimulate a similar mechanism of protective immunity will induce similar molecular signatures. For example, vaccine Y that stimulates long-lived plasma cells that produce high-affinity antibody may stimulate a particular signature, whereas vaccine Z that induces polyfunctional CD8⁺ T cells would stimulate a different signature (Figure A15-2). Vaccine X that induced both types of responses would stimulate a combined signature (Figure A15-2). Other vaccines that relied on opsonophagocytic antibodies for protection may have a different innate signature. Thus, one would have a cluster of signatures that predict various aspects of B cell immunogenicity or T cell immunogenicity. Similarly, there could be a different cluster of signatures that predict protective immunity that is not mediated by T or B cell-dependent mechanisms, but by other mechanisms mediated perhaps by NK cells, DCs, or stress response pathways (Figure A15-2). In this context, our preliminary data with the influenza vaccines suggest that *TNFRSF17*, which was a key predictor of the neutralizing antibody responses to YF-17D (Querec et al., 2009), is also a predictor of the hemagglutinin antibody titers to vaccination with the inactivated influenza vaccine, suggesting that there are likely to be common predictors of antibody responses to many vaccines (unpublished data). This probably underlies common biological mechanisms by which different vaccines could stimulate antibody responses. The identification of such predictive signatures will facilitate not only the rapid screening of vaccines and the development of a vaccine chip, comprising clusters of a few hundred or fewer genes, each cluster being capable of predicting a facet of immunogenicity (Figure A15-2B). Such a chip would therefore be used to predict the immunogenicity of virtually any vaccine. Indeed, in the field of cancer genomics, after several years of false starts, MammaPrint (<http://www.agendia.com>), a prognostic chip for breast cancer, was developed by Agendia and approved by the Food and Drug Administration in the United States. Like the story behind this breast cancer prognostic chip, the development of the vaccine chip will probably require the analysis of hundreds of vaccinees over several clinical trials. However, we have already seen how host gene expression profiles induced after vaccination correlate with, and predict, vaccine immunogenicity and also offer mechanistic insights into immune regulation (Querec et al., 2009). This additional layer of knowledge, translated into an array of functional modules on the vaccine chip, gives us extra power that was not utilized in the earlier, brute-force biomarker hunting.

This is likely to have an impact on several public health-related issues in vaccinology. One major issue is that many common vaccines such as the influenza vaccine (Gardner et al., 2006), pneumococcal vaccine (Jackson and Janoff, 2008), and zoster vaccines induce suboptimal immune responses in a substantial proportion of the elderly, in infants, or in immunocompromised populations such as HIV-infected or transplant patients. Therefore, delineation of signatures of immunogenicity would permit such individuals to be identified prospectively.

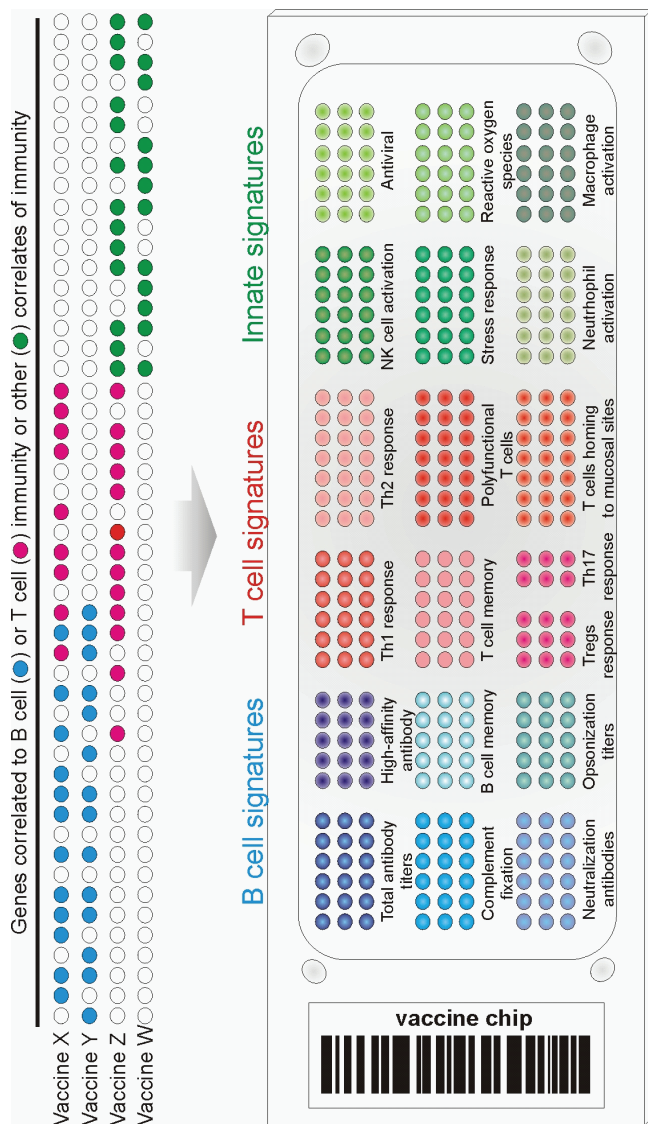


FIGURE A15-2 (Top) Systems biology approaches allow the identification of predictive gene signatures of immunogenicity for many vaccines. Vaccines with similar correlates of protection may or may not share the same gene markers. The identification of predictive signatures of many vaccines would enable the development of a vaccine chip. (Bottom) This chip would consist of perhaps a few hundred genes, subsets of which would predict a particular type of innate or adaptive immune response (e.g., magnitude of effector CD8+ T cell response, frequency of polyfunctional T cells, balance of T helper 1 (Th1), Th2, and Th17 cells, high-affinity antibody titers, and so on). This would allow the rapid evaluation of vaccines for the strength, type, duration, and quality of protective immune responses stimulated by the vaccine. Thus, the vaccine chip is a device that could be used to predict immunogenicity and protective capacity of virtually any vaccine in the future.

In addition, this strategy will help identify nonresponders when vaccinating first responders during an emerging outbreak or when evaluating the efficacy or immunogenicity of untested vaccines (Table A15-1). Furthermore, the predictive signatures could highlight novel correlates or protective immunity and enable the formulation of new hypotheses about the mechanisms underlying vaccine-induced protective immunity.

Systems biology may also be useful in addressing a major challenge in vaccine development: to determine the correlates of protection against a pathogen. The magnitude of the antigen-specific antibody titers is considered to be the primary correlate of protection against most pathogens (Plotkin, 2008) (Table A15-1). For example, antibodies mediate protection against blood-borne viruses such as hepatitis (Jack et al., 1999; Van Damme and Van Herck, 2007) and yellow fever (Lang et al., 1999; Reinhardt et al., 1998; Wheelock and Sibley, 1965); bacteria that secrete toxins that cause diphtheria (Ipsen, 1946) and tetanus (Looney et al., 1956); viruses that infect via mucosal surfaces such as influenza (Dowdle et al., 1973; Mostow et al., 1973) and rotaviruses (Jiang et al., 2008); rabies virus (Nagarajan et al., 2008), which infect neuronal axons; and pneumococcal and meningococcal bacteria, which are leading causes of pneumonia and meningitis (Andreoni et al., 1993; Romero-Steiner et al., 2006). The antigen-specific antibody responses to such vaccines are measured through standardized assays such as ELISAs (which measure binding antibody titers), hemagglutination inhibition, and functional measures of antibody activity such as neutralization and opsonophagocytosis (Table A15-1). Typically, such assays yield a single value, a threshold, above which antibody responses are considered to be protective.

However, despite the widespread use of such antibody assays to measure the efficacy of current vaccines, in the case of many vaccines humoral immunity may not be the only, or even the best, correlate of protection. Furthermore, protective immunity may not even correlate with the humoral immune response. Varicella virus vaccination efficacy is usually determined by measuring antibody titers with serum neutralization or ELISA. However, persistent varicella-specific T cells have been shown to be indicators of protection from varicella virus infection and have been suggested as possible additional or alternative correlates of protection in children and the elderly (Arvin, 2008; Levin et al., 2008). Furthermore, antibody titers to influenza vaccination may be unreliable for predicting risk of influenza illness in the elderly population (McElhaney et al., 2006). On the contrary, elderly individuals that have strong influenza-specific T cell responses are less likely to develop flu regardless of postvaccination antibody titers (McElhaney et al., 2006). Although antibody titers could not distinguish between elderly subjects that did or did not develop flu, those subjects with high IFN- γ :IL-10 ratios following *ex vivo* stimulation of PBMCs with live influenza preparations were more likely to be protected from influenza illness (McElhaney et al., 2006). In addition, patients with high frequencies of CMV-specific T cells are less likely to have reactivation of CMV when they are placed on immunosuppressive drugs

to prevent transplant rejection (Bunde et al., 2005; Sester et al., 2001). In fact, many diseases that are a top priority for vaccine development, such as HIV, TB, and malaria, are believed to require strong T cell responses for protection (Hoft, 2008; Pantaleo and Koup, 2004; Reyes-Sandoval et al., 2009). These realizations have led to interest in measuring T cells as correlates of protection.

However, measuring the functional signature of the T cell response as a correlate of protection is more challenging than assessing antibody titers. First, T cell populations are phenotypically and functionally diverse (e.g., CD8⁺ T cell, CD4⁺, effector memory, central memory, Th1, Th2, and Th17 cells, etc.). Vaccination can induce the proliferation and differentiation of antigen-specific T cells into effector cells that secrete cytokines such as IFN- γ , IL-4, IL-17, IL-10, IL-9, or effector memory cells and central memory cells, all of which play key roles in mediating short- and/ or long-term protective immunity to the pathogen (Harari et al., 2004; Sallusto et al., 1999, 2010) (this issue of *Immunity*). Recent studies have monitored activated T cells in humans phenotypically by measuring upregulation of CD38 and HLA-DR or peptide-MHC tetramer-staining cells (Akondy et al., 2009; Appay et al., 2002; Callan et al., 1998; Morgan et al., 2008). Differentiation into effector and memory phenotypes can be assessed by the expression of markers such as CD45RA, CD62L, CD127, and CCR7 (Akondy et al., 2009; Appay et al., 2002; Callan et al., 1998; Morgan et al., 2008). However, the frequencies of differentiated T cell phenotypes may not be adequate correlates of protection, because these may not necessarily correlate with their functional activity. The functions of T cells can be dependent on the cytokines they secrete (e.g., IFN- γ , IL-2, and TNF- α) or production of perforin, as well as other measures of cell proliferation and cell-mediated cytotoxicity. Thus, there are a variety of T cell functional signatures that can be measured as potential correlates of protection in lieu of the traditional antibody response. Importantly, the assessment of a single parameter of T cell function (e.g., IFN- γ secretion) may not be sufficient as a correlate of protection; however, using a functional signature comprised of two or more types of measurements may provide more specific and reliable correlates of protection (Harari et al., 2004). Finally, it may be necessary to abandon the simple linear functional signature model developed for antibody titers where a predetermined threshold is used as a correlate. Instead of using a set threshold of a single variable to determine vaccine efficacy, so called cocorrelates of protection may be more appropriate where it is the balance among multiple variables that indicates efficacy (Qin et al., 2007). For instance, protection against a pathogen may be achieved when two conditions are satisfied: (1) the frequency of Th1 CD4⁺ effector memory cells meets a given threshold and (2) the magnitude of the neutralizing antibody titers reaches a certain threshold. In individuals in whom the thresholds for each of these conditions are not met, it may be the interaction between various cocorrelates, and not independent levels of each, that provides a functional signature of vaccine efficacy. For instance, in

the control of viruses or intracellular pathogens, the lower the neutralizing antibody titer induced by a vaccine, the higher the cytotoxic T cell response needs to be to enhance the likelihood of protection.

The notion that the innate immune response to vaccination might represent a viable correlate of protection has only recently been considered. Given the pivotal role of the early innate response in regulating the magnitude, quality, and duration of the later adaptive immune responses (Iwasaki and Medzhitov, 2010), specific signatures of innate activation may indicate that the vaccine induced the appropriate quality and sufficient strength of activation to induce protective acquired immunity. As discussed above, it has been shown with yellow fever vaccine 17D that molecular signatures in the blood 3 to 7 days after vaccination, corresponding with vaccine viremia and activation of the innate immune pathways, may be used to predict the peak frequency of activated virus-specific T cells and long-term neutralizing antibody titers (Querec et al., 2009).

How can systems approaches be integrated into the clinical trial framework to identify correlates of protective immunity? At the outset, it is important to clarify a frequent source of confusion that arises regarding correlates of immunogenicity versus correlates of protection. The ultimate goal is of course to determine vaccine-induced signatures a few hours or days after vaccination that can predict whether a given individual will develop long-term protective immunity against the pathogen. The most logical way of addressing this goal is to perform a clinical trial in which vaccinated humans can be challenged with the pathogen and then to identify signatures that would discriminate between those vaccinees who succumbed to the infection versus those who were protected. With very rare exceptions, as in the case of malaria vaccine trials (Vahey et al., 2010), such an approach is clearly untenable ethically and thus alternative approaches must be considered. One alternative approach is to use animal challenge models in which vaccines can be evaluated. Such models, such as the nonhuman primate model for HIV or the ferret model for influenza have greatly accelerated vaccine discovery and offered much insight into the mechanisms of protection (Sui et al., 2010). However, in some cases, opinions vary regarding the relative merits of a given model and how to translate results obtained from such a model into the clinic (Morgan et al., 2008). Therefore, an alternative or even complementary approach is to identify signatures of immunogenicity to the vaccine in humans. This approach relies on the axiom that immune protection against a pathogen is mediated by one or more components of the immune response, which can broadly be divided into the adaptive (antigen-specific B and T cells) and the innate responses. Therefore, if there was a priori knowledge of precisely which component(s) of the immune response (e.g., a combination of persistent neutralizing antibody responses and memory CD8⁺ T cells that migrate to mucosal tissues), then it becomes relatively straightforward to conduct a phase 0 or 1 clinical trial (similar to the yellow fever vaccine trials) in which early predictive

signatures of such responses can be identified (Figure A15-3). Such signatures can then be applied in the clinic to identify vaccinees who will respond suboptimally to the vaccine. But how do we know what types of immune responses are necessary for protection? In many cases, we can be guided by more than a century of immunological wisdom. For example, few immunologists would deny that the induction of persistent neutralizing antibody responses (Table A15-1) and cytotoxic T cells are beneficial to fight most viral infections. In such cases, early signatures that various aspects of T or B cell immunogenicity can be assessed in a high-throughput manner, using a small number of genes (Vaccine Chip) or an ELISA kit that measured protein expression (Figure A15-3).

But what happens in situations in which the types of immune responses required for protection are not readily apparent or where the full range of responses required for optimally effective protection may be unknown? For example, in HIV infections, although neutralizing antibodies and cytotoxic T cells are thought to be important (Letvin, 2007), there is much interest in ascertaining whether there are additional mechanisms that might confer protection. Here, it is interesting to consider how systems approaches may be integrated into phase II and III clinical trials, with a view to identifying new correlates of protection. Two approaches to integrating systems approaches into phase II and III trials are shown in Figure A15-3. Such trials typically involve thousands of participants, and performing high-throughput analyses on all would be prohibitively expensive. In one approach, signatures of various aspects of T and B cell immunogenicity can be established in a smaller phase I trial, and these signatures can be incorporated into a relatively cheap and high-throughput assay that can be used to predict immunogenicity in phase II and III trials (Figure A15-3). The assumption here is that some aspect of the T or B cell response will be protective. In a different approach, blood samples could be collected at a few strategic time points (e.g., days 0 and 7 after vaccination), put straight into RNA lysis buffer, and stored for future use. Once the trial was completed, a retrospective nested case-control study could be performed using the stored samples in which a detailed analysis of innate and adaptive responses could be performed in, say, 50 vaccinees who acquired the disease and 50 vaccinees who did not. The goal would be to identify signatures induced early on that would discriminate between those who were protected by the vaccine versus those who were not. A caveat with this approach is that one would not know whether those vaccinees who didn't acquire the disease were actually protected by the vaccine or simply never encountered the pathogen. However, in many endemic areas of infection (e.g., in a rural area where cholera is endemic and access to clean drinking water is absent), it may be assumed that exposure to infection is high.

A potential benefit of using functional signatures of innate immunity as correlates of protection is that they occur quite early after vaccination compared to the development of memory T cells and antibody responses, which can take weeks, months, or years. Being able to determine vaccine efficacy in a short time is useful for many reasons. The current clinical trial format is very lengthy and

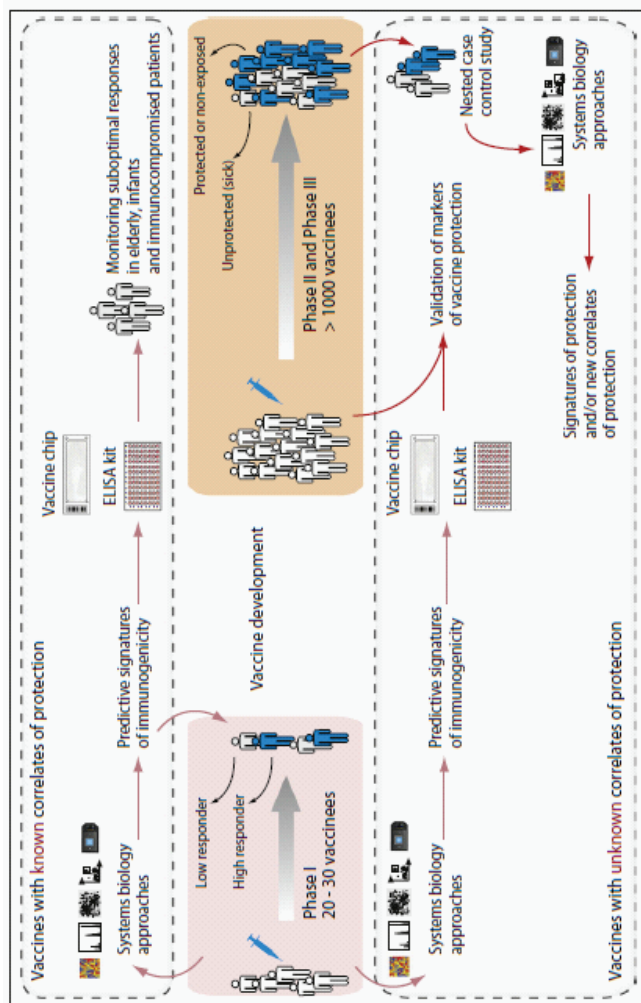


FIGURE A15-3 Integrating systems biology approaches into clinical trials. (Top) For vaccines for which correlates or protection are known (Table A15-1), systems approaches can be used to identify early signatures of protection in a phase I trial. The key genes from these signatures can be incorporated into a vaccine chip or ELISA kit, which can then be used to identify nonresponders or suboptimal responders, particularly in special populations such as immunocompromised patients, the elderly, and infants. (Bottom) For new and emerging vaccines, for which correlates of protection are unknown, signatures that predict various aspects of immunogenicity (e.g., CD8⁺ T cell responses or neutralizing antibody responses) can be assessed in phase I trials. Such signatures can then be incorporated into a vaccine chip or ELISA kit that can then be used in phase II and III trials to determine their capacity to predict protection. Alternatively, a retrospective nested case-control study could be done in a phase II and III trial to identify signatures of protection.

costly and usually offers no insights into why a particular vaccine failed. As such, clinical trials represent a major rate-limiting step in vaccine development. Having a shorter study period increases the probability of retaining all the subjects for the duration of the study, increasing the proportion of subjects that are tracked from vaccination through the final time point. In addition, measuring functional signature of vaccine-induced innate immunity makes high-throughput screening of vaccine candidates more feasible. The short duration of time required to measure innate immune activation relative to the endpoints of acquired immunity means: (1) shorter duration to analyze each batch of vaccine candidates, (2) potentially fewer resources and costs devoted to the early stage analysis of each vaccine candidate, (3) quicker refinement of vaccine formulations and delivery methods, and (4) identification of why a particular vaccine failed (Figure A15-3).

Apart from lack of inducing sufficient protection, another common reason for vaccines to fail is severe side effects. These side effects are often associated with overactivation of certain components of the innate immune system (Gupta et al., 1993; Pulendran et al., 2008). Thus functional signatures of innate immunity may be used to screen adjuvants or as cocorrelates of protection along with parameters of acquired immunity for complete vaccines (antigen + adjuvant). Functional signatures may not only help in the design of protective vaccines but may also help to limit the deleterious side effects.

Finally, systems approaches could also yield biological insights about how vaccines work.

One area that could benefit from systems approaches is delineation of the mechanisms by which adjuvants work. Although the empiric, live attenuated vaccines contain stimuli that activate the innate immune system and, in effect, act as their own adjuvants, recombinant vaccines such as the Hepatitis B vaccine need to be administered with exogenous adjuvants. In the nearly 250 years since the introduction of vaccination, although a great variety of adjuvants have been proposed, until very recently only alum, described by Glenny in 1926, was globally licensed for human use (De Gregorio et al., 2008; Lindblad, 2004). However, alum is a Th2 cell-inducing adjuvant, and does not induce strong Th1 and CTL responses. Thus, there is an urgent need to develop alternative and safe adjuvants that induce different types of immune response that might be optimally effective against different pathogens. Despite its widespread utility, until very recently its mechanism of action has been shrouded in mystery. It has been suggested that alum works by serving as a depot of antigen in the body. It has also been suggested that alum could cause necrosis in the inoculated tissue, which indirectly activates DCs through danger signals in the form of host inflammatory mediators (De Gregorio et al., 2008; Mbow et al., 2010). The details of this mechanism are only now being revealed. Recently it was demonstrated that alum signals via the Nalp3 inflammasome (Eisenbarth et al., 2008; Kool et al., 2008; Li et al., 2008). Thus, DCs or macrophages stimulated *in vitro* with alum

plus LPS induced IL-1 β and IL-18 in a caspase-1- and Nalp3-dependent manner (Eisenbarth et al., 2008; Kool et al., 2008; Li et al., 2008; McKee et al., 2009). Despite the convincing *in vitro* studies, the question of whether Nalp3 is required for the adjuvanticity of alum remains controversial, with some studies demonstrating abrogation of antibody responses in Nalp3-deficient (Nlrp3 $^{-/-}$) mice (Eisenbarth et al., 2008; Li et al., 2008) and other studies showing partial or no effect (Kool et al., 2008; McKee et al., 2009). Thus, the mechanisms by which alum induces Th2 responses are poorly understood, and a systems biological approach (e.g., microarray analyses of signatures in response to an alum-adjuvanted versus unadjuvanted vaccine) is likely to be useful in providing new insights into the mechanism of action of alum. In this context, Mosca et al. performed an elegant study in mice to assess the molecular and cellular signatures of vaccine adjuvants, including the squalene-based oil-in-water emulsion MF59 (Mosca et al., 2008), which was licensed for human use a decade ago. The molecular mechanism of action and the target cells of alum and MF59 are still unknown. By combining microarray and immunofluorescence analysis, Mosca et al. monitored the effects of the adjuvants MF59, CpG, and alum in the mouse muscle. MF59 induced the expression of 891 genes; in contrast, CpG and alum regulated 387 and 312 genes, respectively. Interestingly, there was a core set of 168 genes that were modulated by all adjuvants. Although all adjuvants promoted the recruitment of antigen-presenting cells, MF59 triggered a more rapid influx of CD11b $^{+}$ blood cells compared with other adjuvants. Furthermore, MF59 was the most potent inducer of genes encoding cytokines, cytokine receptors, and adhesion molecules involved in leukocyte migration. Intriguingly, two genes identified by microarrays, JunB and Ptx3, suggested skeletal muscle as a direct target of MF59. Taken together, the authors' interpretation of the data suggests that oil-in-water emulsions are efficient human vaccine adjuvants because they induce an early and strong immunocompetent environment at the injection site by targeting muscle cells. In addition, we have recently applied this approach to identifying a novel mechanism by which adjuvants that induce Th2 responses (e.g., cysteine proteases) program DCs to stimulate Th2 responses (Tang et al., 2010). This involves the induction of reactive oxygen species (ROS) in DCs, which is critical for the induction of Th2 responses (Tang et al., 2010).

These studies demonstrate the utility of systems approaches in understanding the mechanism of action of adjuvants, and in identifying mechanisms that contribute to their toxicity. In addition, emerging work in innate immunity is revealing the mode of action of many adjuvants. Under the brand name AS04, monophosphoryl lipid A (MPL), an LPS derivative and a TLR4 ligand, is used in combination with alum in Cervarix, GlaxoSmithKline's recently approved human papillomavirus vaccine (Hennessy et al., 2010). With the growing number of adjuvants at our disposal to mimic natural infections, we need a frame of reference as to how to use them for maximum efficacy. Turning to the functional

signatures of innate immunity induced by some of our most successful vaccines is beginning to shed light on this area. For example, YF-17D activates multiple TLRs, including TLR 2, 7, 8, and 9, as well as non-TLR PRRs such as RIG-I and MDA-5 (Querec et al., 2006, 2009), which results in the activation of plasmacytoid DCs and myeloid DCs. Similar approaches are being applied to understand innate responses to other vectors such as the attenuated pox vectors modified vaccinia virus Ankara and New York vaccinia (Guerra et al., 2007) and baculovirus-expressed HIV virus-like particles (Buonaguro et al., 2008).

Systems approaches can also shed light on the mechanisms by which vaccines induce a given type of response. As discussed above, one of the key genes in the predictive signature of YF-17D, EIF2AK4, is known to be a critical player in the integrated stress response (Kedersha and Anderson, 2007) and regulates protein synthesis in response to changes in amino acid amounts by phosphorylating the elongation initiation factor 2 (eIF2a) (Figure A15-1). Our recent data demonstrate that immunization of mice deficient in EIF2AK4 with YF-17D results in substantially diminished CD8⁺T cell responses (unpublished data). The precise mechanism of this is under investigation, but this result demonstrates that the integrated stress response plays a key role in regulating adaptive immunity to a viral vaccine.

Finally, it is important to remember that the complex behavior of biological systems cannot be understood by studying parts in isolation (Germain, 2001; Ideker et al., 2001; Kitano, 2002; Weng et al., 1999). Therefore, vaccinologists need to move beyond merely understanding each of the parts of the immune system in isolation to instead understand how the different parts of the immune system interact among themselves. Indeed, a unified model of the cellular and molecular mechanisms of vaccine-induced protective immunity is likely to result from studying different hierarchies of organization with the immune system. In such a hierarchy, the cell can be considered to be the ground level, and zooming into the cell to examine innate receptors and signaling networks offers greater conceptual resolution. In contrast, zooming out from the cell, allows more global views of multicellular cooperation (e.g., between DC subsets) and the influence of tissue microenvironments (e.g., intestine versus lung) (Pulendran et al., 2010). In addition, the immune system, as with all biological systems, has redundancies, feedback and feed-forward regulation, and synergism, which all impact how the instruction of the vaccine is processed (Kitano, 2002). For example, combinatorial triggering of specific combinations of TLRs results in a synergistic production of proinflammatory cytokines via a mechanism dependent on TRIF and MyD88 signaling (Napolitani et al., 2005). Consistent with this, vaccination with nanoparticles containing particular combinations of TLR ligands plus antigens induced a synergistic enhancement in the magnitude and persistence of antigen-specific memory B cells and long-lived plasma cells (unpublished data).

Low-Input, High-Throughput, No Output Biology?

Despite the promise of systems approaches in vaccinology, we may do well to heed the advice of Dr. Sydney Brenner: “The idea that we’ll dissect [cellular] complexity by making lots of measurements is bound to fail. . . . Everyone’s hoping for a magic computer program—experimental data, pharmacogenomics data, the whole lot—and it will come out with the answer. That’s a vague hope. Because I have to tell you, computers are incredibly stupid! It’s better to combine human intelligence with artificial stupidity than the other way around” (Davies, 2008), and “Actually, the orgy of fact extraction in which everybody is currently engaged has, like most consumer economies, accumulated a vast debt. This is a debt of theory, and some of us are soon going to have an exciting time paying it back—with interest, I hope” (Brenner, 1997). The accumulation of a sea of data is but a small stepping stone toward real understanding of biological systems. It is imperative to get beyond colorful heat maps and network maps to an understanding of the functional significance of the molecular signatures of vaccination. This is a daunting challenge because of several intrinsic problems in this approach. These are discussed below.

Conceptual Problems

A major conceptual pitfall lies with the premise that changes in the expression of genes in response to vaccination may necessarily be functionally relevant for generating the immune response to that vaccine. There are many examples where genes that are modulated in response are of no consequence to the biological response to that stimulus because evolution has not had a reason for silencing those genes. Indeed, it is well recognized that gene coexpression only corresponds to causality in very limited cases (Bansal et al., 2007; Schadt et al., 2005). The challenge is to identify true causal relationships among the cooccurring events. One solution is to borrow knowledge from predefined gene modules or pathways. If multiple genes within a module are coordinately regulated by the vaccine, then the likelihood that this module is functionally relevant becomes much higher. Another approach is to combine multiple data types. As Chen et al. (2008) demonstrated, a macrophage-enriched metabolic network, derived by integrating genotyping data and expression data, was causal of obesity traits, whereas each data type alone could not deliver the predictive power. We should be reminded that the current measurements are still a thin slice of immense biological complexity; microarray data, even with a large sample size, may fail to reach any statistical significance (Dixon et al., 2007). The general question is: how much data, what data, at what resolution, at what scale, are needed to explain the immunological phenotypes? This may only be addressed in each individual case through trials and errors. Finally, the results of the analysis have to be validated by functional data via proven techniques, say, gene perturbation or deficient mice. As the study design is closely coupled with computational analysis and

modeling, systems biology is best done in an environment where biologists and computational scientists interact closely.

A second conceptual problem is the premise that we can deduce mechanistic insights about how the vaccine induced immune responses by looking at changes in the expression of genes only in cells isolated from the blood. This is a significant problem because immune response to local vaccinations will be initiated in the draining lymph nodes. However, with many vaccines, such as live viral or bacterial vectors, there is a transient, systemic replication of the vector and, subsequently, a direct activation of blood leukocytes by it. This is likely to produce the profile of gene expression changes observed in the draining lymph nodes, which serves as a surrogate for immunogenicity. Even in the case of nonreplicating vaccines such as the inactivated influenza vaccine, our results demonstrate that signatures of immunogenicity can be ascertained in the blood (unpublished data). An additional problem is that, for many vaccines that induce mucosal immunity, gene expression signatures in the blood may not predict the strength, quality, and duration of mucosal immunity. Sampling mucosal tissues in human vaccinees is wrought with challenges. Clearly further studies are necessary to ascertain the extent to which immunogenicity of mucosal vaccines can be ascertained from the blood.

Technical Problems

One of the key technical issues is that gene expression signatures are prone to artifacts. Since the early studies of cancer expression microarrays, questions have been raised about how robust the gene signatures are (Ein-Dor et al., 2006). Recently, emphasis has been placed on pathway and network analyses because they incorporate prior knowledge into data analysis and are less prone to spurious errors than analyses of individual genes (Chuang et al., 2007; Dinu et al., 2009). This is particularly relevant to immunological studies where signals are often diluted by cell heterogeneity (Haining and Wherry, 2010). In addition, signatures must be validated with additional techniques and independent samples.

Second, when profiling PBMCs, one is looking at signatures from a mixed bag of cells. Therefore, the extent to which the changes in gene expression reflect alterations in the cellular composition of the blood versus de novo induction of gene expression remains uncertain. One solution to this problem is to FACS sort subpopulations of cells and then to evaluate expression profiles in individual cell types. However, this approach is rather laborious and expensive. An alternative approach is to devise computational strategies for assessing cell type-specific gene expression profiles. Recently, Shen-Orr et al. (2010) have devised such an approach using microarray data and relative cell type frequencies. First they validated their approach using predesigned mixtures of cells, and then they applied it to whole-blood gene expression datasets from stable posttransplant kidney transplant recipients and those experiencing acute rejection.

A third challenge lies in the enormous genetic and environmental heterogeneity in human populations and the impact that such heterogeneity may have on vaccine-induced immunity. Therefore, future studies should strive to conduct such research in populations that are uniform with respect to age, gender, ethnicity, and immune status. Furthermore, studies that aim to compare vaccine-induced immunity between different populations (e.g., frail elderly versus healthy adults) are likely to yield many insights into mechanisms that contribute to impaired immunity in given populations.

Fourth, a major challenge concerns data management and integration of the enormous volume of data generated. The timely sharing of these data is important to the research community. A dedicated database service for vaccine-related data, akin to WormBase (Schwarz et al., 2006) and TB database (Reddy et al., 2009), should be created as soon as possible. Public databases for immunology, including InnateDB (Lynn et al., 2008) and Immgen.org (Heng and Painter, 2008), have been well covered by recent reviews (Gardy et al., 2009; Tong and Ren, 2009). In-house databases often become a necessity for high-throughput projects. Integration of multiple data types is usually driven by the specific modeling approach, for instance by naive Bayesian methods (Huttenhower et al., 2009) or by custom algorithms, or combined by biomolecular concepts (Joyce and Palsson, 2006). For example, transcription factor binding data and gene expression are combined under frameworks of transcriptional regulation; metabolites and enzyme expression are combined in metabolic networks. Broader and more definitive immune parameters are desired (Fauci et al., 2008).

Cultural Problems

Finally, the successful application of systems approaches to vaccinology requires a close transdisciplinary collaboration between biologists and computational scientists. It is critical that such individuals engage in active dialog on a daily basis to combine rigorous bioinformatics analyses of the data with biological insights and intuition. Such intimate collaborations could even take place within a single laboratory where, for example, post-docs trained in bioinformatics and biology interact closely.

A Framework for Systems Vaccinology

At the World Economic Forum's annual meeting in Davos this year, Bill Gates pledged \$10 billion for vaccines over the next decade and said that he hoped that the coming 10 years would be the decade of the vaccine. His words symbolize the unique moment we face today in our millennial war with pathogens. For the first time, we have begun to understand the mechanisms by which highly successful vaccines mediate protective immunity and to begin to harness such insights in designing new vaccines against global pandemics. Systems

biology promises to offer a new paradigm in vaccinology. Recently, the National Institute of Allergy and Infectious Diseases (NIAID) initiated a new nationwide initiative to establish a consortium of human immune profiling research centers (National Institute of Allergy and Infectious Diseases, 2010). The purpose of these centers—which together will receive funding up to \$100 million over 5 years—is to characterize the human immune system under normal conditions and to understand how it changes following infection or vaccination to specific viruses and bacteria. Researchers will use the tools of systems biology to follow the global architecture of the immune response to vaccination or infections in humans and integrate information about an individual's genes, proteins, and metabolic components that are perturbed by vaccination or infection. Such studies will be performed in diverse populations with respect to age (including the elderly and children), immune status (including people with autoimmune diseases such as lupus and transplant patients), gender, and ethnicity. In addition, the initiative will provide support for centralized infrastructure to collect, characterize, and store the human samples; for bioinformatic capacity to analyze the large and complex data sets that will be generated; and for the discovery and development of new immune response-monitoring tools and sample-sparing assays. The results of this initiative are likely to have a major impact on vaccinology and generate an unprecedented volume of data on immune responses in humans. However, we must remember Dr. Brenner's admonishment and strive to transcend data and discover knowledge and ultimately understanding. The generation of high-throughput data represents but a stepping stone toward understanding. An essential aspect of this is to integrate mechanistic studies involving models, both animal and human, (e.g., knockout mice, transgenic mice, siRNA knock down of genes in humans cells *in vitro*) that can elegantly validate the functions of genes and proteins picked up in the human immune-profiling studies (Figure A15-4). Therefore, data generated in clinical trials can be mined using bioinformatics tools and used to generate biological hypotheses, which can then be tested with animal models or *in vitro* systems. The insights gained from experimentation will then guide the design and development of new vaccines (Figure A15-4). Such a framework seeks to bridge the so-called gaps between clinical trials and discovery-based science, between human immunology and mouse immunology, and between translational and basic science and offers a seamless continuum of scientific discovery and vaccine invention. That would be emblematic of 21st century vaccinology!

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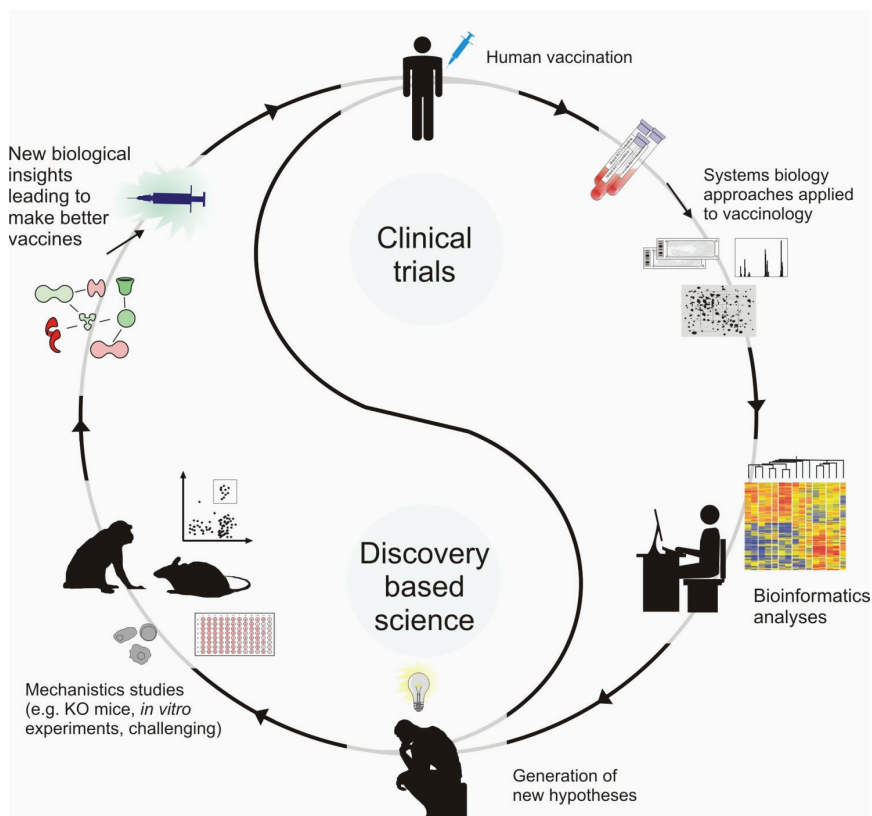


FIGURE A15-4 A framework for systems vaccinology. Systems biology approaches applied to clinical trials can lead to the generation of new hypotheses that can be tested and ultimately lead to developing better vaccines. For example, immune responses to vaccination in clinical trials can be profiled in exquisite depth with technologies such as microarrays, deep sequencing, and proteomics. The high-throughput data generated can be mined using bioinformatics tools and used to create hypotheses about the biological mechanisms underlying vaccine-induced immunity. Such hypotheses can then be tested with animal models or *in vitro* human systems. The insights gained from experimentation can then guide the design and development of new vaccines. Such a framework seeks to bridge the so-called gaps between clinical trials and discovery-based science, between human immunology and mouse immunology, and between translational and basic science and offers a seamless continuum of scientific discovery and vaccine invention.

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A16

SOLVING VACCINE MYSTERIES: A SYSTEMS BIOLOGY PERSPECTIVE**Lydie Trautmann and Rafick-Pierre Sekaly*⁷³

Systems biology has emerged as a promising research strategy that can be applied to vaccine development. This approach can lead to the identification of new mechanisms and predictors of inactivated vaccine immunogenicity.

The search for vaccines against several incurable diseases, including acquired immunodeficiency syndrome, malaria, tuberculosis, and dengue fever, has largely failed, which highlights the need for new vaccine strategies (Rappuoli and Aderem, 2011). In contrast to the empirical development of most efficacious vaccines, these diseases require a rational approach for directing vaccine responses toward different effector cell subsets of the immune system that vary with the nature or cellular tropism of these pathogens (Pulendran and Ahmed, 2011; Steinman and Banchereau, 2007). The lack of well-defined correlates of protection with most vaccines has been a major impediment to the successful development of new vaccines. The failure to identify such correlates has been mostly a consequence of the lack of assays that can measure integrated immune responses and have relied on the evaluation of a limited number of qualitative and quantitative features of effector adaptive immune responses. The efficacy of vaccines is also known to vary considerably in the human population depending on several environmental and genetic parameters, including age, nutrition and pre-existing infections. In this issue of *Nature Immunology*, Nakaya and colleagues use systems biology to identify useful predictors of the efficacy of vaccines against influenza in humans (Nakaya et al., 2011).

Systems biology offers the unique possibility of analyzing the immunological network of complex events and interactions after vaccination (Kitano, 2002). This approach can help accelerate vaccine development by identifying predictors of immunogenicity and previously unknown mechanisms that underlie protective immune responses (Pulendran et al., 2010) (**Fig. A16-1**). Systems biology has been used to identify early gene signatures that can be used to predict the responses of B cells and CD8⁺ T cells after vaccination against yellow fever (Querec et al., 2009; Gaucher et al., 2008; Pulendran, 2009). Such studies have highlighted the importance of inducing a potent and diverse innate immune response for the generation of long-term protective adaptive immunity (Querec et al., 2009). Most of these pathways would not have been identified by con-

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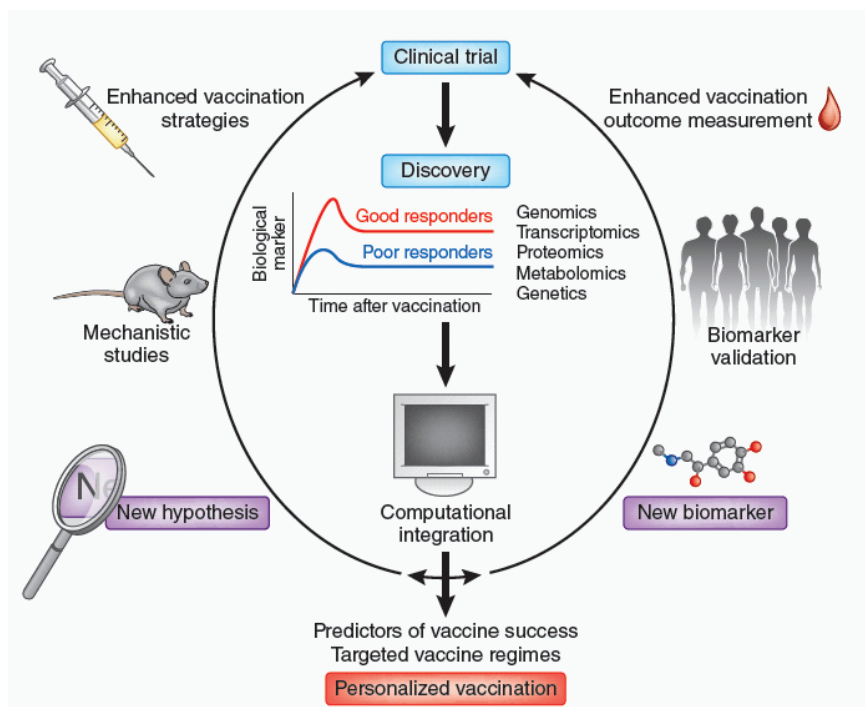


FIGURE A16-1 Systems biology approaches in the vaccine development. Licensed vaccines or new vaccines are tested in clinical trials in humans. Subjects are categorized as good and poor responders to a vaccine on the basis of biological markers. Samples collected are analyzed by one or more ‘-omics’ approaches, and results are integrated by computational methods to generate meaningful data sets. Two outcomes can emerge from these bioinformatic analyses: the generation of new hypotheses that can be tested by mechanistic studies *in vitro* or in animal models and result in enhanced vaccination strategies, or the determination of new biomarkers that can be validated in other clinical trials and result in enhancement of the vaccination outcome measurement. Both of these arms will ‘feed’ new clinical trials that will follow the same path and, after many iterations of the cycle, lead to the development of better vaccination strategies and define better predictors of immunogenicity. This process might allow the generation of personalized vaccination.

ventional immune response–monitoring strategies such as flow cytometry and analysis of cytokine production.

Despite the demonstrated success of systems approaches in predicting the immunogenicity of the vaccine against yellow fever (Querec et al., 2009; Gaucher et al., 2008) (a live replicating virus), the question of whether such approaches would also be useful in predicting the immunogenicity of inactivated vaccines (which do not replicate) has remained unknown. Furthermore, it has been unclear

whether such approaches could be used to predict the immunogenicity of memory responses. The report from Nakaya et al. addresses these questions directly (Nakaya et al., 2011). Using systems biology to analyze the innate and adaptive responses to seasonal influenza vaccination in humans, the authors define early predictors of a vaccine response and provide new insights about the mechanisms that underlie vaccine immunogenicity. Notably, this work describes a new comprehensive approach with which to tackle the challenge of vaccine development, not only because it defines new predictors of the inactivated influenza vaccine but also because it demonstrates and validates the systems biology approach as a powerful tool for predicting vaccine immunogenicity and discovering additional mechanisms of vaccine efficacy (**Fig. A16-1**).

This study compares immune responses induced by two highly efficient vaccines against influenza: the trivalent inactivated vaccine (TIV) and the live attenuated influenza vaccine (LAIV). The transcriptional signatures induced by TIV and LAIV are distinct, but both induce common innate immune response pathways, including an inflammatory response characterized by inflammasome and upregulation of the transcription factor NF- κ B. In contrast, type I interferons, which are a common feature of the two live attenuated vaccines (LAIV and the YF-17D vaccine against yellow fever) and are potent inducers of innate immunity, are not observed after vaccination with TIV, a whole killed virus. Involvement of innate immunity in modulating the response to TIV is further confirmed by the positive correlation between gene-expression pathways associated with natural killer cell signaling early after vaccination (days 3 or 7) and hemagglutination-inhibition (HAI) titers at day 28. This highlights the fact that the innate immune response is diverse and is critical to the development of a strong adaptive immune response and also that the innate immune response can vary with the type of vaccine platform used. Notably, these different innate immune responses lead to protection. Such diversity of innate immune responses could never have been identified by conventional immune response-monitoring assays. Indeed, counting cells of the innate immune response or assessing cytokine profiles failed to predict the immunogenicity of the YF-17D vaccine (Querec et al., 2009). Comparison of the innate immune responses induced by several other vaccines will provide not only a map of the common patterns induced by all successful vaccines but also the characteristics of particular vaccines that could be classified as either the type of vaccine formulation or the kind of immune response induced (Steinman and Banchereau, 2007; Pulendran et al., 2010; Zak and Aderem, 2009). With this knowledge, the design of new vaccine regimens and adjuvants could focus on recapitulating the common patterns required for vaccine efficiency and selective targeting of the specific type of response to be elicited.

Systems biology approaches can also provide insights into the molecular mechanisms that lead to vaccine efficacy. To reach this objective, however, it is necessary to assess the contributions of the various cellular subsets involved in the immune response that contribute to the overall signature in peripheral blood cells.

Previous attempts aimed at deconvoluting these signatures have relied on the extrapolation of cell subset–specific gene-expression profiles from the frequency of these subsets (Shen-Orr et al., 2010). Nakaya et al. propose an alternative solution for deconvoluting gene-expression profiles (Nakaya et al., 2011). This approach is based on the meta-analysis of cell type–specific gene-expression signatures from publicly available microarray studies and does not require assessment of the frequency of specific immune-response cell subsets. Using this approach, the authors confirm that TIV induces the upregulation of genes mostly in B cells, especially antibody-secreting cells. In contrast, LAIV induces genes in T cells, monocytes and natural killer cells. These findings highlight the need to determine if gene-expression changes are due to changes in the distribution of specific cell subpopulations or to the *de novo* induction of gene expression in discrete subsets of cells. Hence, as illustrated by this study, such approaches also provide insight into the mechanism of action of vaccines and could guide the development of specific adjuvants.

To identify predictors of TIV vaccine immunogenicity, the authors use the DAMIP (discriminant analysis via mixed integer programming) model to train and test the predictors of two other independent cohorts vaccinated with TIV. By grouping subjects as ‘good responders’ (an increase of fourfold or more in HAI titers) and ‘poor responders’ (an increase of twofold or less in HAI titers), the authors find that gene signatures at days 3–7 that can be used to predict AI titers at day 28 in the first cohort allow the prediction with 90% accuracy the immunogenicity of the inactivated vaccine in the other cohorts. Of note, some of the genes used to predict the magnitude of the TIV response, including *TNFRSF17* (a gene expressed during B cell maturation), have also been found to be predictors after vaccination with YF-17D.

This report illustrates all the steps and outcomes of systems biology approaches (**Fig. A16-1**). Starting from a comparison of two vaccines (TIV and LAIV) or of good and poor immunogenicity as estimated by HAI titers, the authors generate transcriptomics data that they further analyze by bioinformatics analyses. This study confirms the ability of such approaches to tackle complex network interactions that have never been studied before in humans in an unbiased way. For example, five members of the leukocyte immunoglobulin-like receptor family with high expression in monocytes and myeloid dendritic cells at day 3 after vaccination are found by the DAMIP model to be predictors of immunogenicity, which suggests a role for receptors of the innate immune system in regulating adaptive antibody responses. It is still unknown if the best predictors of vaccine immunogenicity would emanate from innate or adaptive immune responses. It is also possible that such predictors would be identified from the analysis of immune responses occurring in tissues or at mucosal sites. The ultimate goal would be to be able to predict if a particular subject would respond to a given vaccine by doing a simple cost-effective assay, such as quantitative

PCR analysis of a drop of blood early after vaccination. The second outcome of this approach is the generation of unexpected hypotheses. This study finds that expression of the gene encoding CaMKIV, an enzyme involved in Ca²⁺ signaling, is inversely correlated with HAI titers induced by TIV at day 28. The role of CaMKIV in the regulation of antibody response has never been studied before (Racioppi and Means, 2008). The authors demonstrate that TIV induces phosphorylation of CaMKIV and further demonstrate a critical role for CaMKIV in regulating antibody responses, using mice deficient in CaMKIV.

The pioneering approach of Nakaya et al. has led to the generation of several new hypotheses that will pave the way for fundamental research focused on the identification of the mechanisms of protection induced by vaccines and will lead to the improvement of vaccination strategies (Nakaya et al., 2011). Additional studies using similar systems biology approaches to identify determinants of vaccine and adjuvant efficacy will undoubtedly result in the discovery of previously unknown biological mechanisms by which various vaccines trigger and modulate the effector and memory arms of the immune response and thus revitalize immunology. Furthermore, the identification of signatures that can be used to predict vaccine immunogenicity will be of immense value in the early identification of people who respond suboptimally to the vaccine. This in turn will accelerate the pace of clinical trials and allow the design of new vaccine strategies for incurable diseases and personalized vaccine regimens for immunocompromised populations.

Competing Financial Interests

The authors declare no competing financial interests.

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A17

SYSTEMS BIOLOGY OF VACCINATION FOR SEASONAL INFLUENZA IN HUMANS⁷⁴

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Here we have used a systems biology approach to study innate and adaptive responses to vaccination against influenza in humans during three consecutive influenza seasons. We studied healthy adults vaccinated with trivalent inactivated influenza vaccine (TIV) or live attenuated influenza vaccine (LAIV). TIV induced higher antibody titers and more plasmablasts than LAIV did. In subjects vaccinated with TIV, early molecular signatures correlated with and could be used to accurately predict later antibody titers in two independent trials. Notably, expression of the kinase CaMKIV at day 3 was inversely correlated with later antibody titers. Vaccination of CaMKIV-deficient mice with TIV induced enhanced antigen-specific antibody titers, which demonstrated an unappreciated role for CaMKIV in the regulation of antibody responses. Thus, systems approaches can be used to predict immunogenicity and provide new mechanistic insights about vaccines.

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Annual vaccination is one of the most effective methods for preventing influenza (Sasaki et al., 2007). At present, two types of vaccines for seasonal influenza are licensed for use in the USA: trivalent inactivated influenza vaccine (TIV), given by intramuscular injection; and live attenuated influenza vaccine (LAIV), administered intranasally. These vaccines contain three strains of influenza viruses that are usually changed annually on the basis of the results of global influenza surveillance data (Fiore et al., 2010). The efficacy of a vaccine against influenza, therefore, depends on the match of antigenicity between the vaccine and circulating influenza strains (Sasaki et al., 2008). Additionally, other factors such as the age and immunocompetence of vaccinees, as well as preexisting amounts of antibody derived from prior infection or vaccination, contribute to mechanisms that mediate the efficacy of vaccines against influenza (Sasaki et al., 2007; Fiore et al., 2010; Zeman et al., 2007).

Systems vaccinology has emerged as an interdisciplinary field that combines systems-wide measurements plus network and predictive modeling applied to vaccinology (Pulendran and Nakaya, 2010). A systems biology approach has been used to identify early gene signatures that correlate with and can be used to predict later immune responses in humans vaccinated with the live attenuated vaccine YF-17D against yellow fever (Querec et al., 2009; Gaucher et al., 2008). YF-17D is one of the most successful vaccines ever developed (Pulendran, 2009; Monath, 2005); it stimulates polyvalent innate immune responses (Querec et al., 2006) and adaptive immune responses (Barrett and Teuwen, 2009) that can persist for decades after vaccination (Barrett and Teuwen, 2009). Although systems biology approaches have been used to predict the immunogenicity of YF-17D (Querec et al., 2009; Gaucher et al., 2008), which is a live replicating virus, the extent to which such approaches can be applied to the prediction of the immunogenicity of inactivated vaccines is unknown. Furthermore, it remains unclear whether systems approaches can be used to predict the immunogenicity of recall responses. In the case of influenza, the immune response to vaccination is greatly enhanced by the past history of the vaccine recipient, both by prior infections and vaccinations. Notably, whether such approaches can provide insight into the immunological mechanisms of action of vaccines and help with the discovery of new correlates of protective immunity is untested. To address these issues, we did a series of clinical studies during the annual influenza seasons in 2007, 2008 and 2009, in which we vaccinated healthy young adults with TIV. Our goal was to undertake a detailed characterization of the innate and adaptive responses to vaccination with TIV to identify putative early signatures that correlated with or could be used to predict later immunogenicity and to obtain new insight into the mechanisms that underlie immunogenicity.

The results of our studies demonstrate that systems biology approaches can indeed be used to predict the immunogenicity of an inactivated vaccine such as TIV with up to 90% accuracy. Notably, the expression at day 3 of one of the genes in the predictive signature, encoding the kinase CaMKIV, was inversely correlated with plasma hemagglutination-inhibition (HAI) antibody titers at day 28. Vaccina-

tion of CaMKIV-deficient (*Camk4^{-/-}*) mice with TIV induced enhanced antigen-specific antibody titers, which demonstrated an unappreciated role for CaMKIV in the regulation of antibody responses. Together our results demonstrate the utility of systems biology not only in the prediction of vaccine immunogenicity but also in offering new insight into the molecular mechanism of influenza vaccines.

Results

Antibody Responses Induced by TIV and LAIV

We evaluated the antibody responses of 56 healthy young adults vaccinated with either LAIV ($n = 28$) or TIV ($n = 28$) during the 2008 influenza season. We determined HAI titers for each of the three influenza strains in LAIV and TIV in the plasma of vaccinees at baseline (day 0) and at 28 d after vaccination. We calculated the magnitude of antibody responses to the vaccine (HAI response) as the maximum difference between the HAI titer at day 28 and the baseline titer (day 0) for any of the three influenza strains contained in the vaccine (**Fig. A17-1a**). The mean HAI response of subjects vaccinated with TIV was sixfold higher than that of those vaccinated with LAIV (**Fig. A17-1a**), consistent with many published reports (Sasaki et al., 2007; Johnson et al., 1985; Beyer et al., 2002). Furthermore, among the subjects vaccinated with TIV, there was considerable variation in the magnitude of the HAI response (>100-fold; **Fig. A17-1a**). According to the US Food and Drug Administration Guidance for Industry document for this field (US Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research, 2007), seroconversion can be defined by an HAI titer of 1:40 or more and a minimum fourfold increase in antibody titer after vaccination. Thus, we operationally classified the vaccinees as ‘low HAI responders’ or ‘high HAI responders’ based on whether or not a fourfold increase occurred after vaccination (**Fig. A17-1a**). Most of the subjects vaccinated with TIV (22 of 28) were classified as high HAI responders; only six were classified as low HAI responders. In contrast, most subjects vaccinated with LAIV (24 of 28) were classified as low HAI responders and only four were classified as high HAI responders (**Fig. A17-1a**).

Antibodies are produced by antibody-secreting B cells in the blood (plasmablasts) or bone marrow and secondary lymphoid organs (fully differentiated plasma cells). High frequencies of antigen-specific plasmablasts in the blood within a few days of vaccination, reaching a peak at day 7, have been documented (Wrammert et al., 2008). To determine whether the early plasmablast response to influenza vaccination correlated with the later HAI response, we assessed the frequency of influenza-specific plasmablasts at baseline and 7 d after vaccination (**Fig. A17-1b,c**). As reported before (Wrammert et al., 2008), we observed rapid clonal expansion of influenza-specific plasmablasts 7 d after vaccination with TIV, as measured by enzyme-linked immunospot (ELISPOT) assay (**Fig. 1b**) and by flow cytometry (**Fig. A17-1c**). We further found that the population expansion of

circulating plasmablasts secreting immunoglobulin G (IgG) was also greater in subjects vaccinated with TIV than in those vaccinated with LAIV (**Fig. A17-1b,c**). We obtained similar results for IgA-secreting plasmablasts at day 7 after vaccination (**Supplementary Fig. 1a**), and a very good correlation was evident between the frequency of plasmablasts as measured by ELISPOT and their frequency as measured by flow cytometry (**Fig. A17-1d** and **Supplementary Fig. 1b**).

As we detected a very low HAI response after vaccination with LAIV, we considered only subjects vaccinated with TIV in further correlation analyses. There was a modest positive correlation between the number of IgG-secreting plasmablasts at day 7 and the HAI response at day 28 after vaccination (**Fig. A17-1e**). Because the frequency of plasmablasts returns to a barely detectable amount by day 14 after vaccination (Wrarmert et al., 2008), this correlation suggested that the later antibody response was associated with early circulation of plasmablasts in the blood of vaccinees (Sasaki et al., 2008). However, given the modest correlation ($r = 0.43$), there was clearly a need for more robust correlates of immunogenicity.

Molecular Signatures of Influenza Vaccines

We first determined whether TIV and LAIV induced molecular signatures that were detectable in the blood. To identify such signatures of immunogenicity, we first measured by multiplex assay the concentrations of key cytokines in the plasma of vaccinees on days 0, 3 and 7 after vaccination (**Supplementary Fig. 2a**). We selected ten cytokines or chemokines on the basis of their importance as key mediators of host immune responses (CCL5 (RANTES), interleukin 1 α , interferon- α 2 (IFN- α 2), CCL3 (MIP-1 α), CCL11 (eotaxin), interleukin 12 subunit p70, IFN- γ , interleukin 1 β , CXCL10 (IP-10) and CCL2 (MCP-1)). Among those, only the chemokine CXCL10 (IP-10) was significantly induced by TIV on day 3 relative to its expression on day 0 ($P = 0.0189$ (t -test); **Supplementary Fig. 2b**). None of those cytokines were significantly induced or repressed by vaccination with LAIV. The concentration of CXCL10 (IP-10) at day 3 relative to its baseline concentration was negatively correlated to the HAI response at day 28 after vaccination (**Supplementary Fig. 2c**), which suggested possible involvement of CXCL10 (IP-10) in the antibody response. However, the correlation coefficient was modest ($r = -0.48$), which again emphasized the need for more robust correlates of immunogenicity.

To determine in an unbiased way the expression changes induced by vaccination against influenza on a genome-wide scale, we did microarray analysis using peripheral blood mononuclear cells (PBMCs) collected from all 56 vaccinees on days 0, 3 and 7 after vaccination. We calculated the change in expression by subtracting the \log_2 expression value at day 0 from its corresponding value day 3 or 7, and we filtered out genes if we observed no increase or decrease greater than 25% (1.25-fold) in at least 20% of the vaccinees. After that step, we applied three independent statistical tests to the remaining genes and considered only genes identified by all three analyses as being differently expressed.

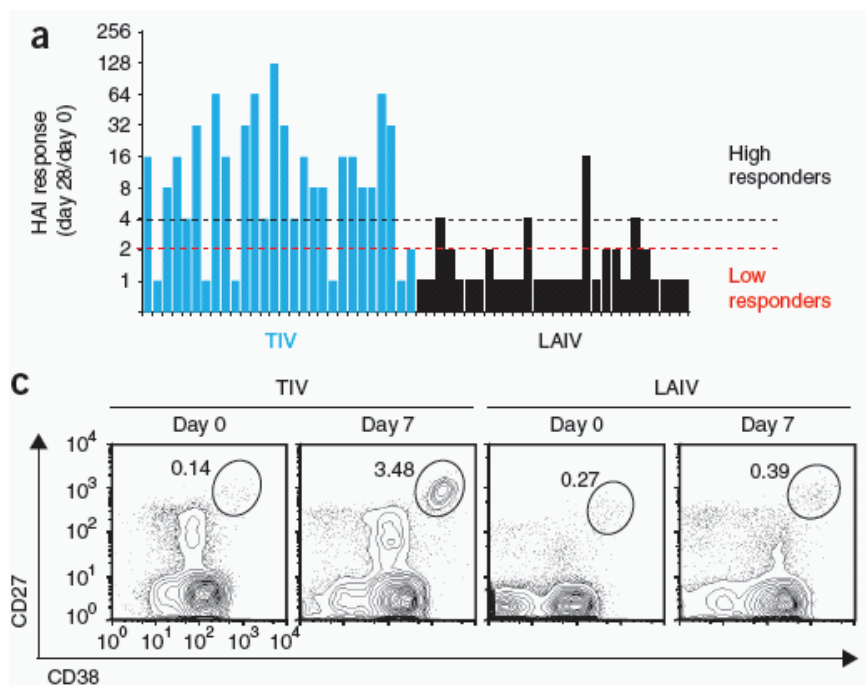
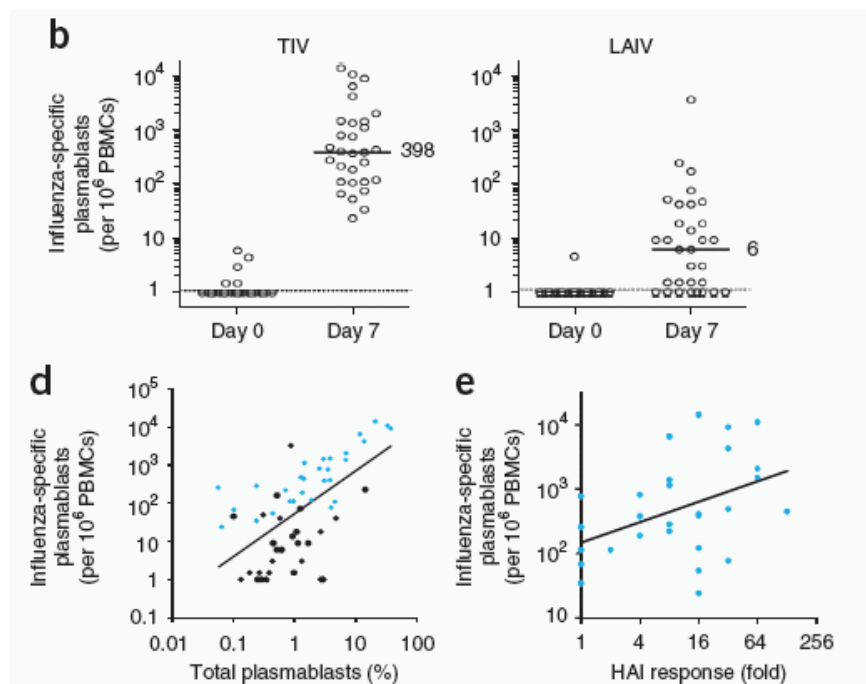


FIGURE A17-1 Analysis of humoral immunity to influenza vaccination. **(a)** HAI titers in plasma on day 28 after vaccination with TIV or LAIV, relative to baseline (day 0); results are the highest HAI response among all three influenza strains in the vaccine: low responders, no increase above twofold; high responders, fourfold or more above baseline. $P < 0.0001$, mean HAI response, TIV versus LAIV (t -test). **(b)** ELISPOT assay of influenza-specific IgG-secreting plasmablasts among PBMCs from all vaccinees at 0 and 7 d after vaccination. Each symbol represents an individual donor; small horizontal lines indicate the median (numbers adjacent median values); dotted lines are the limit of detection. **(c)** Flow cytometry analysis of plasmablasts in the plasmablast gate (CD3⁻CD20^{lo-}negCD19⁺CD27^{hi}CD38^{hi}) in blood from subjects vaccinated with TIV or LAIV. Numbers

Transcriptome analysis of vaccinees showed that LAIV and TIV induced very different gene signatures (**Supplementary Fig. 3a**). However, the expression of 1,445 probe sets was altered similarly by both vaccines (**Supplementary Fig. 3a**). Among these common ‘differentially expressed genes’ (DEGs), ingenuity pathway analysis identified a network composed of several genes related to inflammatory and antimicrobial responses (**Supplementary Fig. 3b**; complete list of DEGs after vaccination with TIV or LAIV, **Supplementary Table 1**). This indicated that processes related to innate immunity may have influenced the immunogenicity of each vaccine. The expression of several interferon-related genes was altered after vaccination with LAIV but not after vaccination with TIV



adjacent to outlined areas indicate percent cells in the plasmablast gate. (d) Frequency of plasmablasts, assessed by flow cytometry, versus the number of influenza-specific IgG-secreting plasmablasts, assessed by ELISPOT, at day 7 after vaccination with TIV (blue) or LAIV (black). $r = 0.58$ (Pearson); $P < 0.0001$ (for Pearson correlation; two-tailed test). (e) Influenza-specific IgG-secreting plasmablasts at day 7 versus the antibody response at day 28 after vaccination with TIV. $r = 0.43$ (Pearson); $P = 0.02$ (for Pearson correlation; two-tailed test). Data are from one experiment with 56 subjects assayed in duplicate (a), 61 subjects assayed in duplicate (b) or 59 subjects assayed once (c) or were generated from data in a–c (d,e).

(Fig. A17-2). Type 1 interferons are central components of the innate immune response to virus16. Therefore, the higher expression of type I interferon-related genes may be attributed to the replication competence of LAIV. Our analysis identified genes encoding molecules closely associated with the interferon signaling pathways, such as STAT1, STAT2, TLR7, IRF3 and IRF7 (Fig. A17-2a). Notably, the difference in expression for many interferon-related genes was greatest at day 3 after immunization with LAIV (Fig. A17-2a).

We also compared the gene signatures of the two influenza vaccines with that of another live attenuated vaccine, the YF-17D vaccine against yellow fever6. For consistency with that publication (Querec et al., 2009), we applied the same

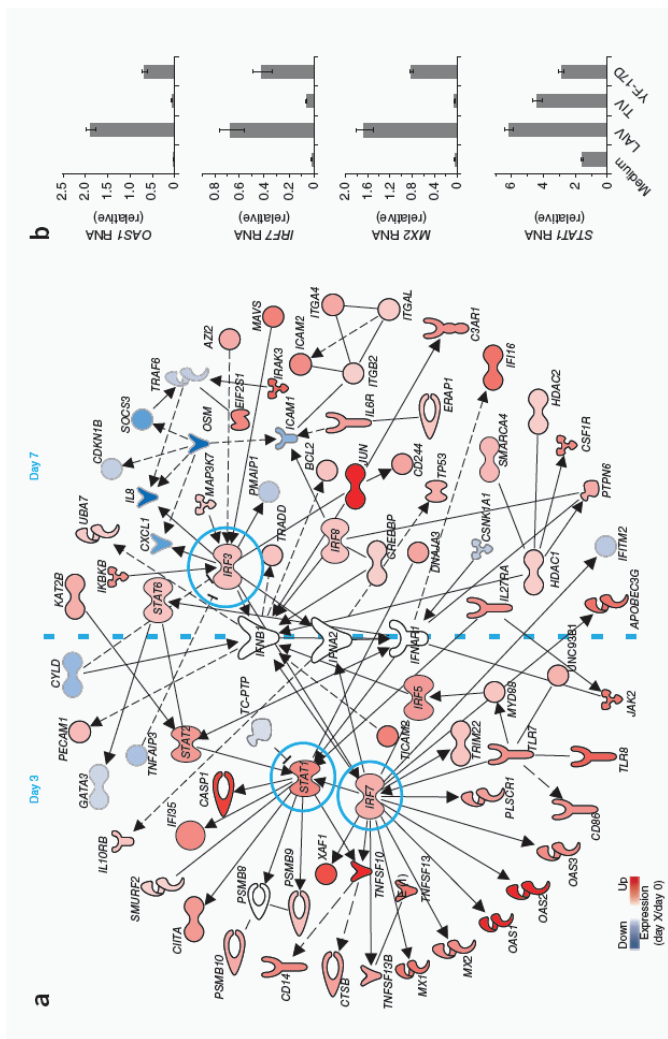


FIGURE A17-2 Molecular signature induced by vaccination with LAIV. **(a)** Interferon-related genes upregulated (Up) or downregulated (Down) on day 3 or 7 ('X' in key) after vaccination with LAIV relative to their expression at day 0 (colors in key); solid lines indicate direct interactions; dashed lines indicate indirect interactions. **(b)** Quantitative RT-PCR confirmation of the induction of key interferon-related genes (*OAS1*, *IRF7*, *MX2* and *STAT1*) in PBMCs obtained from healthy subjects and left unstimulated (Medium) or stimulated for 24 h *in vitro* with LAIV, TIV or YF-17D; results are normalized to the expression of *GAPDH* (glyceraldehyde phosphate dehydrogenase) and are presented relative to those of unstimulated PBMCs. Data are representative of one experiment **(a)** or three independent experiments with one subject each **(b)**; error bars, s.d.).

stringency and criteria to identify genes with differences in expression in subjects vaccinated with YF-17D, as follows: we filtered out genes if we found no increase or decrease in expression (on day 3 or 7 relative to baseline) greater than 1.41-fold in at least 60% of the vaccinees; we used one-way analysis of variance with the Benjamini and Hochberg false-discovery-rate method with a cutoff of 0.05; and genes had to have a difference in expression in both YF-17D trials (Querec et al., 2009). However, this time we did the analysis at the level of the probe set instead of defining genes based on the UniGene database (National Center for Biotechnology Information). Although subjects vaccinated with YF-17D had a gene-expression profile distinct from that of those vaccinated against influenza, many interferon-related genes were commonly induced by YF-17D and LAIV (data not shown). RT-PCR analysis of RNA from PBMCs stimulated *in vitro* with LAIV, TIV or YF-17D confirmed that interferon-related genes were upregulated 24 h after treatment with LAIV or YF-17D but not after stimulation with TIV (**Fig. A17-2b**). Together these data demonstrated that vaccination with TIV or LAIV induced distinct molecular signatures in the blood.

Molecular Signatures of Sorted Cell Subsets

We did microarray analyses of the gene-expression profiles of PBMCs isolated from the blood of vaccinees at baseline and at days 3 and 7 after vaccination. One confounding variable here was that the observed transcriptional changes may have resulted from new induction of gene expression or may have simply reflected the changing cellular composition of the PBMC compartment. To overcome this issue, we used the approach of isolating and identifying the genomic signatures of each subset in the PBMC pool. We did microarray experiments with the following four different cell types, obtained from subjects vaccinated with LAIV ($n = 6$) or TIV ($n = 6$) and sorted by flow cytometry: CD19⁺ B cells, CD14⁺ monocytes, CD11c^{hi}CD123^{lo} myeloid dendritic cells (DCs) and CD123^{hi} CD11c^{lo} plasmacytoid DCs. We extracted, amplified and labeled total RNA from 96 sorted cell samples at baseline and day 7 and hybridized the RNA on microarray chips (**Supplementary Fig. 4a**). We did significance analysis of microarrays (Tusher et al., 2001) for each subset, separately comparing the values at day 7 with the corresponding baseline values. This approach identified hundreds to thousands of probe sets with differences in expression after vaccination with TIV or LAIV (**Supplementary Fig. 4b** and **Supplementary Table 2**), which demonstrated that vaccines against influenza produced global expression changes for each cell type.

In subjects vaccinated with TIV, myeloid DCs and B cells had the most DEGs (**Supplementary Fig. 4b**). Notably, there was an enrichment for DEGs associated with plasmablasts (**Supplementary Fig. 4c**). However, because a substantial proportion of plasmablasts die after being frozen and thawed (data not shown), the DEGs observed in the B cell compartment were probably an underestimation of the DEGs associated with plasmablasts (**Fig. A17-3**). Nevertheless,

we were still able to identify DEGs related to antibody-secreting cells (ASCs) and the unfolded protein response in sorted B cells after immunization with TIV (**Supplementary Fig. 4c,d**). To cope with the large amount of immunoglobulin proteins that are produced, ASCs must greatly increase the function of their secretion machinery, which may lead to the accumulation of misfolded proteins in the endoplasmic reticulum (Iwakoshi et al., 2003a,b). In response to such stress, the cells activate intracellular signal-transduction pathways and the unfolded protein response, which protects the cells by enhancing the capacity of the secretory apparatus and by diminishing the endoplasmic reticulum load (Ron and Walter, 2007). After vaccination with TIV, upregulation of genes encoding two transcription factors, XBP-1 and ATF6B, which are central orchestrators of the unfolded protein response, was detectable in sorted B cells but not in PBMCs (**Supplementary Fig. 4d**).

In subjects vaccinated with LAIV, in contrast to results obtained with those vaccinated with TIV, the plasmacytoid DC subset generated the most DEGs (**Supplementary Fig. 4b**). Of the many interferon-related genes induced by LAIV (**Fig. A17-2a**), we found that 37 were induced in at least one subset of the sorted cells. Of those, 17 and 14 were upregulated in monocytes and plasmacytoid DCs, respectively (**Supplementary Fig. 4e**). In addition, there were 44 interferon-related genes that were induced or repressed in at least one subset of the sorted cells but not in the PBMCs (**Supplementary Fig. 4e**). Most were upregulated in myeloid and plasmacytoid DCs (**Supplementary Fig. 4e**). These data suggest that antigen-presenting cells may be important in the innate response to vaccination with LAIV. The large number of interferon-related genes ‘missing’ from the PBMC analysis may have been due to the fact that myeloid DCs and plasmacytoid DCs together represent <1% of total PBMCs (Ueda et al., 2003).

The observations reported here indicated the type of information that can be obtained by examination of the gene-expression profiles of sorted cell types. However, evaluating the gene-expression signatures of individual subsets of cells isolated by flow cytometry presents a considerable challenge. The practical use of such an approach is very limited, both logistically (that is, the need to use freshly isolated samples to prevent the ‘preferential’ loss of certain cell types, such as plasmablasts and effector T cells) and financially (that is, the need for large numbers of gene chips). Therefore, as described below, we devised an alternative strategy.

Meta-Analysis of Cell Type-Specific Signatures

Human PBMCs consist of many different cell types, each with a distinct transcriptome. A published study has demonstrated the use of a deconvolution method to analyze cell type-specific gene expression differences in complex tissues (Shen-Orr et al., 2010). We devised an independent strategy to discern cell type-specific transcriptional signatures with the results of the PBMC microarray analyses. We did a meta-analysis of publicly available microarray studies in

which the gene-expression profiles of isolated individual cell types of PBMCs (such as T cells, B cells, monocytes, natural killer cells and so on) or B cell subsets (such as naive, memory and germinal center B cells and ASCs from blood or tonsils) had been analyzed (**Supplementary Fig. 5a,b**). To avoid issues of cross-platform normalization and probe selection, we used only samples hybridized to Affymetrix Human Genome U133 Plus 2.0 Arrays or Affymetrix Human Genome U133A Arrays in our meta-analysis. Additionally, for each study, we manually removed samples based on the severity of the disease or treatment and/or the method of cell purification (samples and studies, **Supplementary Table 3**). We included in our meta-analysis microarray data of flow cytometry–sorted plasmacytoid and myeloid DCs obtained from PBMCs of subjects before and after vaccination with TIV or LAIV (**Supplementary Fig. 4a**). We compared the expression profile of a given cell subset with the expression profile of all other subsets by *t*-test ($P < 0.05$; mean change, over twofold). We designated a gene as having high expression in a particular cell type by determining the number of times the gene was upregulated in the cell type by all possible pairwise comparisons with its expression in other cell types (**Supplementary Fig. 5b** and **Supplementary Methods**). We then compared the genomic signatures of cells of the immune response obtained by this approach (**Fig. A17-3a** and **Supplementary Table 4**) with the genomic signatures of subjects vaccinated against influenza.

Our meta-analysis confirmed that the group of genes upregulated by TIV was enriched for genes with high expression in B cells (**Fig. 3b**) and, among those, genes with high expression in ASCs (**Fig. A17-3c**). We prepared a heat map of the genes upregulated in ASCs after vaccination with TIV (**Fig. A17-3d**). Among the genes upregulated were those encoding ‘antibody parts’ (rearranged variable-diversity-joining immunoglobulin gene segments) and several other genes encoding parts of immunoglobulins (*IGH@*, *IGHE*, *IGHG3*, *IGHG1* and *IGHD*), as well as *TNFRSF17* (which encodes BCMA, a member of the BAFF-BLyS family of receptors (Avery et al., 2003), and whose expression has been shown before to be a key feature of the best predictive signatures of neutralizing antibody responses to YF-17D6). These results confirmed the results obtained by flow cytometry and ELISPOT, with which we observed a greater frequency of IgG⁺ and IgA⁺ ASCs in the blood of vaccinees at day 7 after vaccination with TIV (**Fig. A17-1** and **Supplementary Fig. 1**).

In addition to the ASC signature, we observed a signature composed of several genes encoding molecules that orchestrate the unfolded protein response (Iwakoshi et al., 2003b; Ron and Walter, 2007) (data not shown). The large number of XBP-1 target genes with differences in expression after vaccination was consistent with a role for XBP-1 in orchestrating the differentiation of plasma cells (Iwakoshi et al., 2003b). Among those, genes such as *ATF6*, *MANF*, *CREB3*, *PDIA4*, *DNAJB11*, *HSP90B1*, *HERPUD1* and *DNAJB9* encode molecules that are already known to be involved in the unfolded protein response (Park et al., 2010; Liu and Li, 2008; Apostolou et al., 2008).

In contrast to results obtained for TIV, analysis of the transcriptional signature induced by LAIV by meta-analysis showed considerable enrichment for genes with high expression in T cells and monocytes (**Fig. A17-3e**). We also found many genes with high expression in natural killer cells, although these results did not reach statistical significance (data not shown). Among the interferon-related genes upregulated after vaccination with LAIV (**Fig. A17-2a**), most had high expression in monocytes and natural killer cells (data not shown). That result was similar to our microarray analysis of flow cytometry–sorted cells obtained from subjects vaccinated with LAIV, in which most interferon-related genes with differences in expression in PBMCs and at least one cell subset had high expression in monocytes (**Supplementary Fig. 4e**). These results indicate that the innate immune responses can have an important role in the mechanism of action of this live attenuated virus vaccine.

Signatures That Correlate With the Antibody Response

Vaccination with TIV induced considerable variation in the magnitude of the HAI response (**Fig. A17-1a**). To gain insight into the potential mechanisms underlying that variation and to identify gene signatures with which we could predict the magnitude of the HAI response, we searched for early gene signatures that correlated with the B cell responses at days 7 and 28 after vaccination with TIV (complete list, **Supplementary Table 5**). Pearson correlation analysis identified 600–1,100 probe sets that correlated, either directly or inversely, with the magnitude of the HAI response (**Fig. A17-4a**). Among those were several genes known to be regulated by XBP-1 and to be involved in the differentiation of plasma cells and the unfolded protein response (**Fig. A17-4b**).

Ingenuity pathway analysis of the genes that were either positively or negatively correlated with HAI titers showed enrichment for genes related to the cell-mediated immune response and to the infection mechanism and inflammatory response, respectively (**Supplementary Fig. 6a,b**). The identification of genes such as *TLR5*, *CASP1*, *PYCARD*, *NOD2* and *NAIP* suggested previously unknown mechanistic links between host innate immunity and humoral responses to influenza vaccination. In fact, research has shown that a candidate vaccine against influenza composed of a recombinant fusion protein linking influenza antigens to the Toll-like receptor 5 ligand flagellin may induce potent immunogenicity in mice (Huleatt et al., 2008) and humans (Treanor et al., 2010). In addition, canonical pathways, such as T cell receptor antigen receptor signaling and CTLA-4 signaling in cytotoxic T lymphocytes, included many of the genes present in the cell-mediated immune response network and were among those with the highest enrichment score by ingenuity pathway analysis (**Supplementary Fig. 7a,b**). Although further experimentation is needed, these data indicated a possible association between cellular responses and humoral responses to vaccination with TIV (He et al., 2006). Among the top canonical pathways enriched

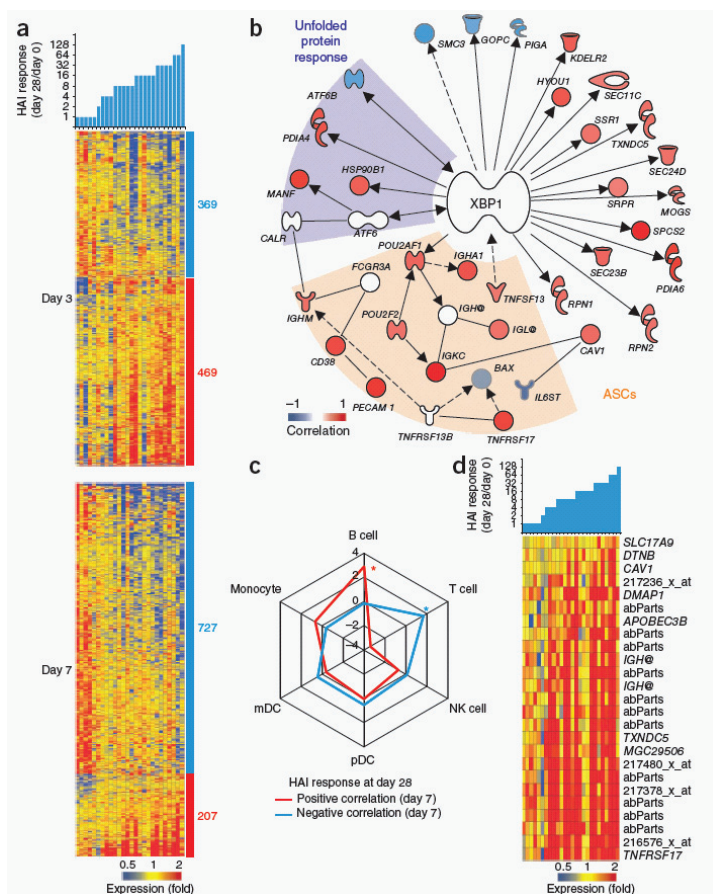


FIGURE A17-4 Molecular signatures that correlate with titers of antibody to TIV. (a) Heat map of probe sets (rows) and subjects (columns) whose baseline-normalized expression at day 3 (top) or day 7 (bottom) correlated with baseline-normalized antibody response at day 28 after vaccination with TIV (colors in map indicate gene expression at day 3 or 7 relative to expression at day 0). Right margin, number of probe sets with negative correlation (blue) or positive correlation (red). Probe sets that correlated with the HAI response on both day 3 and day 7 were considered ‘day 7’. $P < 0.05$ (Pearson). (b) HAI response–correlated genes associated with the unfolded protein response (purple shading) or ASC differentiation (tan shading) and/or regulated by XBP-1 (solid and dashed lines as in **Fig. A17-2a**). $P < 0.05$ (Pearson). (c) Enrichment for genes (among those with high expression in any PBMC subset) whose expression on day 3 or 7 after vaccination with TIV was positively or negatively correlated with HAI titers (cutoff, $P < 0.05$ (Pearson)). $*P < 10^{-10}$ (two-tailed Fisher’s exact test). (d) Heat map of probe sets with high expression in B cells and ASCs whose baseline-normalized expression correlated with the baseline-normalized HAI response. $P < 0.05$ (Pearson). Data are representative of one experiment with 28 subjects.

for genes positively correlated to HAI response (**Supplementary Fig. 7c**), we found networks associated with innate immunity, such as the natural killer cell signaling network, and network for the production of nitric oxide and reactive oxygen species in macrophages (**Supplementary Fig. 7d**). Our analysis also showed that the expression of interferon-related genes (including those encoding the receptors for interferon- α and interferon- γ) on day 3 after vaccination was correlated to the HAI response (**Supplementary Fig. 8**), which suggested a link between the interferon response and the antibody response (Le Bon et al., 2006).

Next we compared the genes whose expression correlated with the HAI response at day 28 after vaccination of subjects with TIV with the genomic signatures of the cells of the immune response defined by our meta-analysis. This approach showed that the set of genes positively correlated to HAI response was enriched for genes with high expression in B cells (**Fig. A17-4c**) and, more specifically, in the ASC subset (**Fig. A17-4d**). The genes with negative correlation to the HAI response were substantially enriched among the genes with high expression in T cells (**Fig. A17-4c**), which supported the identification of the T cell pathways by ingenuity pathway analysis (**Supplementary Fig. 7a,b**). Together these data demonstrated the identification of early signatures that correlated with later HAI titers induced by TIV.

Molecular Signatures to Predict Antibody Responses

Once we had delineated signatures that correlated with the magnitude of HAI response, our next step was to identify the minimum sets of genes we could use to predict such a response. Ideally, such sets of genes must be able to be used to accurately classify high responders versus low responders in additional and independent TIV trials. For this, we used DAMIP (discriminant analysis via mixed integer programming [Lee, 2007; Brooks and Lee, 2010]), which is a very powerful supervised-learning classification method for predicting various biomedical and ‘biobehavioral’ phenomena (Lee, 2007; Brooks and Lee, 2010).

In initial analyses, we classified the subjects vaccinated with TIV into two ‘extreme’ groups: very low HAI responders, and very high HAI responders. The former group consisted of subjects with an increase of twofold or less in HAI titers against any of the three influenza strains of the vaccine (**Fig. A17-1a**). The latter group consisted of subjects with an increase of eightfold or more in the HAI response for at least one of the three influenza strains of the vaccine. We did not analyze subjects with intermediate HAI response (between twofold and eightfold) and subjects for whom microarray data were not available at either day 3 or day 7 after vaccination ($n = 7$). We used that trial (the 2008–2009 trial) to train the DAMIP model to establish an unbiased estimate of correct classification. We used a second, independent trial to evaluate the predictive accuracy of the classification rules identified in the first trial (**Fig. A17-5a**). The second trial (the 2007–2008 trial) consisted of the microarray gene-expression profiles of subjects

($n = 9$) vaccinated with TIV in the previous year. With this approach, DAMIP model identified 12 sets of genes containing two to four genes each (each set associates with one predictive rule) from 2008–2009 trial with a tenfold cross-validation accuracy over 90%. The resulting ‘blind prediction’ accuracy of the 2007–2008 trial (predicting low or high responders) was over 90%. Furthermore, some of the 271 sets of discriminatory genes offered an accuracy of over 90% in both tenfold cross-validation in the training trial and ‘blind prediction’ accuracy (**Fig. A17-5a** and **Supplementary Table 6**).

We then used real-time RT-PCR to confirm that 44 genes from the DAMIP gene signatures encoded molecules with potential biological relevance and/or utility as a predictor of influenza vaccine immunogenicity. We found a significant positive correlation ($r = 0.679$; $P = 3.25 \times 10^{-12}$) for changes in expression on day 3 or 7 relative to baseline expression as detected by microarray and RT-PCR (**Fig. A17-5b** and **Supplementary Table 7**), which confirmed the correctness of the microarray data. More notably, that result gave us confidence to test some of the candidate predictors of immunogenicity in a third and independent influenza vaccine trial (**Fig. A17-5a**). We collected RNA from PBMCs of subjects ($n = 30$) vaccinated with TIV during the 2009–2010 influenza season and analyzed this RNA by real-time RT-PCR. We then used the expression of the 44 genes selected from the initial DAMIP gene signatures to confirm their utility in predicting the magnitude of antibody response in this third TIV trial (**Fig. A17-5a**). To avoid the identification of ‘over-trained’ rules, we re-ran the DAMIP analysis using the 2008–2009 trial as the training set and the 2007–2008 and 2009–2010 trials as the blind predictive sets. This approach identified 47 sets of genes; some of these we used to correctly classify >85% of the vaccines as being very low HAI responders or very high HAI responders in any of the three trials (**Supplementary Table 8**).

Because seroconversion after vaccination is widely defined as a fourfold increase in HAI titers (Sullivan et al., 2010), we ran an additional DAMIP analysis using a cutoff of fourfold to classify the vaccinees (**Fig. A17-5a**). Thus, we classified subjects with an increase of fourfold or greater in the HAI titers after vaccination as ‘high responders’ and those with an increase of twofold or less as ‘low responders’. With the 2008–2009 trial as a training set and 2007–2008 and 2009–2010 trials as blind predictive sets, the DAMIP model generated 42 sets of gene signatures (**Fig. A17-5c**), each composed of three to four discriminatory genes, some of which had an unbiased estimate of correct classification above 85%, as determined by tenfold cross-validation and blind prediction (**Supplementary Table 9**).

One of the genes present in the DAMIP gene signatures, *TNFRSF17*, was also identified in DAMIP models used to predict antibody responses to vaccination with YF-17D6.

Among the genes in the TIV DAMIP models, we found five members of the leukocyte immunoglobulin-like receptor family (**Supplementary Table 9**). These genes are expressed by immune-response cells of both myeloid and lymphoid lineages and the molecules they encode are thought to have an immunomodulatory

role in the innate and adaptive immune systems by regulating T cells and autoimmunity (Anderson and Allen, 2009; Thomas et al., 2010; Brown et al., 2004). Our meta-analysis showed that these genes had high expression in monocytes and myeloid DCs at day 3 after vaccination (data not shown). These results and the presence of five members of this family among markers of antibody responses to influenza vaccination raised the possibility of previously unknown roles for these innate immune receptors in regulating antibody responses.

CaMKIV Regulates the Antibody Response

To demonstrate that the gene signatures identified in our study could be used to generate new hypotheses, we selected one gene in the predictive signature, *CAMK4*, for functional confirmation experiments. CaMKIV is involved in several processes of the immune system, such as T cell development (Krebs et al., 1997; Wang et al., 2001; Anderson and Means, 2002), inflammatory responses (Illario et al., 2008; Sato et al., 2006) and the maintenance of hematopoietic stem cells (Kitsos et al., 2005). However, nothing is known about the possible role of CaMKIV in B cell responses.

The change in *CAMK4* expression on day 3 after vaccination with TIV was negatively correlated with the antibody response on day 28 after vaccination in two independent trials (**Fig. A17-6a**). Additionally, the change in *CAMK4* expression was negatively correlated with the population expansion of IgG-secreting plasmablasts at day 7 (**Fig. A17-6b**), which suggested a possible role for CaMKIV in the regulation of antibody responses to vaccination against influenza.

In vitro stimulation of mouse splenocytes with TIV resulted in phosphorylation of CaMKIV (**Fig. A17-6c**), which suggested that this vaccine may trigger activation of CaMKIV. That finding was further demonstrated in human PBMCs, in which *in vitro* stimulation with influenza vaccine resulted in phosphorylation of CaMKIV as early as 2 h after stimulation (**Fig. A17-6d**). The mechanism by which this occurs remains to be identified.

To check if CaMKIV regulates the antibody response to influenza vaccine, we immunized wild-type and *Camk4*^{-/-} mice with TIV and measured serum concentrations of IgG1 and IgG2c on days 7, 14 and 28 after vaccination (**Fig. A17-6e**). After immunization, *Camk4*^{-/-} mice had a significantly greater antibody response than that of wild-type mice (**Fig. A17-6e**). The biggest difference was on day 7, with 3- to 6.5-fold higher antibody titers in *Camk4*^{-/-} mice than in wild-type mice (**Fig. A17-6e**). These results supported our prediction based on the microarray results and suggested that CaMKIV is important in the regulation of B cell response.

Discussion

Despite the great success of vaccines, little is understood about the mechanisms by which effective vaccines stimulate protective immune responses. Two

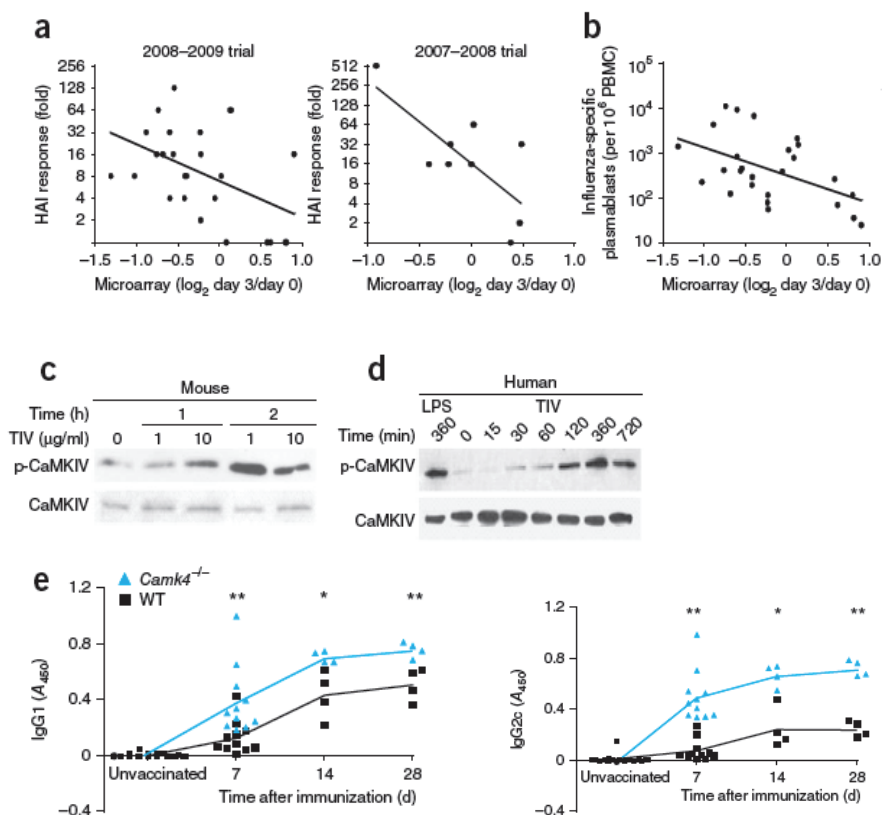


FIGURE A17-6 CaMKIV regulates the antibody response to vaccines against influenza. (a) HAI response at day 28 versus microarray analysis of *CAMK4* mRNA in PBMCs at day 3 after vaccination with TIV in the 2008–2009 trial (left; $r = -0.47$ (Pearson); $P = 0.016$ (for Pearson correlation; two-tailed test) or the 2007–2008 trial (right; $r = -0.73$ (Pearson); $P = 0.024$ (for Pearson correlation; two-tailed test)). (b) ELISPOT analysis of influenza-specific IgG-secreting plasmablasts at day 7 versus microarray analysis of *CAMK4* mRNA on PBMCs at day 3 after vaccination with TIV. (c) Immunoblot analysis of the phosphorylation (p-) of mouse CaMKIV after *in vitro* stimulation of splenocytes for 1 or 2 h with various doses of TIV (above lanes). (d) Immunoblot analysis of the phosphorylation of CaMKIV after *in vitro* stimulation of human PBMCs for 0–720 min with lipopolysaccharide (LPS) or TIV. (e) Serum antigen-specific IgG1 (top) and IgG2c (bottom) responses of wild-type and *Camk4*^{-/-} mice at days 7, 14 and 28 after immunization with TIV (symbols represent individual mice), presented as absorption at 450 nm (A_{450}). * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test). Data are representative of one trial each with 26 subjects (2008–2009) or 9 subjects (2007–2008); (a), one experiment with 26 subjects (b), three experiments (c,d) or at least four independent experiments (e).

developments are beginning to offer such understanding: increasing appreciation of the key roles of the innate immune system in sensing vaccines and tuning immune responses, and emerging advances in systems biology (Pulendran and Ahmed, 2011). A systems biology approach has been used to obtain a global picture of the immune responses in humans to vaccine YF-17D against yellow fever, one of the most successful vaccines ever developed. This approach has identified unique biomarkers (molecular signatures) used to predict the magnitude of the antigen-specific CD8⁺ T cell and antibody responses induced by YF-17D6,7 and has resulted in the formulation of new hypotheses about the mechanism of action of this vaccine. However, whether such an approach could have broad utility in the identification of signatures of immunogenicity of other kinds of vaccines, particularly inactivated vaccines, and whether such signatures would be informative about the underlying mechanisms of immunity remain unknown. To address these issues, we did a series of studies over three consecutive influenza seasons. The goal of these studies was to analyze in detail the innate and adaptive immune responses to vaccination with two vaccines against influenza, TIV and LAIV, to identify early molecular signatures that can be used to predict later immune responses and to obtain insight into the mechanisms that underlie immunogenicity. According to guidelines established by the US Food and Drug Administration (US Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research, 2007), seroconversion can be defined as an HAI titer of 1:40 or more and a minimum increase of fourfold in antibody titer after vaccination. However, it often takes several weeks after vaccination to achieve this titer; therefore, the ability to predict seroconversion just a few days after vaccination and identify nonresponders would be of great value from a public health perspective. We thus used systems biology approaches to identify early signatures that we used to predict HAI titers 4 weeks after vaccination. To accomplish this goal, we used an interdisciplinary approach, including gene-expression profiling by microarray, RT-PCR and computational methods, combined with cellular and molecular biological approaches, as well as experiments involving genetically deficient mice. Our data have demonstrated that such a systems biology approach can indeed be used not only to identify predictive signatures but also to obtain new insights about the immunological mechanisms involved.

Although the clinical effectiveness of both vaccines is similar, LAIV induces lower serum antibody response in adults than does TIV (Sasaki et al., 2007, 2008; Moldoveanu et al., 1995). This probably reflects the lower ‘take’ of LAIV because of preexisting mucosal IgA that can neutralize the virus (Beyer et al., 2002). Nevertheless, our microarray analysis identified a large number of genes with differences in expression, most related to the type I interferon response, in the PBMCs of subjects vaccinated with LAIV. Future studies should focus on analyzing changes in the transcriptome of the nasal mucosa after vaccination with LAIV and how that correlates with or can be used to predict local antibody responses.

Among the genes induced by vaccination with TIV, we found enrichment

for genes with high expression in ASCs. This result may have reflected the rapid proliferation of plasmablasts at day 7 after vaccination (Wrarmert et al., 2008); however, our microarray analysis of B cells sorted from subjects vaccinated against influenza indicated that the changes in expression observed in PBMCs could also have been derived from real transcriptional changes in B cells. The transcription factor XBP-1, which is essential for the differentiation of ASCs and the unfolded protein response (Iwakoshi et al., 2003a), and its target genes were upregulated after vaccination with TIV and correlated with IgG and HAI responses. The genes identified by our study may offer new opportunities for studying the complex mechanisms involved in the unfolded protein response and its link to ASC differentiation (Iwakoshi et al., 2003a).

A key question was whether the signatures that can be used to predict the T cell and B cell response to one vaccine can also be used to predict such responses to another vaccine. Notably, of the 133 genes present in the 271 DAMIP gene signatures that we used to predict the antibody response to vaccination with TIV, 7 were also predictors of the antibody response to vaccination with the YF-17D vaccine against yellow fever⁶. Key genes in the predictive signatures were *TNFRSF17*, which encodes BCMA, a receptor for the B cell growth factor BlyS (known to have a key role in B cell differentiation; Avery et al., 2003), and *CD38*, which encodes a surface protein important in lymphocyte development (Shubinsky and Schlesinger, 1997; Deaglio et al., 2001). BCMA belongs to a family of molecules (BAFF, APRIL, BAFF-R and TACI) that regulate the differentiation of plasma cells and antibody production (Avery et al., 2003). Notably, there were strong correlations between the expression of genes encoding APRIL, BAFF-R and TACI and the magnitude of the HAI titers in response to vaccines against influenza and the magnitude of neutralizing antibody response to YF-17D (data not shown), which suggested that this network may be critically involved in regulating antibody responses to different vaccines. The functional relevance of this network in mouse models remains to be determined. It also remains to be seen whether this network represents a common predictor of antibody responses induced by many vaccines.

A second issue was whether the data generated from such studies would be useful in providing new biological insights into the regulatory mechanisms that underlie vaccine immunogenicity. Our experiments with *Camk4*^{-/-} mice demonstrated that such data can indeed identify unexpected biological targets, which can be mechanistically confirmed by mouse models. Although the data demonstrated a potent role for CaMK4 in regulating antibody responses to vaccines against influenza, further work is needed to delineate the cellular mechanisms involved.

Third, whether signatures that can be used to predict immunogenicity can also be used to predict efficacy must be considered. Several studies have shown that serum HAI antibody concentrations correlate with protection against influenza (Clements et al., 1986; Potter and Oxford, 1979; Hirota et al., 1997). Seroconversion after vaccination, commonly defined as an increase of fourfold in HAI titers (Sullivan et al., 2010), represents a useful surrogate for vaccine ef-

ficacy when applied to a population. However, this parameter may not provide the optimal prediction of protection in an individual vaccinee or a group of vaccinees. In addition, protective concentrations of antibody may vary according to the prevalent virus subtype and laboratory doing the assay (Belshe, 2004). Therefore, we used a more stringent parameter (an increase of eightfold or more in HAI response) to classify subjects with very high antibody responses. Using this cutoff in our analyses, the DAMIP method was able to identify gene signatures that we could use to predict the antibody response induced by vaccination with TIV. We confirmed the validity of these gene signatures in three independent trials, which demonstrated the robustness of our approach. To meet the definition of seroconversion in the US Food and Drug Administration Guidance for Industry document for this field (an HAI titer of 1:40 or more and a minimum increase of fourfold in antibody titer after vaccination) (US Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research, 2007), we reran the DAMIP analysis using an increase of fourfold as a cutoff for defining high HAI responders. Again, the DAMIP method was able to identify sets of three to four discriminatory genes with an unbiased estimate of correct classification up to 90% for the three influenza trials. However, the generality of our findings in terms of using gene signatures in PBMCs to predict the immunogenicity and/or efficacy of other vaccines such as mucosal vaccines must be tested. It is likely that different signatures could be generated by analysis of mucosal tissues.

Finally, although the main goal of our study was a proof-of-concept demonstration of the feasibility of this approach in predicting vaccine immunogenicity, (rather than a demonstration of cost effectiveness), in ascertaining the predictive value of our signature in the 2009–2010 trial, we used a PCR-based assay (instead of an assay with gene-expression chips) of only a handful of genes. This demonstrated the feasibility of designing a cost-effective, PCR-based ‘vaccine chip’ that can be used to predict the immunogenicity of vaccines. Thus, we have shown how systems biology approaches can be applied to elucidate the molecular mechanisms of influenza vaccines. We envision that the predictive signatures of influenza vaccine-induced antibody responses may have implications in vaccine development, in the monitoring of suboptimal immune responses (in the elderly, infants or immunocompromised populations) or perhaps in identifying new correlates of protection.

Methods

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession codes. *GEO: microarray data, GSE29619.*

Note: Supplementary information is available on the Nature Immunology website.

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Author Contributions

H.I.N. did all the experiments and analyses in **Figures A17-2–6** and **Supplementary Figures 2–8**; J.W., G.-M.L., M.M. and V.K. did the analyses in **Figure A17-1** and **Supplementary Figure 1**; E.K.L. did the DAMIP model analyses in **Figure A17-5**; L.R., A.R.M., S.P.K. and N.K. did the mouse experiments in **Figure A17-6**; W.N.H. helped with the microarray analyses in **Supplementary Figure 4**; S.L. assisted with the bioinformatics analyses of the data in **Figure A17-3**; A.A. did the microarray analysis of samples from the 2007 influenza annual season; S.M.-K., K.E.K., R.E. and A.K.M. assisted with the collection and processing of samples; K.S. measured HAI titers; R.A. helped conceive of and design the study and supervised the studies in **Figure A17-1** and **Supplementary Figure 1**; B.P. conceived of the study and designed and supervised the experiments and analyses in **Figures A17-1–6** and **Supplementary Figures 1–8**; and B.P. and H.I.N. wrote the paper.

Competing Financial Interests

The authors declare no competing financial interests.

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A18

**SYNTHETIC SYSTEMS AS MICROBIAL THREATS:
PREDICTABILITY OF LOSS-OF-FUNCTION
MUTATIONS IN ENGINEERED SYSTEMS**

Sean C. Sleight,⁸⁷ Bryan A. Bartley, and Herbert M. Sauro⁸⁸

Abstract

One problem with engineered genetic circuits and metabolic pathways in synthetic microbes is their stability over evolutionary time in the absence of selective pressure. We recently measured the evolutionary stability dynamics and determined the loss-of-function mutations for a wide variety of BioBrick-assembled genetic circuits in order to develop simple design principles for engineering mutationally robust genetic circuits (Sleight *et al.*, 2010b). In this report, we focus on experiments performed with the LuxR receiver device, T9002, and reengineered versions of this circuit. T9002 loses function in fewer than 20 generations and the mutation that repeatedly causes its loss of function is a deletion between two homologous transcriptional terminators. To measure the effect between transcriptional terminator homology levels and evolutionary stability, we reengineered six versions of T9002 with a different transcriptional terminator at the end of the circuit. When there is no homology between terminators, the evolutionary half-life of this circuit is significantly improved by more than twofold and is independent of the expression level. Removing all homology between terminators and decreasing expression level fourfold increases the evolutionary half-life over 17-fold. We also investigated the predictability of loss-of-function mutations between nine replicate evolved populations. T9002 circuits reengineered to have no sequence similarity between terminators effectively removes a certain class of mutations from occurring, where the most common loss-of-function mutation is a deletion between 8-base pair (bp) scar sequences to remove the *luxR* promoter. Finally, we evolved T9002 and a reengineered version in media with different levels of inducer to measure the relationship between expression level and evolu-

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tionary stability. The results show that on average, like other circuits we studied, evolutionary half-life exponentially decreases with increasing expression levels.

Introduction

Experimental evolution is a powerful system for studying evolution in the laboratory over short or long timescales (Elena and Lenski, 2003). For these experiments, normally bacteria or other microbes are propagated over multiple generations in certain environmental conditions to understand the phenotypic and genetic differences between evolved strains and their progenitors (Herring et al., 2006; Riehle et al., 2005; Sleight and Lenski, 2007). Experimental evolution experiments also allow for the study of parallel genetic or phenotypic changes between replicate evolved populations. When multiple evolved populations converge on a similar phenotype, it is often a strong indicator of evolutionary adaptation, as opposed to the product of genetic drift (Bull et al., 1997; Colosimo et al., 2005; Sleight et al., 2008). Understanding convergent evolution is of interest because it increases our ability to predict the evolution of unknown strains in different environments.

Synthetic biologists use engineering principles to design and construct new organisms that do not exist in nature (Endy, 2005). Often these synthetic organisms are programmed with genetic circuits that are constructed from biological parts such as promoters, ribosome binding sites, coding sequences, and transcriptional terminators called BioBricks (Endy, 2005; Shetty et al., 2008). The MIT Registry of Standard Biological Parts (called “the Registry” from here on) maintains over 3,000 BioBricks encoded on plasmids that are available to researchers with a wide variety of functions, from bacterial photography, to quorum sensing, to odor production and sensing. BioBricks are widely available for the design of more complex systems but in general are not well characterized (Canton et al., 2008; Endy, 2005). The most well characterized part to date is a cell-to-cell communication receiver device (Canton et al., 2008), which was provided with a published prototype “biological part data sheet” containing information engineers would need to use it in their own designs. One of the figures in this data sheet describes the reliability of this circuit over evolutionary time. Connecting the receiver device to a green fluorescent protein (GFP)-reporter device causes this circuit to repeatedly lose function in fewer than 100 generations due to a deletion mutation between transcriptional terminators that are repeated in both the receiver and reporter devices.

From a synthetic biologist’s perspective, ideally synthetic organisms will be robust to environmental conditions and mutations to allow for optimal function over evolutionary time. However, the genetic circuits encoded on plasmids in synthetic organisms are often unstable over evolutionary time if there is no selective pressure to maintain function of the circuit. This loss of function occurs because a cell in the population that has a mutation in one of its plasmids may have a slight growth advantage. When this cell divides, the daughter cell may acquire

more copies of the mutant plasmid, giving it a greater growth advantage. Over multiple generations, the functional cells in the population may be outcompeted by nonfunctional cells, unless these evolutionary events are controlled.

From the public health perspective, if a synthetic microbe escaped the laboratory into the environment, how long would it survive and how many generations would it take before its synthetic function was lost over evolutionary time? Could a synthetic pathogenic strain evolve to become more virulent over time? To our knowledge, these questions are largely unexplored, but from this perspective we hope that synthetic cells will not be robust to environmental conditions and mutations. As a first step toward understanding the evolutionary stability dynamics and predictability of loss-of-function mutations in genetic circuits, we evolved replicate populations of *Escherichia coli* carrying several different genetic circuits for multiple generations (Sleight et al., 2010b).

In this study, we found that a wide variety of loss-of-function mutations are observed in BioBrick-assembled genetic circuits including point mutations, small insertions and deletions, large deletions, and insertion sequence (IS) element insertions that often occur in the scar sequence between parts. Promoter mutations are selected more than any other biological part. We also found that, on average, evolutionary half-life exponentially decreases with increasing expression levels. Surprisingly, one particular circuit that was expressed at a low level due to having a weak promoter was able to maintain function for 500 generations, but most circuits lost function within 100 generations. We were able to reengineer genetic circuits to be more mutationally robust using a few simple design principles: high expression of genetic circuits comes with the cost of low evolutionary stability, avoid repeated sequences, and the use of inducible promoters increases stability. Interestingly, inclusion of an antibiotic resistance gene within the circuit does not ensure evolutionary stability, but a reengineered version of this circuit may improve stability when certain environmental conditions are used.

In this report, we focus on the results of this study from experiments performed with the LuxR receiver device, T9002, and six reengineered versions of this circuit. We then discuss the relevance of these results for emerging microbial pathogens and future directions.

Results

Loss-of-Function Mutations and Evolutionary Stability Dynamics in the T9002 Circuit

We first measured the evolutionary stability dynamics of the T9002 genetic circuit (Canton et al., 2008) propagated in *Escherichia coli* MG1655 in order to determine the loss-of-function mutations that cause its instability. High-copy plasmids were used instead of low- or medium-copy plasmids to maximize selective pressure so that evolution would occur more rapidly since replication and expression of genetic circuits encoded on high-copy plasmids will increase

metabolic load and lower fitness. Cells with a low metabolic load (e.g., cells with mutant plasmids) have greater fitness than cells with a higher metabolic load (e.g., cells with functional plasmids) (unpublished results). Therefore, we expect that mutants will be able to rapidly outcompete functional cells that have a high expression level. However, other factors besides expression level will play a role in this evolutionary process such as mutation rate and the metabolic load associated with plasmid replication.

T9002 is a Lux receiver circuit that expresses *luxR* that activates GFP expression when the inducer AHL is added to the media (Figure A18-1). The evolutionary stability dynamics were measured by serial propagation with a dilution factor that allows for about 10 generations per day. Figure A18-2 shows the evolutionary stability dynamics of the T9002 circuit propagated in high-input (with AHL) and low-input (without AHL) conditions. From different time points in the experiment, the low- and high-input populations were induced with AHL to measure their normalized expression (here measured by fluorescence divided by cell density) over time. The low-input evolved populations slowly lose their function to about 50 percent of the maximum after 300 generations. The evolved populations in high-input conditions rapidly lose their function in fewer than 30 generations. The dynamics of this evolutionary stability are described below in Figure A18-5. No functional clones were observed after 30 generations as determined by measurement of individual colonies. The mutation that repeatedly causes loss of function in the high-input evolved populations is a deletion between two homologous transcriptional terminators (Figure A18-3), the same mutation described by Canton et al. (2008). This mutation evidently occurs at such a high rate that mutants quickly take over the population. In fact, Canton et al. (2008) were unable to isolate a population derived from a single isolate that did not already carry the deletion. The mutant plasmid was transformed back into the progenitor and was shown not to fluoresce after induction with AHL.

Evolution Experiment with Reengineered Circuits

Based on the results of the previous experiments, we reengineered the T9002 circuit to test various predictions of evolutionary stability and mutational predictability. The loss-of-function mutation was a deletion that repeatedly occurred between two homologous transcriptional terminators. Mutations and genetic rearrangements can occur due to misalignment of homologous sequences during replication (termed “replication slippage”) (Lovett, 2004). Deletion mutations between repeated sequences are known to be dependent on repeat length, proximity, and homology level (Bzymek and Lovett, 2001). These deletions are *recA*-independent if the repeat length is less than 200 bp (Bi and Liu, 1994; Lovett, 2004), as is the case with the repeated terminators in the T9002 circuit. Thus, we reengineered the last terminator of T9002 with various terminators available in the Registry to measure the effect of terminator homology level and orientation with evolutionary stability (Figure A18-4). We predicted that we could increase

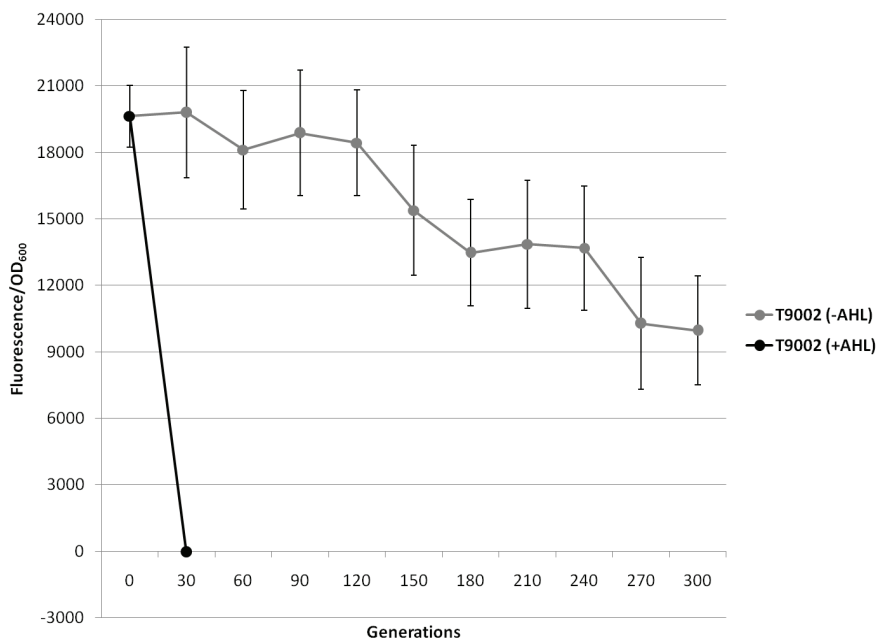


FIGURE A18-2 Evolutionary stability dynamics of T9002 evolved under low-input (-AHL) and high-input (+AHL) conditions. Low- and high-input evolved populations are shown with solid gray circles and solid black circles, respectively. Evolved populations at different time points were grown with AHL to measure relative GFP levels. Relative fluorescence normalized by OD is plotted versus generations. Error bars represent one standard deviation from the mean of nine independently evolved populations.

SOURCE: This figure is taken from the *Journal of Biological Engineering*, 4(1), 12. doi: 10.1186/1754-1611-4-12.

evolutionary robustness by decreasing the mutation rate of this deletion. Furthermore, although there have been several studies on recombination between repeated sequences, this phenomenon has not been studied in the context of synthetic biology using genetic circuits constructed from BioBricks. For instance, we do not know the effect of using various BioBrick terminators with different homology levels in the same circuit. The use of different terminators will become increasingly important when more complex circuits are constructed and BioBricks become even more widespread in the community. Also, many of the studies on recombination between repeated sequences use antibiotic resistance genes to measure recombination rates and may not relate to actual functioning circuits.

Since we learned from previous experiments that evolutionary stability dynamics of genetic circuits have high variability between replicate populations, we evolved nine independent populations of each reengineered circuit for at least 300 generations. Three experimental replicate populations of three independent

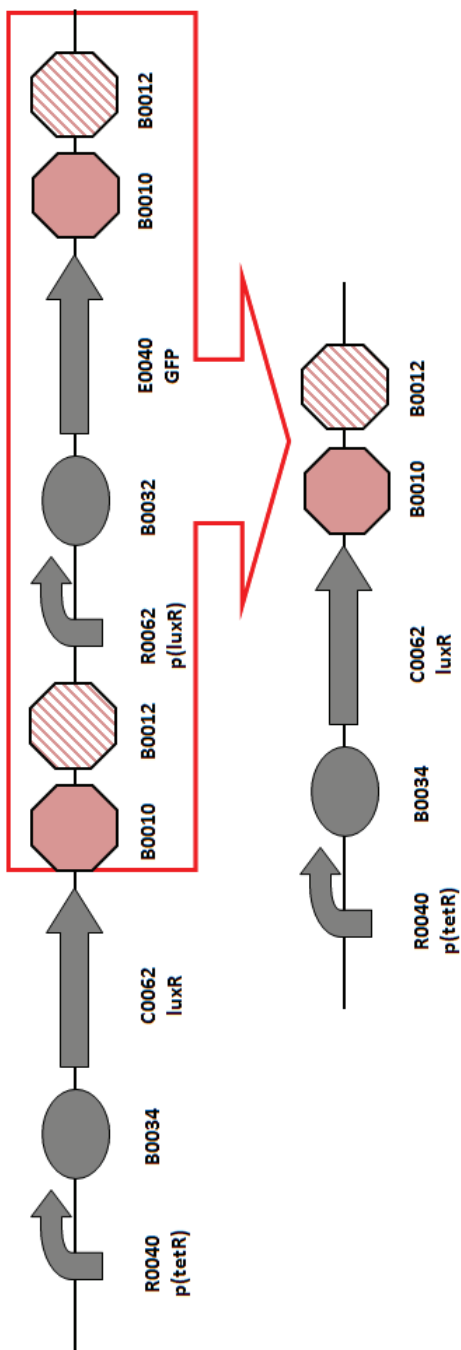


FIGURE A18-3 T9002 loss-of-function mutation. This circuit repeatedly has a deletion between homologous repeated terminators after 30 generations in the high-input evolved populations. This is the same mutation found by Canton et al. (2008).
 SOURCE: This figure is taken from the *Journal of Biological Engineering*, 4(1), 12. doi: 10.1186/1754-1611-4-12.

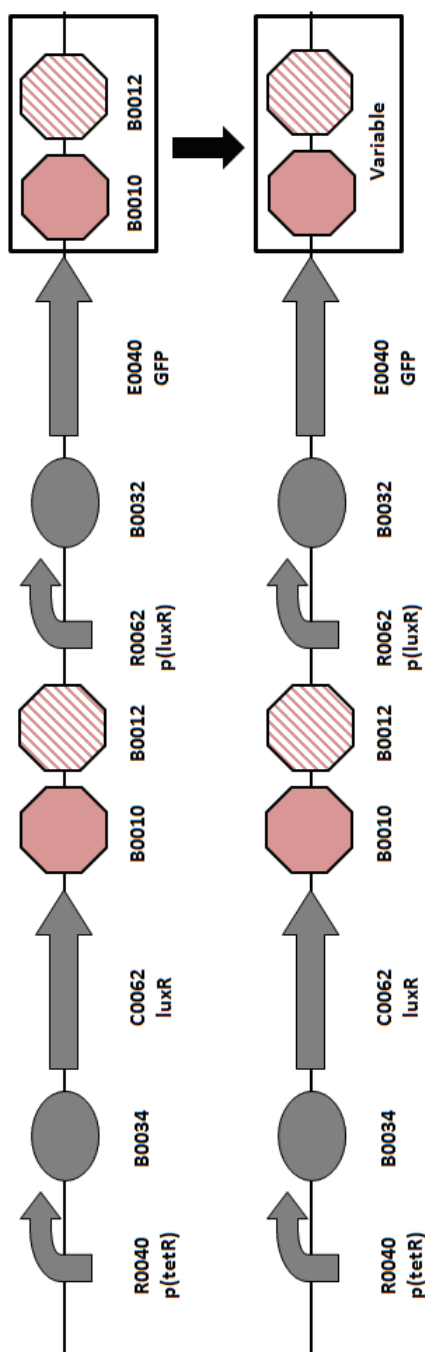


FIGURE A18-4 T9002 reengineering. T9002 reengineering involves changing the second double transcriptional terminator with varying degrees of homology and orientation to the first double transcriptional terminator.
 SOURCE: This figure is taken from the *Journal of Biological Engineering* 4(1), 12. doi: 10.1186/1754-1611-4-12.

transformants were used to test for independent mutational events. A single transformant may have a mutation at a low level that will eventually sweep through the population, so if only one transformant was used, the same mutation may show up in all replication populations. For each of the nine populations in every circuit, the evolutionary half-life was measured to quantitate the number of generations until the expression level had decreased to half of its initial level (Table A18-1). Plasmids from a single clone from each evolved population were then sequenced after the population level had decreased to less than 10 percent of the original expression level, or after 500 generations, whichever came first.

Reengineered T9002 Circuits with Different Transcriptional Terminators: Loss-of-Function Mutations and Evolutionary Stability Dynamics

Figure A18-4 shows the schematic for reengineering the last transcriptional terminator in the T9002 circuit. The evolutionary stability dynamics for six reengineered T9002 circuits and the original T9002 circuit are shown in Figure A18-5. Figure A18-6 shows the types of mutations that occurred in each of the nine replicate evolved populations. Finally, Figure A18-7 shows the most common mutations for each reengineered circuit. The six reengineered circuits are labeled T9002-A through T9002-F in Figure A18-5 and are color coded to correspond to the same circuit mutations shown in Figure A18-7. These circuits were all propagated with the inducer AHL to allow for GFP expression.

In the following paragraphs, the loss-of-function mutations and evolutionary stability dynamics for the original T9002 circuit and for each reengineered circuit are described in detail.

T9002 The original T9002 circuit decreases rapidly to about 30 percent of the original level after only 10 generations and all function is lost by 20 generations (Figure A18-5). The same deletion between homologous terminators as was observed in previous experiments (Figure A18-3) was found in all nine replicate populations (Figure A18-6). The evolutionary half-life of this circuit was found to be about 7.1 generations on average (Table A18-1).

TABLE A18-1 Evolutionary Half-Life of T9002 and Reengineered T9002 Genetic Circuits

Circuit	Evolutionary Half-life	SD
T9002	7.056	3.657
T9002-A	5.563	1.687
T9002-C	6.650	3.250
T9002-D	56.986	6.188
T9002-E	16.701	4.966
T9002-F	125.443	47.664

NOTE: The mean evolutionary half-life for nine independently evolved populations is shown for each genetic circuit with the standard deviation (SD). See the Methods section on page 414 for details.

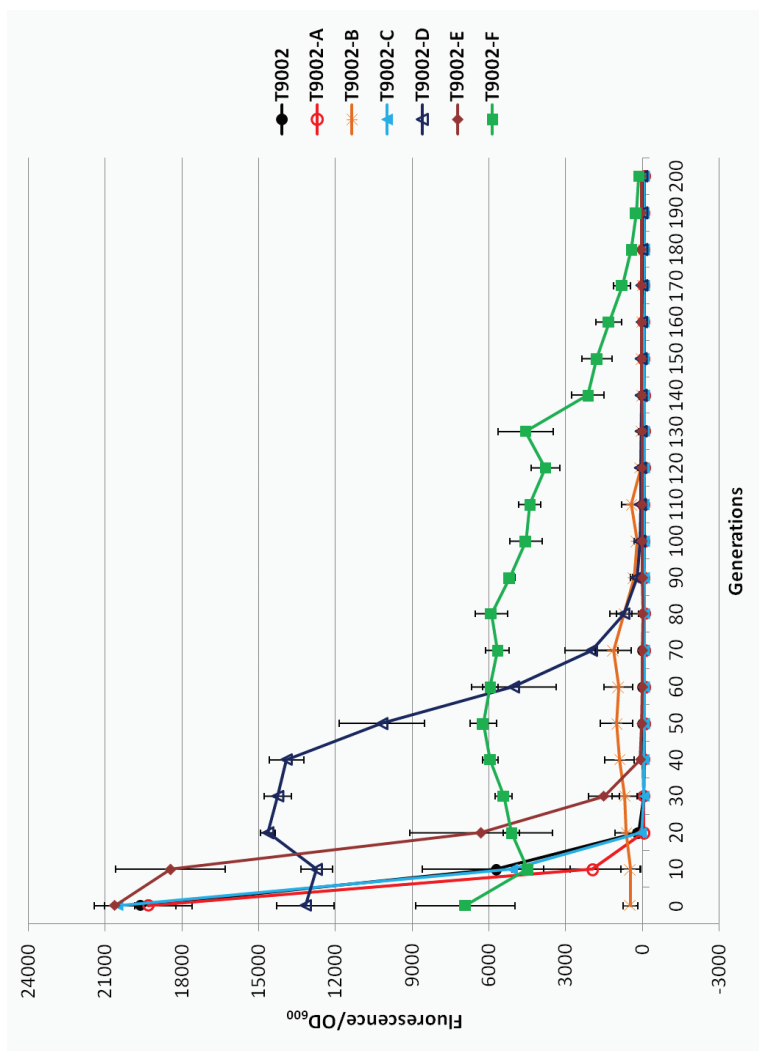


FIGURE A18-5 Evolutionary stability dynamics of T9002 and reengineered T9002 circuits. The fluorescence/OD₆₀₀ is plotted versus the generations for T9002 (solid black circles) and T9002 reengineered circuits (various shapes and colors) under high-input (+AHL) conditions. Error bars represent one standard deviation from the mean of nine independently evolved populations. SOURCE: This figure is taken from the *Journal of Biological Engineering* 4(1), 12. doi: 10.1186/1754-1611-4-12.

	1	2	3	4	5	6	7	8	9
T9002	1	1	1	1	1	1	1	1	1
T9002-A	1	1	1	1	1	1	2	2	1
T9002-B	1	1	1	1	1	1	1	1	1
T9002-C	1	1	1	1	1	1	1	1	1
T9002-D	1	2	1	1	2	1	1	1	1
T9002-E	1	1	1	2	1	1	1	3	1
T9002-F	1	1	2	1	2	2	1	3	2

- Deletion between homologous sequences (terminators)
- Point mutation
- Small insertion or deletion
- IS element insertion
- Deletion between homologous sequences (operators or scars)
- No mutation

FIGURE A18-6 Loss of mutations in nine independently evolved populations. For nine independently evolved populations, colored boxes correspond to the mutation legend below the table. The most common mutation for a particular type of mutation is labeled with “1” in the boxes above and less common mutations are labeled with increasing numbers. SOURCE: This figure is taken from the *Journal of Biological Engineering* 4(1), 12. doi: 10.1186/1754-1611-4-12.

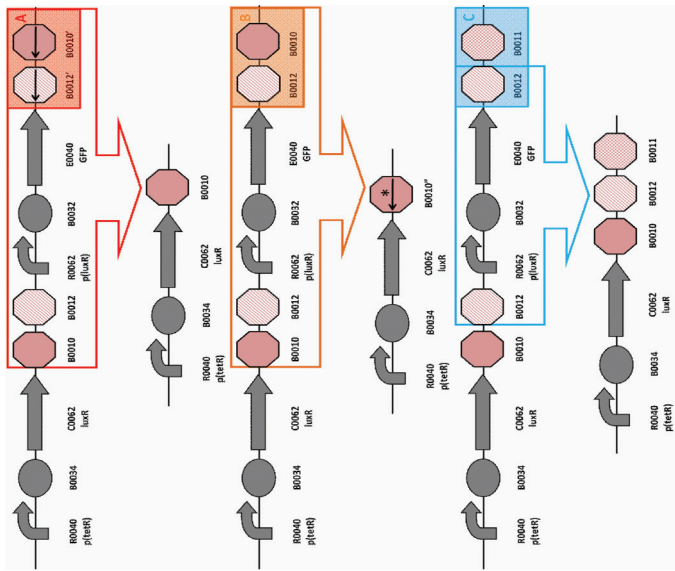
T9002-A The final double terminator in T9002 was reengineered in the reverse complementary orientation (called B0025 in the Registry) to make T9002-A. The stability of this circuit has approximately the same expression level and stability dynamics as T9002, but it has an evolutionary half-life of about 5.6 generations (Figure A18-5, Table A18-1). This decreased stability may be because the terminator in the reverse orientation is more likely to cause replication slippage. Since the expression level is similar to T9002 and therefore the metabolic load should be roughly equivalent, the difference in stability is primarily due to an increased mutation rate. Seven of nine replication populations have a deletion between the first B0010 terminator and the reverse complement of B0010 (Figures A18-6 and A18-7). This effect likely occurs because B0010 has a long hairpin structure, so one-half of B0010 can interact with the other half of the reverse complementary B0010 sequence during DNA replication since they have perfect homology. Two of the nine populations had a deletion that formed a triple terminator of B0010-B0012-B0010.

T9002-B The T9002-B circuit was reengineered to rearrange the B0010 and B0012 terminators to have B0012 first and then B0010. The rearrangement de-

creases the expression level to almost zero initially and this expression drifts up over time and then decreases to zero (Figure A18-5). For this circuit, evolutionary half-life measurements are essentially meaningless due to the randomness of low expression. Notice that other reengineered T9002 circuits also have decreased expression levels relative to T9002, presumably due to weaker terminator hairpin structures having increased mRNA degradation (Smolke and Keasling, 2002) or transcriptional readthrough that can decrease plasmid copy number (Stueber and Bujard, 1982). Others have observed that removal of transcriptional terminators can decrease expression levels in general (Kaur et al., 2007). All nine populations have the same deletion between B0010 terminators (Figures A18-6 and A18-7). Because the B0010 terminator is an inexact hairpin (there are some mismatches), one-half of the first B0010 interacts with the other half of the second B0010 terminator, causing a hybrid B0010 terminator (Figure A18-7).

T9002-C The T9002-C circuit was reengineered to have B0012 and B0011 as the final double terminator instead of B0010 and B0012. This circuit has nearly identical stability dynamics as T9002, with an evolutionary half-life of about 6.7 generations on average (Figure A18-5, Table A18-1). All nine populations have the same deletion between B0012 terminators that make a triple terminator of B0010-B0012-B0011 (Figures A18-6 and A18-7). Since the expression level and stability dynamics are roughly equivalent to that of T9002, the mutation rate between repeated B0010-B0012 terminators (129 bp) is probably about the same as that between repeated B0012 terminators (41 bp). Interestingly, no significant stability difference was observed between T9002-C (41-bp homology) and T9002 or T9002-A (both 129-bp homology), despite having similar expression levels. This result suggests that shortening the repeated regions of homologous terminators did not increase evolutionary robustness, contrary to what we expected.

T9002-D The T9002-D circuit has the same final B0012-B0011 terminator but is the reverse complement of this sequence. The inclusion of this terminator decreases the initial expression level to about 65 percent of T9002 (Figure A18-5). The evolutionary half-life of this circuit is about 57 generations (Figure A18-5, Table A18-1). Also, the slope of the stability plot is decreased relative to other circuits with higher expression (T9002, T9002-A, T9002-C, and T9002-E) and the stability lag (time for expression to decrease to zero along the x axis) is increased (Figure A18-5). In contrast to other circuits with repeated terminators, only three of nine have deletions between homologous terminators, forming a hybrid B0012 terminator (Figures A18-6 and A18-7). This result is probably because, unlike T9002-C, the second B0012 is the reverse complement, and therefore the only homology in this circuit is the 8-bp hairpin structure having complementary sequences; the rest of the terminator has a loop structure of noncomplementary sequences. In other words, B0010 has sufficient homology in either the forward or reverse orientation to cause replication slippage, but in B0012 replication slippage is more likely to occur only in the forward orientation. The other mutations



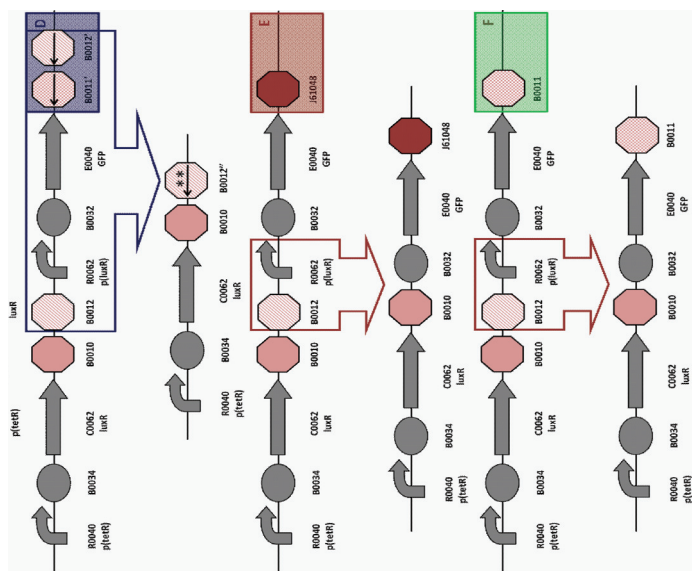


FIGURE A18-7 Most common loss-of-function mutations in reengineered T9002 circuits. The original circuit is shown above the loss-of-function mutation circuit for each of the six reengineered T9002 circuits.

SOURCE: This figure is taken from the *Journal of Biological Engineering* 4(1), 12. doi: 10.1186/1754-1611-4-12.

in this circuit are composed of point mutations, short insertions or deletions, deletions between scar sequences, or IS mutations (Figure A18-6).

T9002-E The T9002-E circuit, like the T9002-F circuit, was reengineered to have no homology between terminator sequences. This circuit has the highest initial expression level on average probably because J61048 is a very strong terminator, but has similar expression relative to the T9002, T9002-A, and T9002-C circuits (Figure A18-5). Its evolutionary half-life is about 16.7 generations (Figure A18-5, Table A18-1). Thus, relative to other circuits with similar expression levels, it is the most evolutionary robust circuit, having over twofold higher stability than T9002. When the evolutionary half-life is measured for the nine replicate populations, this evolutionary half-life difference compared to T9002 is highly significant (one-tailed *t*-test, $p = 0.0003$). Notice that the stability slope is similar to that for T9002, T9002-A, and T9002-C circuits, but the stability lag is increased by about 10 generations. This difference in lag is likely due to a decreased mutation rate since mutations are more random compared to the other similar expression-level circuits (Figure A18-6). The most common mutation is a deletion between repeated scar sequences that removes the *luxR* promoter and effectively inactivates the circuit function (Figure A18-7).

T9002-F The T9002-F circuit was reengineered with the B0011 terminator, so it also has no homology between terminator sequences. The B0011 is evidently a weak terminator since its initial expression level is about fourfold lower than that of T9002. Its stability dynamics show that it is the most evolutionary robust of the reengineered T9002 circuits, with an evolutionary half-life of about 125 generations (Figure A18-5, Table A18-1). This result indicates that decreasing homology levels and expression through terminator reengineering increased the evolutionary half-life of this circuit over 17-fold relative to T9002. Like T9002-E, the mutations in each of the nine populations are mostly random (Figure A18-6). Also like T9002-E, the most common mutation is a deletion between repeated scar sequences that removes the *luxR* promoter driving GFP expression (Figure A18-7). Since T9002-E and T9002-F likely have similar mutation rates with zero terminator homology, the large stability difference between these circuits can be explained by expression levels alone.

Overall, excluding T9002-B, three of the five reengineered T9002 circuits are more evolutionary robust than the original circuit. The order of evolutionary robust genetic circuits is as follows: T9002-F > D > E > A = C = T9002. This increase in evolutionary robustness can be attributed to decreased expression levels (due to the terminator reengineering) and to decreased mutation rate between homologous transcriptional terminators. The reengineered circuits with homologous transcriptional terminators almost always have deletions between homologous regions, whereas circuits without homology have mutations in other locations in the circuit. Reengineering this circuit to remove all homology effectively removes a certain class of mutations from occurring. The T9002-E circuit is more

evolutionarily robust than other circuits with similar expression levels, which is likely due to decreased mutation rate alone. Thus, evolutionary robustness can be increased by removing long repeated sequences from genetic circuits, but even short 8-bp scar sequences have the potential for replication slippage.

Evolutionary Half-Life Versus Initial Expression Level in T9002 and T9002-E Circuits Evolved with Different Inducer Concentrations

From previous results (Figure A18-5, Table A18-1), we noticed that circuits with a high initial expression level tended to have low evolutionary stability. Also, particular circuits with high mutation rates had lower stability compared to circuits with lower mutation rates. To test the relationship between initial expression level and evolutionary half-life directly, we evolved the T9002 (high mutation rate) and T9002-E (lower mutation rate) circuits propagated with different AHL concentrations.

Figure A18-8 shows the mean initial expression level versus mean evolutionary half-life for eight replicate populations from three different AHL concentrations in T9002 (black) and T9002-E (red). An exponential fit of these mean data points gives a much higher r^2 value than a linear fit (>0.1) in both cases. T9002 has an r^2 value of 0.954 compared to the r^2 value of 0.955 in T9002-E. The curve for T9002 is shifted to the left from T9002-E due to its higher mutation rate (expression alone cannot account for the shift), but as expression is decreased the evolutionary half-life difference between these two circuits also decreases. This decrease may be because, at high expression levels, the fitness difference between the progenitor and mutant cells is the highest, and therefore mutants outcompete functional cells in the population more quickly.

Discussion

Genetic circuits lose function over evolutionary time and are found to have a wide variety of mutations that cause their loss of function. Circuits with repeated sequences almost always have deletions between these sequences, but the effect of repeat length is not well understood. In one reengineered T9002 circuit, shortening the length of homology from 129 to 41 bp did not significantly increase evolutionary stability. Stability was only increased when there was no homology whatsoever between transcriptional terminators. Mutations between repeated sequences without perfect homology in the case of some reengineered T9002 circuits are usually, but not always, predictable.

In circuits without repeated sequences, mutations are more random between evolved replicate populations. In other circuits we studied (not shown here), mutations that remove promoter function are most often selected for among all the genetic circuits tested (Sleight et al., 2010b). This result is likely because promoter mutations remove the metabolic load at both the transcriptional and translational levels. Mutations within RBSs are not found and mutations in

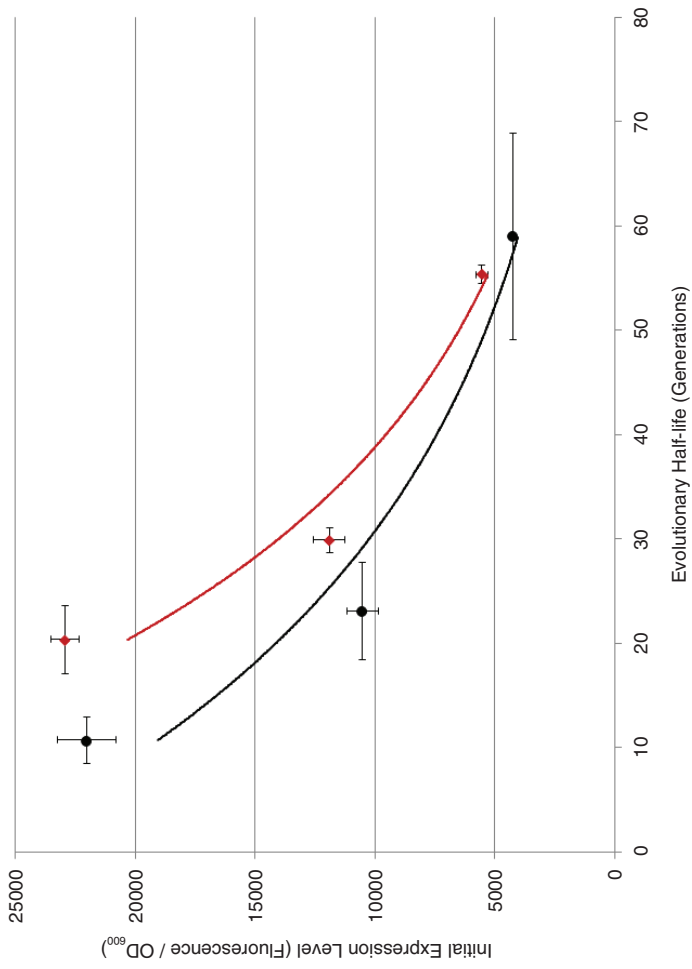


FIGURE A18-8 Evolutionary half-life versus initial expression level in T9002 and T9002-E circuits evolved with different inducer concentrations. Evolutionary half-life versus initial expression level is plotted for T9002 (solid black circles) and T9002-E (solid dark red diamonds) circuits. An exponential fit for the mean of each evolutionary half-life versus initial expression data point is shown by the black (T9002) or red (T9002-E) line. Error bars for both the x and y axes represent one standard deviation from the mean of eight independently evolved populations. SOURCE: This figure is taken from the *Journal of Biological Engineering*, 4(1), 12. doi: 10.1186/1754-1611-4-12.

coding sequences are rare except when that coding sequence is an activator of transcription downstream (as in the case of the *luxR* coding sequence in T9002). In the case of T9002, removing homology between transcriptional terminators only shifts the mutation to one that often removes function of the *luxR* promoter or *luxR* coding sequence instead.

What is needed is a method to predict the evolutionary stability of circuits from the properties of their parts, but the emergent behaviors of circuits will likely make prediction difficult. At the very least, publishing the evolutionary stability properties of simple circuits in future data sheets may allow engineers to calculate the expected evolutionary stability of more complex circuits. This calculation would likely require software (Chandran et al., 2009) and mathematical modeling (Chandran et al., 2008) that analyzes each part individually and the entire DNA sequence as a whole to determine the expected evolutionary stability. This calculation would also require standardization for methods to measure evolutionary stability, and the methods described here are not necessarily the best way. On the other hand, more general methods may be developed that focus less on design of the circuit and more on design of the environment to impose a selective pressure for function of the circuit (Bayer et al., 2009). Design of a selective environment is ideal, but it is difficult to do when the output of most circuits (e.g., GFP) is not linked to survival or growth rate. A cell-sorter device that sorts between functional and nonfunctional cells may help with this issue, but it may not be practical for performing routine experiments.

From our results of what types of mutations are selected for in genetic circuits and the evolutionary stability dynamics, a few simple design principles can be proposed when engineering circuits. The first principle is that high expression of genetic circuits comes with the cost of low evolutionary stability. Although exceptions to this rule certainly occur, a genetic circuit with high expression correlates with a large metabolic load and therefore is predicted to have decreased cellular fitness. When the fitness difference between the functional and nonfunctional cells in the population is large, evolutionary stability will decrease quickly. Therefore, the initial expression level of the circuit is likely to be a good predictor of evolutionary stability when a circuit with high mutational robustness is desired. Using a low- or medium-copy plasmid will help with stability as long as the expression level does not need to be high. For more complex circuits where a high expression level is needed for proper functioning of the circuit, decreasing expression level then comes at the cost of changing the function of the circuit.

The second design principle is to avoid repeated sequences. This principle may be obvious, but nearly every circuit in the Registry with more than one coding sequence has repeated B0015 terminators. When a circuit has a high metabolic load (higher than T9002) and repeated sequences on a high-copy-number plasmid, when using MG1655 as the host strain, the circuit will almost always lose function during overnight growth (unpublished results). Reengineering the T9002 circuit to have two different transcriptional terminators (T9002-E) does significantly increase evolutionary half-life by more than twofold and is inde-

pendent of expression levels. However, since this circuit has high expression, this improvement only results in an increase of about 10 generations. Decreasing the expression level along with the mutation rate will increase the evolutionary half-life about 17-fold, as seen in the T9002-F circuit. This result suggests that simple ways to increase evolutionary stability can be used without changing the function of the circuit. For more complex circuits, the community will need to identify many more terminators than those that currently exist in the Registry to design circuits without repeated sequences.

The third design principle is that use of inducible promoters generally increases evolutionary stability (not shown here) (Sleight et al., 2010b). This principle may or may not be significant depending on the circuit used. Inducible circuits are likely more stable due to decreased metabolic load and are preferred since expression can be controlled and fine-tuned, though in some circumstances a constitutive promoter may be desired. Therefore, the use of inducible promoters can be thought of as one extra precaution to maximize evolutionary stability, but expression levels and repeated sequences should first be considered.

We emphasize that the design principles proposed may not be general since only relatively simple circuits were tested in this study. Evolutionary stability should be measured in larger and more complex circuits to understand if these design principles apply. Furthermore, these simple design principles should not necessarily be all used simultaneously. A researcher may not want only to design circuits that have low expression, have no repeated regions, and use a promoter that is inducible. For instance, if recombination sites are needed in the circuit, then repeated or inverted sequences may be impossible to avoid. In addition to the design for the proper function of the circuit, the design for evolutionary robustness should be carefully considered. For this, we need to think about the probability of mutations occurring when the expression level, and therefore metabolic load, is high. In this study, removing repeated regions often shifts mutations to the promoter, and putting a selection on the promoter often shifts the mutation to the chromosome (not shown here) (Sleight et al., 2010b).

Thus, mutations are unavoidable without a selective pressure, but evolutionary stability can likely be improved in the future by better design of selective environments where the circuit is linked to survival and/or growth rate, understanding of mutation rates in genetic circuits, fitness differences between functional and nonfunctional cells, and improvements to the host strain that decrease mutation rates or buffer metabolic loads more efficiently. Another way to improve evolutionary stability is to engineer an error detection and correction circuit that will correct mutations, but it will need careful design since this circuit itself will be prone to mutation. Recently, a technique called ClChE was developed to insert and amplify (increase copy number of) genetic circuits on the chromosome to avoid the problem of maintaining evolutionary stability of function on plasmids (Tyo et al., 2009). This is a powerful method that should be used, but it is also somewhat tedious to insert and amplify circuits on the chromosome. There are also examples of chromosomal genes that are evolutionarily unstable when not

under selection (Cooper and Lenski, 2000; Cooper et al., 2001) and therefore chromosomally integrating synthetic circuits will only delay this problem. Designing mutationally robust genetic circuits, therefore, is somewhat of an art form at the moment besides a few simple design rules, but it should be seen as something the engineer can eventually control.

From the opposite perspective, if a synthetic microbe is released into the environment, we need to hope it will not be mutationally robust. For this, we need to be able to predict how it will evolve if it is able to survive and compete with microbes in their natural environment. One circuit with a weak constitutive promoter was stable for 500 generations in the presence of ampicillin (Sleight et al., 2010b), so it is theoretically possible for circuits to maintain their function for many generations in the right environmental conditions. For the T9002 circuits described here, if the cells carrying these plasmids escaped into the environment, there would likely not be any ampicillin to maintain the plasmid itself, so plasmid stability may follow similar dynamics, as shown by Lenski and Bouma (1987). As far as circuit stability goes, since it is unlikely there will be AHL in the environment to keep circuit function on, function of the circuit will likely decay with similar dynamics as that shown in Figure A18-2 in the T9002 populations evolved without AHL. A circuit with a lower expression level and lower mutation rate (such as T9002-F) will decay more slowly, but it really depends on the interaction between circuit function and the selective pressure in a particular environment.

When thinking about the evolutionary trajectory of a synthetic microbe, if the circuit function imparts a selective advantage over natural microbes, it should be of concern. For example, it is unlikely that GFP will provide a fitness boost in a particular environment, but a different circuit that allows for the degradation of a carbon source may allow cells with this function to have a growth advantage and permanently change an ecosystem. More worrisome is the release of a synthetic microbe that evolves to become pathogenic by acquiring genes from a virus, for instance. Here, one could imagine cells with T9002 mutating out the GFP part of the circuit and inserting a virulence factor instead. Then, the new cells would express the virulence factor only if AHL was present—a remote possibility if there are other microbes that use this molecule for quorum sensing, but still possible. The results shown in this report illustrate the evolution of genetic circuits when no selective pressure is imposed to maintain function of the circuit, but it is unknown what will happen in a natural environment where real selective pressures exist. The study of natural pathogen evolution in *E. coli* and other pathogens in combination with evolutionary studies in synthetic biology will allow us to understand the risks involved.

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Methods

Circuit Engineering and Use of Strains

All circuits were either obtained from the Registry of Standard Biological Parts or engineered using the Clontech In-Fusion PCR Cloning Kit with the specific methods described by Sleight et al. (2010a). All circuits are encoded on the pSB1A2 plasmid, a high-copy-number plasmid (100-300 plasmids/cell) with an ampicillin resistance gene. Plasmids were transformed into strains via chemical transformation. *Escherichia coli* MG1655 was the strain used for all circuits described.

Evolution Experiment

For each engineered circuit, plasmid DNA that had been fully sequenced was transformed into either MG1655 competent cells. Three individual transformant colonies were grown overnight at 37°C, shaking at 250 rpm in +100 µg/mL ampicillin. Freezer stocks were saved of these cultures in 15 percent glycerol and stored at -80°C. These freezer stocks were streaked out on LB +100 µg/mL ampicillin plates with appropriate inducer (1×10^{-7} M AHL for T9002 circuits) and grown overnight at 37°C. Three colonies were chosen from each transformant (nine total colonies) and inoculated into 1.5 mL LB + 100 µg/mL ampicillin media in Eppendorf deep-well plates sealed with a Thermo Scientific gas-permeable membrane to allow for maximum oxygen diffusion. T9002 circuit cultures were supplemented with the inducer 1×10^{-7} M 3OC₆HSL (AHL). Cultures were propagated with a serial dilution scheme using a 1:1000 dilution to achieve about 10 generations per day ($\log_2 1000 = 9.97$). Evolved populations were grown for 24 hours at 37°C, shaking at 250 rpm. Freezer stocks (with 15 percent glycerol) of each population were saved at -80°C every day for future study. All replicate populations were evolved in parallel to minimize experimental variability.

Evolutionary Stability Measurements

Every 24 hours, cell density (OD₆₀₀) and fluorescence (excitation wavelength, 485/15; emission wavelength, 516/20) of evolved populations were measured in a Biotek Synergy HT plate reader. Twenty-four hours was used as the measurement time point because the rate of change of GFP was closest to zero

(i.e., closest to steady state). Evolved populations thus spent about 8-12 hours in lag or exponential phase and the remaining time in stationary phase. For each time point, all populations were thoroughly mixed and 200 μL was transferred into black, clear-bottom 96-well plates (Costar). OD was subtracted from blank media to measure the OD without background. Fluorescence was subtracted from the R0011 + E0240 circuit with a mutation in the promoter to measure background fluorescence. Fluorescence was then divided by OD to measure the normalized expression (fluorescence/OD₆₀₀).

Plasmid Sequencing

At appropriate evolutionary time points, usually when circuit function had decreased to less than 10 percent of the original expression level, or 500 generations, the evolved populations were streaked out on LB + 100 $\mu\text{g/mL}$ ampicillin plates, supplemented with 1×10^{-7} M AHL. Colonies were visualized for fluorescence on a Clare Chemical Dark Reader Transilluminator. Nonfluorescing colonies, or weakly fluorescing colonies if no nonfluorescing colonies were present, were grown overnight in 5 mL of LB + 100 $\mu\text{g/mL}$ ampicillin. Plasmids were extracted using the Qiagen Miniprep Kit or glycerol stocks were sent to the University of Washington High-Throughput Genomics Unit facility (<http://www.htseq.org>). Purified plasmid DNA was sequenced using the VF2 (5'-TGC-CACCTGACGTCTAAGAA-3') and VR (5'-ATTACCGCCTTTGAGTGAGC-3') primers specific to the pSB1A2 vector (about 100 bp on either side of the circuit) or primers specific to the circuit.

Quantitative Analysis of Evolutionary Half-Life

Evolutionary half-life was calculated for each independently evolved population. First, the slope and y -intercept were calculated using the two normalized expression (fluorescence/OD₆₀₀) data points on either side of the half-maximum expression value on the evolutionary stability plot. A linear regression on those two data points was performed using the equation $y = ax + b$, where y is the half-maximum initial expression, a is the slope of the two data points, b is the y -intercept of the two data points, and solving for x gives the evolutionary half-life. This method gave a very accurate half-life estimate in terms of generations and was a better estimate than using third-order polynomial equations, which we also calculated.

Experiment to Measure Evolutionary Half-Life Versus Initial Expression Level in T9002 and T9002-E Circuits Evolved with Different Inducer Concentrations

This experiment was performed as described earlier in the Evolution Experiment section, except that eight replicate populations were propagated with different inducer concentrations. The results of this experiment are shown in Figure

A18-8. The AHL concentrations used were 1×10^{-7} M (high-expression-level data point on the far left side of Figure A18-7a), 2×10^{-9} M (medium expression level), and 1×10^{-9} M (low expression level). The evolutionary half-life for individual evolved populations was determined as described earlier in the Quantitative Analysis of Evolutionary Half-Life section. For each inducer concentration, the mean evolutionary half-life versus initial-expression-level data point was plotted. These data points were fit to an exponential curve since this relationship always had the highest r^2 value.

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A19

ISOPRENOID PATHWAY OPTIMIZATION FOR TAXOL PRECURSOR OVERPRODUCTION IN ESCHERICHIA COLI⁸⁹

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Taxol (paclitaxel) is a potent anticancer drug first isolated from the *Taxus brevifolia* Pacific yew tree. Currently, cost-efficient production of Taxol and its analogs remains limited. Here, we report a multivariate-modular approach to metabolic-pathway engineering that succeeded in increasing titers of taxadiene—the first committed Taxol intermediate—approximately 1 gram per liter (~15,000-fold) in an engineered *Escherichia coli* strain. Our approach partitioned the taxadiene metabolic pathway into two modules: a native upstream methylerythritol-phosphate (MEP) pathway forming isopentenyl pyrophosphate and a heterologous downstream terpenoid-forming pathway.

⁸⁹From Ajikumar, P.K., et al. 2010. Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science* 330: 70-74. Reprinted with permission from AAAS.

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Systematic multivariate search identified conditions that optimally balance the two pathway modules so as to maximize the taxadiene production with minimal accumulation of indole, which is an inhibitory compound found here. We also engineered the next step in Taxol biosynthesis, a P450-mediated 5 α -oxidation of taxadiene to taxadien-5 α -ol. More broadly, the modular pathway engineering approach helped to unlock the potential of the MEP pathway for the engineered production of terpenoid natural products.

Taxol (paclitaxel) and its structural analogs are among the most potent and commercially successful anticancer drugs (Kingston, 2007). Taxol was first isolated from the bark of the Pacific yew tree (Wani et al., 1971), and early-stage production methods required sacrificing two to four fully grown trees to secure sufficient dosage for one patient (Suffness and Wall, 1995). Taxol's structural complexity limited its chemical synthesis to elaborate routes that required 35 to 51 steps, with a highest yield of 0.4% (Nicolaou et al., 1994; Holton et al., 1994; Walji and MacMillan, 2007). A semisynthetic route was later devised in which the biosynthetic intermediate baccatin III, isolated from plant sources, was chemically converted to Taxol (Holton et al., 1995). Although this approach and subsequent plant cell culture-based production efforts have decreased the need for harvesting the yew tree, production still depends on plant-based processes (Frense, 2007), with accompanying limitations on productivity and scalability. These methods of production also constrain the number of Taxol derivatives that can be synthesized in the search for more efficacious drugs (Roberts, 2007; Goodman and Walsh, 2001).

Recent developments in metabolic engineering and synthetic biology offer new possibilities for the overproduction of complex natural products by optimizing more technically amenable microbial hosts (Tyo et al., 2007; Ajikumar et al., 2008). The metabolic pathway for Taxol consists of an upstream isoprenoid pathway that is native to *Escherichia coli* and a heterologous downstream terpenoid pathway (fig. S1). The upstream methylerythritol-phosphate (MEP) or heterologous mevalonic acid (MVA) pathways can produce the two common building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), from which Taxol and other isoprenoid compounds are formed (Ajikumar et al., 2008). Recent studies have highlighted the engineering of the above upstream pathways to support the biosynthesis of heterologous isoprenoids such as lycopene (Farmer and Liao, 2000; Alper et al., 2005), artemisinic acid (Chang and Keasling, 2006; Martin et al., 2003), and abietadiene (Morrone et al., 2010; Leonard et al., 2010). The downstream taxadiene pathway has been reconstructed in *E. coli* and *Saccharomyce cerevisiae* together with the overexpression of upstream pathway enzymes, but to date titers have been limited to less than 10 mg/liter (Huang et al., 2001; Engels et al., 2008).

The above rational metabolic engineering approaches examined separately either the upstream or the downstream terpenoid pathway, implicitly assuming that modifications are additive (a linear behavior) (Farmer and Liao, 2000;

Morrone et al., 2010; Yuan et al., 2006). Although this approach can yield moderate increases in flux, it generally ignores nonspecific effects, such as toxicity of intermediate metabolites, adverse cellular effects of the vectors used for expression, and hidden pathways and metabolites that may compete with the main pathway and inhibit the production of the desired molecule. Combinatorial approaches can overcome such problems because they offer the opportunity to broadly sample the parameter space and bypass these complex nonlinear interactions (Yuan et al., 2006; Jin and Stephanopoulos, 2007; Wang et al., 2009). However, combinatorial approaches require high-throughput screens, which are often not available for many desirable natural products (Klein-Marcuschamer, 2007).

Considering the lack of a high-throughput screen for taxadiene (or other Taxol pathway intermediate), we resorted to a focused combinatorial approach, which we term “multivariate-modular pathway engineering.” In this approach, the overall pathway is partitioned into smaller modules, and the modules’ expression are varied simultaneously—a multivariate search. This approach can identify an optimally balanced pathway while searching a small combinatorial space. Specifically, we partition the taxadiene-forming pathway into two modules separated at IPP, which is the key intermediate in terpenoid biosynthesis. The first module comprises an eight-gene, upstream, native (MEP) pathway of which the expression of only four genes deemed to be rate-limiting was modulated, and the second module comprises a two-gene, downstream, heterologous pathway to taxadiene (Figure A19-1). This modular approach allowed us to efficiently sample the main parameters affecting pathway flux without the need for a high-throughput screen and to unveil the role of the metabolite indole as inhibitor of isoprenoid pathway activity. Additionally, the multivariate search revealed a highly nonlinear taxadiene flux landscape with a global maximum exhibiting a 15,000-fold increase in taxadiene production over the control, yielding 1.02 ± 0.08 g/liter (SD) taxadiene in fed-batch bioreactor fermentations.

We have further engineered the P450-based oxidation chemistry in Taxol biosynthesis in *E. coli* to convert taxadiene to taxadien-5 α -ol and provide the basis for the synthesis of subsequent metabolites in the pathway by means of similar cytochrome P450 (CYP450) oxidation chemistry. Our engineered strain improved taxadiene-5 α -ol production by 2400-fold over the state of the art with yeast (Dejong et al., 2006). These advances unlock the potential of microbial processes for the large-scale production of Taxol or its derivatives and thousands of other valuable terpenoids.

The multivariate-modular approach in which various promoters and gene copy-numbers are combined to modulate diverse expression levels of upstream and downstream pathways of taxadiene synthesis is schematically described in fig. S2. A total of 16 strains were constructed in order to widen the bottleneck of the MEP pathway as well as optimally balance it with the downstream taxadiene pathway. (Materials and methods are available as supporting material on *Science Online*.) The dependence of taxadiene accumulation on the upstream pathway for constant values of the downstream pathway is shown in Figure A19-2A, and the

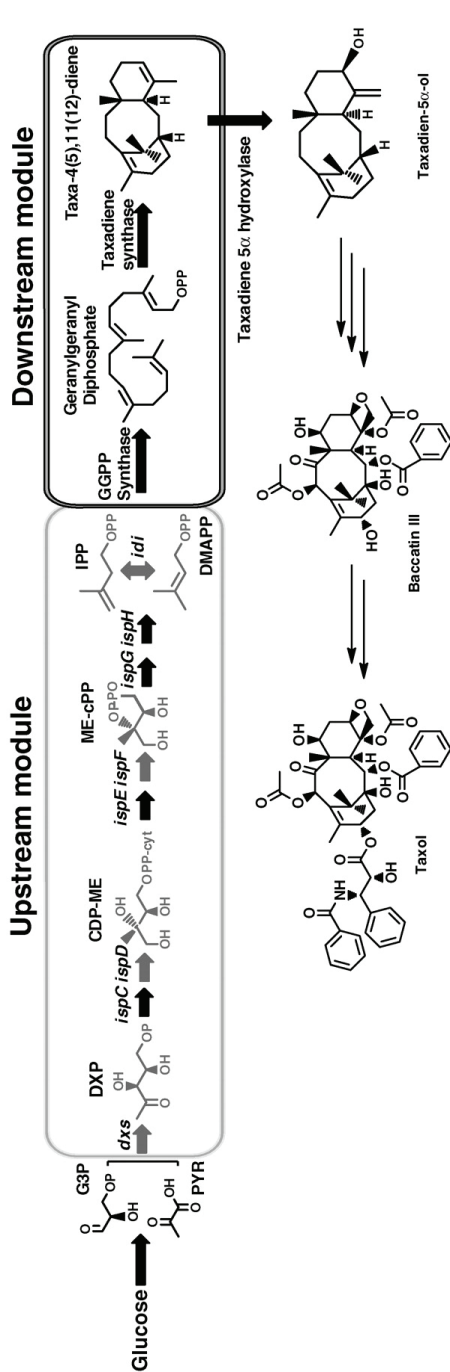


FIGURE A19-1 Multivariate-modular approach for isoprenoid pathway optimization. To increase the flux through the upstream MEP pathway, we targeted reported enzymatic bottlenecks (*dxs*, *idi*, *ispD*, and *ispF*) (gray) for overexpression by an operon (*dxs-idi-ispDF*) (Yuan et al., 2006). To channel the overflow flux from the universal isoprenoid precursors, IPP and DMAPP, toward Taxol biosynthesis, we constructed a synthetic operon of downstream genes GGPP synthase (G) and taxadiene synthase (T) (Walker and Croteau, 2001). Both pathways were placed under the control of inducible promoters in order to control their relative gene expression. In the *E. coli* metabolic network, the MEP isoprenoid pathway is initiated by the condensation of the precursors glyceraldehyde-3 phosphate (G3P) and pyruvate (PYR) from glycolysis. The Taxol pathway bifurcation starts from the universal isoprenoid precursors IPP and DMAPP to form geranylgeranyl diphosphate, and then the taxadiene. The cyclic olefin taxadiene undergoes multiple rounds of stereospecific oxidations, acylations, and benzylation to form the late intermediate Baccatin III and side chain assembly to, ultimately, form Taxol.

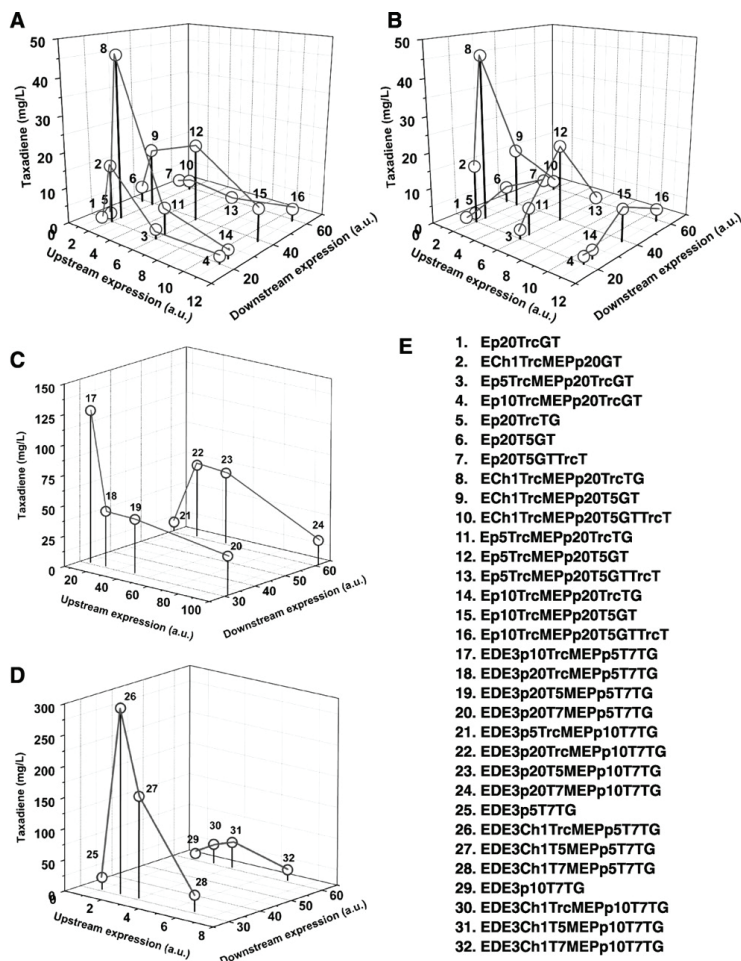


FIGURE A19-2 Optimization of taxadiene production through regulating the expression of the upstream and downstream modular pathways. **(A)** Response in taxadiene accumulation to changes in upstream pathway strengths for constant values of the downstream pathway. **(B)** Dependence of taxadiene on the downstream pathway for constant levels of upstream pathway strength. **(C)** Taxadiene response from strains (17 to 24) engineered with high upstream pathway overexpressions (6 to 100 a.u.) at two different downstream expressions (31 a.u. and 61 a.u.). **(D)** Modulation of a chromosomally integrated upstream pathway by using increasing promoter strength at two different downstream expressions (31 a.u. and 61 a.u.). **(E)** Genotypes of the 32 strain constructs whose taxadiene phenotype is shown in Figure A19-2, A to D. E, *E. coli* K12MG1655 Δ recA Δ endA; EDE3, *E. coli* K12MG1655 Δ recA Δ endA with DE3 T7 RNA polymerase gene in the chromosome; MEP, *dxs-idi-ispDF* operon; GT, GPPS-TS operon; TG, TS-GPPS operon; Ch1, 1 copy in chromosome; Trc, Trc promoter; T5, T5 promoter; T7, T7 promoter; p5, pSC101 plasmid; p10, p15A plasmid; and p20, pBR322 plasmid.

dependence on the downstream pathway for constant upstream pathway strength is shown in Figure A19-2B (table S1, calculation of the upstream and downstream pathway strength from gene copy number and promoter strength). As the upstream pathway expression increases in Figure A19-2A from very low levels, taxadiene production also rises initially because of increased supply of precursors to the overall pathway. However, after an intermediate value further upstream pathway increases cannot be accommodated by the capacity of the downstream pathway. For constant upstream pathway expression (Figure A19-2B), a maximum in downstream expression was similarly observed owing to the rising edge to initial limiting of taxadiene production by low expression levels of the downstream pathway. At high (after peak) levels of downstream pathway expression, we were probably observing the negative effect on cell physiology of the high copy number.

These results demonstrate that dramatic changes in taxadiene accumulation can be obtained from changes within a narrow window of expression levels for the upstream and downstream pathways. For example, a strain containing an additional copy of the upstream pathway on its chromosome under *Trc* promoter control (strain **8**) (Figure A19-2A) produced 2000-fold more taxadiene than one expressing only the native MEP pathway (strain **1**) (Figure A19-2A). Furthermore, changing the order of the genes in the downstream synthetic operon from GT (GGPS-TS) to TG (TS-GGPS) resulted in a two- to threefold increase (strains **1** to **4** as compared with strains **5**, **8**, **11**, and **14**). Altogether, the engineered strains established that the MEP pathway flux can be substantial if an appropriate range of expression levels for the endogenous upstream and synthetic downstream pathway are searched simultaneously.

To provide ample downstream pathway strength while minimizing the plasmid-born metabolic burden (Jones et al., 2000), two new sets of four strains each were engineered (strains **17** to **20** and **21** to **24**), in which the downstream pathway was placed under the control of a strong promoter (*T7*) while keeping a relatively low number of five and 10 plasmid copies, respectively. The taxadiene maximum was maintained at high downstream strength (strains **21** to **24**), whereas a monotonic response was obtained at the low downstream pathway strength (strains **17** to **20**) (Figure A19-2C). This observation prompted the construction of two additional sets of four strains each that maintained the same level of downstream pathway strength as before but expressed very low levels of the upstream pathway (strains **25** to **28** and **29** to **32**) (Figure A19-2D). Additionally, the operon of the upstream pathway of the latter strain set was chromosomally integrated (fig S3). Not only was the taxadiene maximum recovered in these strains, albeit at very low upstream pathway levels, but a much greater taxadiene maximum was attained (~300 mg/liter). We believe that this significant increase can be attributed to a decrease in the cell's metabolic burden.

We next quantified the mRNA levels of 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) and taxadiene synthase (*TS*) (representing the upstream and downstream pathways, respectively) for the high-taxadiene-producing strains (**25** to

32 and **17** and **22**) that exhibited varying upstream and downstream pathway strengths (fig. S4, A and B) to verify our predicted expression strengths were consistent with the actual pathway levels. We found that *dxs* expression level correlates well with the upstream pathway strength. Similar correlations were found for the other genes of the upstream pathway: *idi*, *ispD*, and *ispF* (fig. S4, C and D). In downstream TS gene expression, an approximately twofold improvement was quantified as the downstream pathway strength increased from **31** to **61** arbitrary units (a.u.) (fig. S4B).

Metabolomic analysis of the previous strains led to the identification of a distinct metabolite by-product that inversely correlated with taxadiene accumulation (figs. S5 and S6). The corresponding peak in the gas chromatography–mass spectrometry (GC-MS) chromatogram was identified as indole through GC-MS, ^1H , and ^{13}C nuclear magnetic resonance (NMR) spectroscopy studies (fig. S7). We found that taxadiene synthesis by strain **26** is severely inhibited by exogenous indole at indole levels higher than ~ 100 mg/liter (fig. S5B). Further increasing the indole concentration also inhibited cell growth, with the level of inhibition being very strain-dependent (fig. S5C). Although the biochemical mechanism of indole interaction with the isoprenoid pathway is presently unclear, the results in fig. S5 suggest a possible synergistic effect between indole and terpenoid compounds of the isoprenoid pathway in inhibiting cell growth. Without knowing the specific mechanism, it appears that strain **26** has mitigated the indole's effect, which we carried forward for further study.

In order to explore the taxadiene-producing potential under controlled conditions for the engineered strains, fed-batch cultivations of the three highest taxadiene accumulating strains (~ 60 mg/liter from strain **22**; ~ 125 mg/liter from strain **17**; and ~ 300 mg/liter from strain **26**) were carried out in 1-liter bioreactors (Figure A19-3). The fed-batch cultivation studies were carried out as liquid-liquid two-phase fermentation using a 20% (v/v) dodecane overlay. The organic solvent was introduced to prevent air stripping of secreted taxadiene from the fermentation medium, as indicated by preliminary findings (fig. S8). In defined media with controlled glycerol feeding, taxadiene productivity increased to 174 ± 5 mg/liter (SD), 210 ± 7 mg/liter (SD), and 1020 ± 80 mg/liter (SD) for strains **22**, **17**, and **26**, respectively (Figure A19-3A). Additionally, taxadiene production significantly affected the growth phenotype, acetate accumulation, and glycerol consumption [Figure A19-3, B and D, and supporting online material (SOM) text]. Clearly, the high productivity and more robust growth of strain **26** allowed very high taxadiene accumulation. Further improvements should be possible through optimizing conditions in the bioreactor, balancing nutrients in the growth medium and optimizing carbon delivery.

Having succeeded in engineering the biosynthesis of the “cyclase phase” of Taxol for high taxadiene production, we turned next to engineering the oxidation-chemistry of Taxol biosynthesis. In this phase, hydroxyl groups are incorporated by oxygenation at seven positions on the taxane core structure, mediated by CYP450-dependent monooxygenases (Kaspera and Croteau, 2006). The first

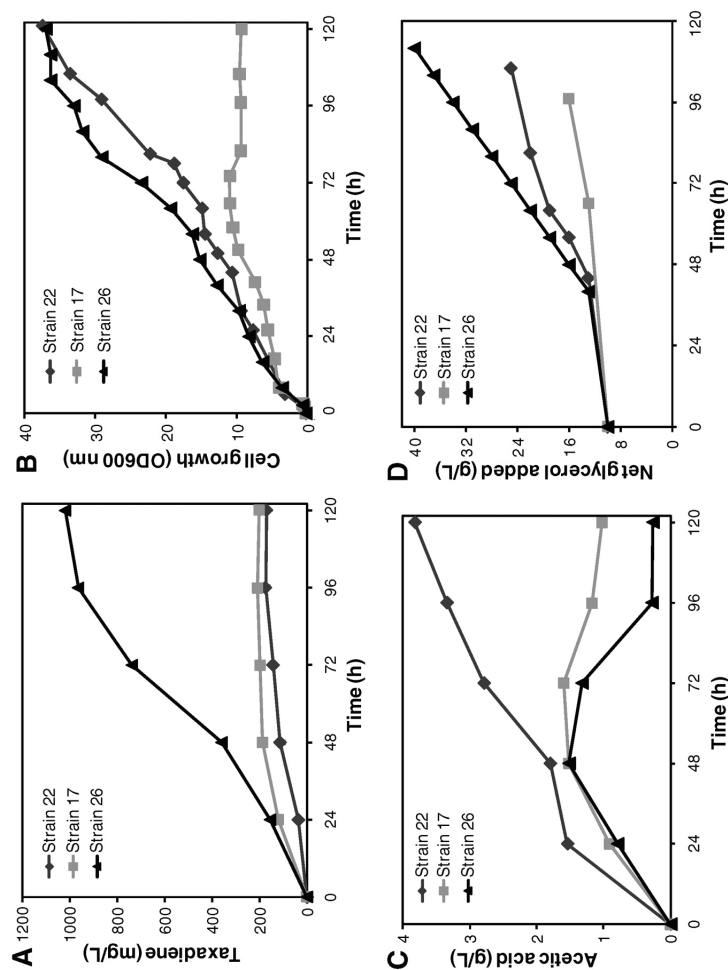


FIGURE A19-3 Fed-batch cultivation of engineered strains in a 1-liter bioreactor. Time courses of (A) taxadiene accumulation, (B) cell growth, (C) acetic acid accumulation, and (D) total substrate (glycerol) addition for strains 22, 17, and 26 during 5 days of fed-batch bioreactor cultivation in 1-liter bioreactor vessels under controlled pH and oxygen conditions with minimal media and 0.5% yeast extract. After glycerol depletes to ~0.5 to 1 g/liter in the fermentor, 3 g/liter of glycerol was introduced into the bioreactor during the fermentation. Data are mean of two replicate bioreactors.

oxygenation is the hydroxylation of the C5 position, followed by seven similar reactions en route to Taxol (fig. S1) (Jennewein et al., 2004a). Thus, a key step toward engineering Taxol-producing microbes is the development of CYP450-based oxidation chemistry *in vivo*. The first oxygenation step is catalyzed by a CYP450, taxadiene 5 α -hydroxylase, which is an unusual monooxygenase that catalyzes the hydroxylation reaction along with double-bond migration in the diterpene precursor taxadiene (Figure A19-1).

In general, functional expression of plant CYP450 in *E. coli* is challenging (Schuler and Werck-Reichhart, 2003) because of the inherent limitations of bacterial platforms, such as the absence of electron transfer machinery and CYP450-reductases (CPRs) and translational incompatibility of the membrane signal modules of CYP450 enzymes because of the lack of an endoplasmic reticulum. Recently, through transmembrane (TM) engineering and the generation of chimera enzymes of CYP450 and CPR, some plant CYP450s have been expressed in *E. coli* for the biosynthesis of functional molecules (Chang and Keasling, 2006; Leonard and Koffas, 2007). Still, every plant CYP450 has distinct TM signal sequences and electron transfer characteristics from its reductase counterpart (Nelson, 1999). Our initial studies were focused on optimizing the expression of codon-optimized synthetic taxadiene 5 α -hydroxylase by N-terminal TM engineering and generating chimera enzymes through translational fusion with the CPR redox partner from the *Taxus* species, *Taxus* CYP450 reductase (TCPR) (Figure A19-4A) (Jennewein et al., 2004a, 2005; Leonard and Koffas, 2007). One of the chimera enzymes generated, At24T5 α OH-tTCPR, was highly efficient in carrying out the first oxidation step, resulting in more than 98% taxadiene conversion to taxadien-5 α -ol and the by-product 5(12)-Oxa-3(11)-cyclotaxane (OCT) (fig. S9A).

Compared with the other chimeric CYP450s, At24T5 α OH-tTCPR yielded twofold higher (21 mg/liter) production of taxadien-5 α -ol (Figure A19-4B). Because of the functional plasticity of taxadiene 5 α -hydroxylase with its chimeric CYP450's enzymes (At8T5 α OH-tTCPR, At24T5 α OH-tTCPR, and At42T5 α OH-tTCPR), the reaction also yields a complex structural rearrangement of taxadiene into the cyclic ether OCT (fig. S9) (Rontein et al., 2008). The by-product accumulated in approximately equal amounts (~24 mg/liter from At24T5 α OH-tTCPR) to the desired product taxadien-5 α -ol.

The productivity of strain **26**-At24T5 α OH-tTCPR was significantly reduced relative to that of taxadiene production by the parent strain **26** (~300 mg/liter), with a concomitant increase in indole accumulation. No taxadiene accumulation was observed. Apparently, the introduction of an additional medium copy plasmid (10-copy, p10T7) bearing the At24T5 α OH-tTCPR construct disturbed the carefully engineered balance in the upstream and downstream pathway of strain **26** (fig S10). Small-scale fermentations were carried out in bioreactors so as to quantify the alcohol production by strain **26**-At24T5 α OH-tTCPR. The time course profile of taxadien-5 α -ol accumulation (Figure A19-4C) indicates alcohol production of up to 58 ± 3 mg/liter (SD) with an equal amount of the

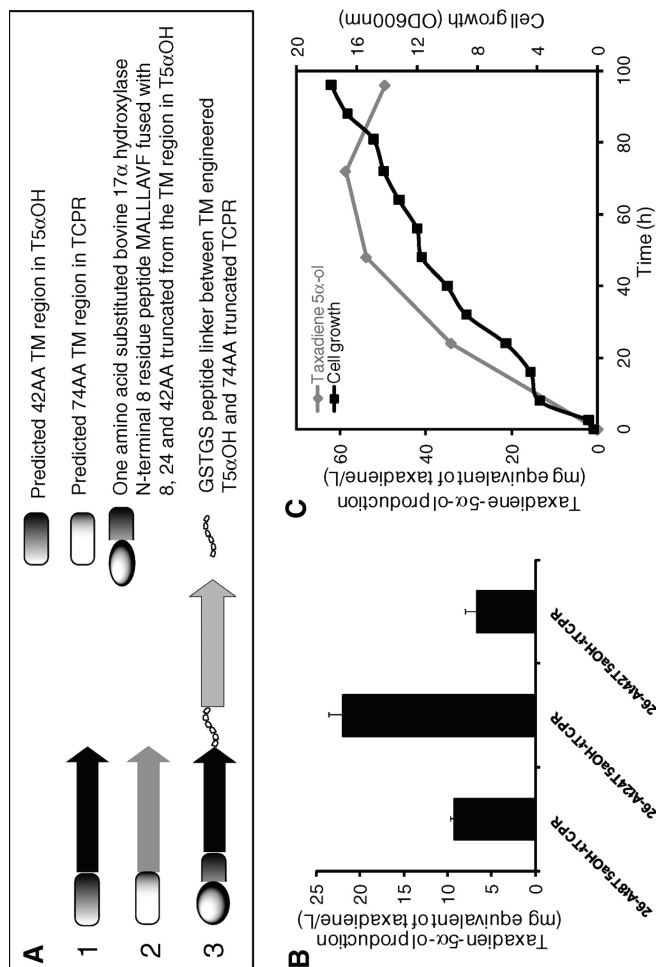


FIGURE A19-4 Engineering Taxol P450 oxidation chemistry in *E. coli*. (A) TM engineering and construction of chimera protein from taxadien-5 α -ol hydroxylase (T5 α OH) and Taxus cytochrome P450 reductase (TCPR). The labels **1** and **2** represent the full-length proteins of T5 α OH and TCPR identified with 42 and 74 amino acid TM regions, respectively, and **3** represents chimera enzymes generated from three different TM engineered T5 α OH constructs [A18T5 α OH, A124T5 α OH, and A142T5 α OH] constructed by fusing an 8-residue synthetic peptide MALLAVF (A) to 8, 24, and 42AA truncated T5 α OH through a translational fusion with 74AA truncated TCPR (tTCPR) by use of linker peptide GSTGS. (B) Functional activity of A18T5 α OH-tTCPR, A124T5 α OH-tTCPR, and A142T5 α OH-tTCPR constructs transformed into taxadiene producing strain **26**. Data are mean \pm SD for three replicates. (C) Time course profile of taxadien-5 α -ol accumulation and growth profile of the strain **26**-A124T5 α OH-tTCPR fermented in a 1-liter bioreactor. Data are mean of two replicate bioreactors.

OCT by-product produced. The observed alcohol production was approximately 2,400-fold higher than previous production in *S. cerevisiae* (Dejong et al., 2006).

The MEP pathway is energetically balanced and thus overall more efficient in converting either glucose or glycerol to isoprenoids (fig. S11). Yet, during the past 10 years many attempts at engineering the MEP pathway in *E. coli* in order to increase the supply of the key precursors IPP and DMAPP for carotenoid (Yuan et al., 2006; Farmer and Liao, 2001), sesquiterpenoid (Martin et al., 2003), and diterpenoid (Morrone et al., 2010) overproduction met with limited success. This inefficiency was attributed to unknown regulatory effects associated specifically with the expression of the MEP pathway in *E. coli* (Martin et al., 2003). Here, we provide evidence that such limitations are correlated with the accumulation of the metabolite indole, owing to the non-optimal expression of the pathway, which inhibits the isoprenoid pathway activity. Taxadiene overproduction (under conditions of indole-formation suppression), establishes the MEP pathway as a very efficient route for biosynthesis of pharmaceutical and chemical products of the isoprenoid family (fig. S11). One simply needs to carefully balance the modular pathways, as suggested by our multivariate-modular pathway-engineering approach.

For successful microbial production of Taxol, demonstration of the chemical decoration of the taxadiene core by means of CYP450-based oxidation chemistry is essential (Kaspera and Croteau, 2006). Previous efforts to reconstitute partial Taxol pathways in yeast found CYP450 activity limiting (Dejong et al., 2006), making the At24T5 α OH-rTCPR activity levels an important step to debottleneck the late Taxol pathway. Additionally, the strategies used to create At24T5 α OH-rTCPR are probably applicable for the remaining monooxygenases that will require expression in *E. coli*. CYP450 monooxygenases constitute about one half of the 19 distinct enzymatic steps in the Taxol biosynthetic pathway. These genes show unusually high sequence similarity with each other (>70%) but low similarity (<30%) with other plant CYP450s (Jennewein et al., 2004b), implying that these monooxygenases are amenable to similar engineering.

To complete the synthesis of a suitable Taxol precursor, baccatin III, six more hydroxylation reactions and other steps (including some that have not been identified) need to be effectively engineered. Although this is certainly a daunting task, the current study shows potential by providing the basis for the functional expression of two key steps, cyclization and oxygenation, in Taxol biosynthesis. Most importantly, by unlocking the potential of the MEP pathway a new more efficient route to terpenoid biosynthesis is capable of providing potential commercial production of microbially derived terpenoids for use as chemicals and fuels from renewable resources.

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Materials and Methods

SOM Text

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A20

PROGRAMMING CELLS: TOWARDS AN AUTOMATED 'GENETIC COMPILER'⁹³

*Kevin Clancy*⁹⁴ and *Christopher A Voigt*⁹⁵

One of the visions of synthetic biology is to be able to program cells using a language that is similar to that used to program computers or robotics. For large genetic programs, keeping track of the DNA on the level of nucleotides becomes tedious and error prone, requiring a new generation of computer-aided design (CAD) software. To push the size of projects, it is important to abstract the designer from the process of part selection and optimization.

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The vision is to specify genetic programs in a higher-level language, which a genetic compiler could automatically convert into a DNA sequence. Steps towards this goal include: defining the semantics of the higher-level language, algorithms to select and assemble parts, and biophysical methods to link DNA sequence to function. These will be coupled to graphic design interfaces and simulation packages to aid in the prediction of program dynamics, optimize genes, and scan projects for errors.

Introduction

Numerous genetic circuits have been built that encode functions that are analogous to electronic circuits (Stricker et al., 2008; Ham et al., 2006; Voigt, 2006). When multiple circuits are connected to sensors and actuators, this forms a genetic program. For example, we constructed an ‘edge detector’ program that combines a ANDN gate, light sensor, and cell–cell communication that give bacteria the ability to draw the edge between the light and dark regions of an image projected onto a plate (Salis and Kaznessis, 2006). Other genetic programs have been built that combine circuits to produce a push-on/push-off circuit (Lou et al., 2009+), implement a counter (Friedland et al., 2009++), and reproduce predator–prey dynamics (Balagadde et al., 2008). These represent toy systems, but the implementation of such programs in applications for industrial biotechnology is inevitable.

Automated DNA synthesis gives genetic engineers an unprecedented design capacity (Czar et al., 2008). This technology enables the specification of every basepair for long sequences, without having to be concerned about the path to construction. Together with methods to rapidly combine genetic parts (Gibson et al., 2009++; Shetty et al., 2008) and assembly methods that scale to whole genomes (Gibson et al., 2008, 2010; Wang et al., 2009), the problem of DNA construction has far outpaced our capacity for design (Purnick and Weiss, 2009+). A good example of this is the 2006 UCSF iGEM team to build a ‘remote-controlled bacterium.’ DNA synthesis was used to build the first construct (requiring a few weeks), but after four years of additional tinkering, the paper will be submitted in 2010.

Our ability to design programs has been hampered by three problems. First, there is a lack of good, robust genetic circuits that can be easily connected. Second, there are few design rules that are sufficiently quantitative to be carried out algorithmically. Modeling can be helpful before the experiments to determine the topologies and parameter regimes required to obtain a particular function. However, simulations cannot be used to ‘reach down’ to the DNA and suggest a specific mutation or select a part. Third, the frequency of mistakes in the DNA sequence increases quickly with size. Currently, to scan for potential errors (e.g. transposon insertion sites or putative internal promoters), it requires the running

of multiple (usually web-based) programs. There is no unified software package to date that addresses all of these issues.

The creation of a simulation environment for genetic engineering is complicated by the diversity of cellular functions. When studying natural networks, there is a feeling of ‘peeling an onion,’ where there are seemingly endless redundancies and classes of biochemical interactions. Even within the Registry of Standard Biological Parts (www.partsregistry.org), there are a wide variety of cellular functions: from enzymes and transcription factors to multi-gene gas vesicles and secretion chaperones. Each specific problem requires its own style of simulation; a dynamic program may be well satisfied by sets of differential equations, pattern formation by cellular automata, and enzymes by metabolic flux analysis. It would be daunting to create a simulation package that could encompass all of this diversity.

To reduce the problem complexity and to frame recent computational work, we introduce the concept of a ‘Genetic Compiler,’ whose inputs are high-level instructions (equivalent to VHDL or Verilog) and whose output is a DNA sequence. The sequence can be sent to a company for DNA synthesis or a robot for automated assembly. The problem is constrained by focusing on genetic programs that encode a desired logical or dynamical function, which can be integrated into many applications in biotechnology (Figure A20-1). This avoids the application-specific portions of the problem; for example, building a butanol sensor a particular metabolic pathway. It is distinct from tools for protein or metabolic engineering (Cho et al., 2007).

The scope of this review is on the underlying algorithms and biophysical methods that would power such a compiler (Figure A20-2). Realizing this goal will require: 1. Libraries of reliable genetic circuits designed specifically to be part of a CAD program, 2. the definition of a higher-level language, 3. algorithms to assemble circuits according to a specified program, 3. biophysical methods to connect and optimize circuits, 4. simulation programs to debug the program dynamics, 5. algorithms for DNA assembly and experimental design. The scope has been limited to exclude several topics that are crucial to synthetic biology, but have been well-reviewed elsewhere, notably codon optimization and tools from systems biology and metabolic engineering (Cho et al., 2007; Richardson et al., 2010; Villalobos et al., 2006).

Main Text

Robust combinatorial logic

Combinatorial logic is implemented by Boolean circuits and is the basis for digital computing. It is used to build circuits that apply Boolean algebra on a set of inputs to transform them into a set of desired outputs. Simple circuits can be layered in different configurations in order to achieve a computational operation.

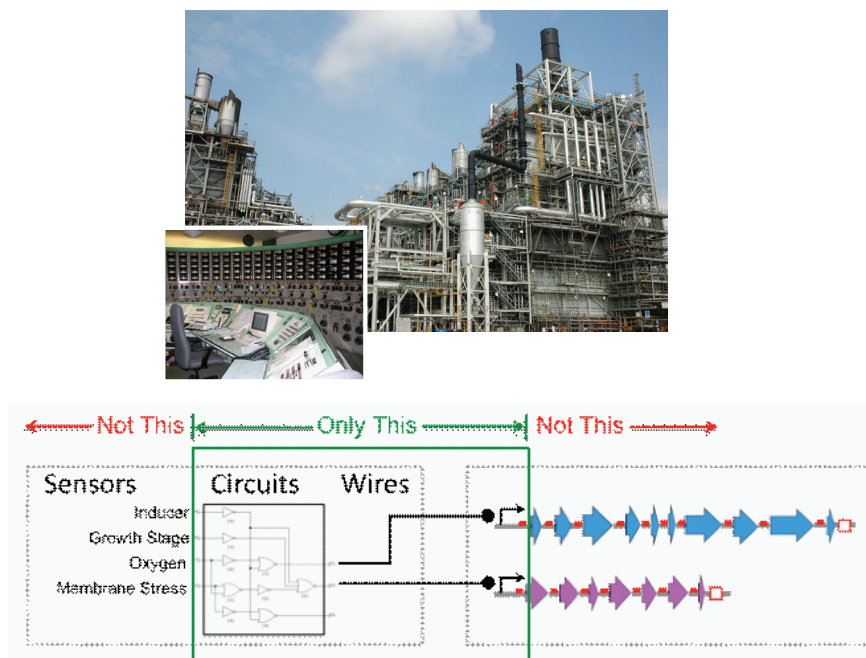


FIGURE A20-1 The compiler is focused on assembling the circuitry that links the inputs and outputs of a larger project. The inputs include genetic sensors that can respond to diverse signals, such as temperature, light, stress, or metabolites. The circuitry encodes the logic and dynamics. The output of the circuits control actuators, such as a metabolic pathway, the activation of cell–cell communication, or a stress response. The inputs and outputs tend to be problem-specific and the diversity of biological applications makes this difficult to encompass in a single simulation package. By contrast, the circuitry can be reconfigured to build programs relevant to diverse problems in biotechnology.

This has enabled the automated design that underlies VLSI. The ability for digital circuits to be flexibly used and easily captured by CAD comes at a cost of speed, design size, and power (Sarpeshkar, 2010++). There are ongoing efforts to design the core biochemistry that would act as genetic logic gates (Salis and Kaznessis, 2006; Ramalingam et al., 2009; Kinkhabwala and Guet, 2008; Cox et al., 2007; Rackham and Chin, 2006; Kramer et al., 2005; Benenson, 2009; Sharma et al., 2008+; Dueber et al., 2003; Anderson et al., 2007; Rinaudo et al., 2007; Buchler et al., 2003). There are several important considerations in designing genetic gates to be used in CAD:

- *Scalability.* Each circuit in a project needs to be orthogonal because all of the circuits operate based on biochemical interactions within the cell. A circuit is scalable when the underlying genetic parts can be swapped

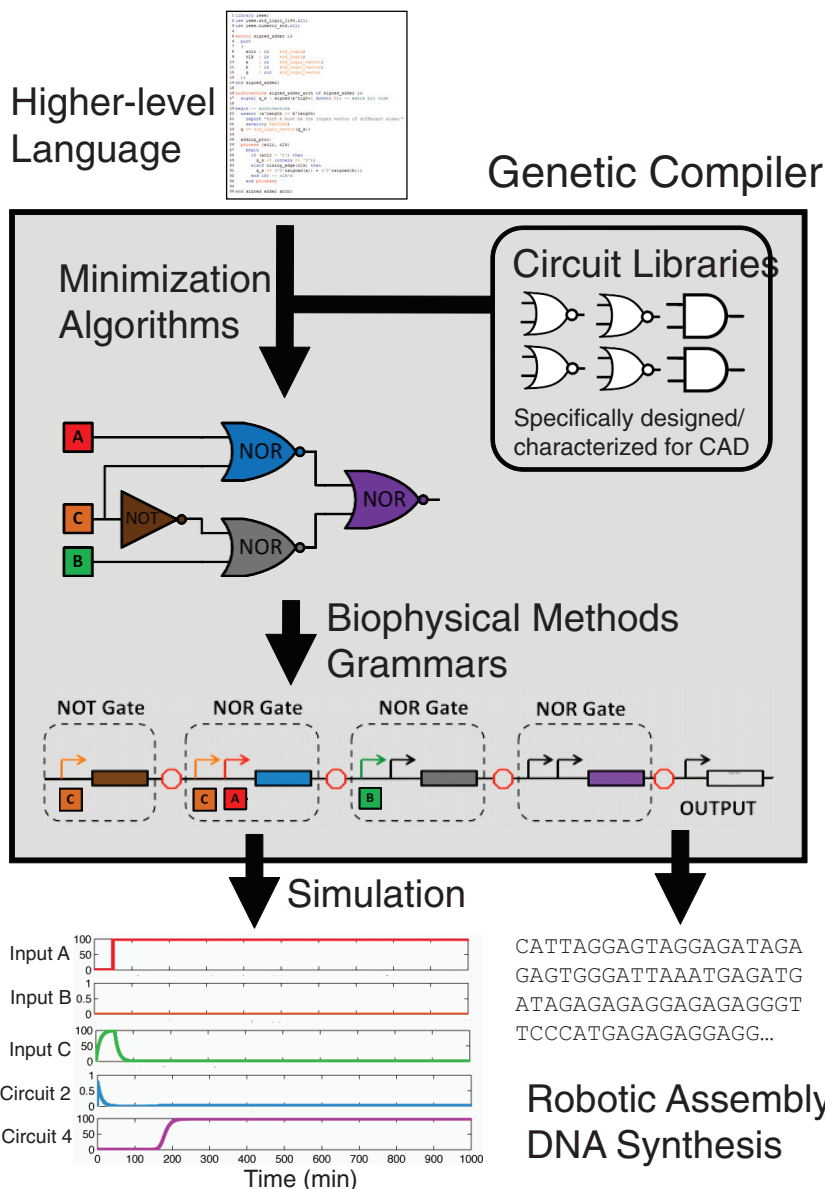


FIGURE A20-2 A genetic compiler. The compiler automatically converts a higher-level language to a DNA sequence. Ideally, the designer would be completely blind to these steps (gray box). Simulations aid the debugging of the program, but the debugging would occur within the higher-level language.

to create orthogonal circuits. For example, orthogonal NOT gates could be built based on new repressor-operator pairs (Yokobayashi et al., 2002). By contrast, an AND gate that we constructed is not scalable because its underlying parts do not lend themselves to making multiple gates (Anderson et al., 2007).

- *Extensibility.* In order to layer genetic circuits, the inputs and outputs of the circuit have to have the same signal carrier (Canton et al., 2008). Put simply, for circuits to be connected, the inputs and the outputs need to be the same biochemical form (phosphorylation, transcription, etc.). For transcriptional circuits, it has been proposed that the flux of RNAPs on DNA could serve this purpose (Canton et al., 2008). In practice, this can be implemented by making the circuit inputs and the outputs be promoters. Several examples of this are a NOT gate (Yokobayashi et al., 2002) and an AND gate (Anderson et al., 2007). This is in contrast to circuits where, for example, the inputs are small molecules and the output is the activation of a riboswitch (Sharma et al., 2008+).
- *Modularity.* The inputs and outputs of the circuits need to be able to be easily changed. Considering CAD, the ease has to be sufficient for a computer to be able to reliably perform this function. Promoters, protein–protein interaction domains, and RNA are sufficiently modular for this purpose (Sharma et al., 2008+). Some biological systems that are considered modular—such as the protein domains of bacterial two-component systems—are not sufficiently modular for CAD. This is because small libraries are required to identify a functional chimera, as opposed to design rules that can be implemented by a machine.
- *Robustness.* The circuits must remain functional over large regions of parameter space. This is particularly important when connecting multiple circuits in series, where robust circuits will allow for more uncertainty while still producing a functional connection (Salis and Kaznessis, 2006; Salis et al., 2009). The circuits also have to be non-toxic and have minimal impact on host processes (Tan et al., 2009++).
- *Speed.* The circuits also need to be reasonably fast. For example, a three-layer cascade of NOT gates required 20 min for each layer to compute (Hooshangi et al., 2005), but a DNA inversion switch requires 4 h (Ham et al., 2006). Even 20 min may be too slow for some applications, thus requiring logic to be implemented by phosphorelays or protein–protein interactions, which can occur in milliseconds (Groban et al., 2009).

Higher-level languages and their deconstruction

A higher-level language abstracts the designer from the details of the implementation. In electronics, it enables the CPU operations to be hidden. In synthetic biology, it would hide the details of the molecular biology and DNA sequence, and possibly even the choice of circuits. Impeding the development of a higher-

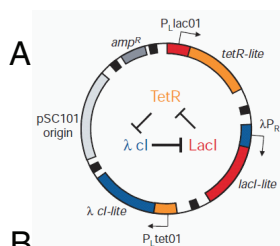
level language in synthetic biology is the diversity of cellular functions. This can be partially overcome by limiting the language to describe problems that are common to many applications, while leaving the application-specific aspects unabstracted (Figure A20-1). There are two areas that have shown promise in moving towards such a language. First, vocabularies and grammars are being developed that implement design rules and can be used to describe genetic parts and their combination. This will form the backbone of the higher-level language. Second, there are a number of algorithms that can be borrowed from electrical engineering, notably logic minimization, which can convert the language into integrated circuits.

The semantics of genetic programs

A higher-level language in synthetic biology needs a vocabulary and grammar to represent genetic parts and the rules underlying how they are combined to build circuits and programs (Cai et al., 2007). A vocabulary captures how genetic functions are described and how the data underlying a part is stored and accessed. A rule is an enforced relationship between parts. For example, an expression cassette is defined as needing a promoter, cistron, and terminator and a cistron requires a RBS sequence and gene (Cai et al., 2009+).

An example of a program that describes a well-known genetic oscillator (the ‘repressilator’) is shown in Figure A20-3. At first glance, this appears unnecessarily complex compared to the more familiar plasmid visualization. However, it becomes advantageous as the designs get larger and more complex. Plasmid construction programs (like Vector NTI or ApE) toggle between the plasmid map for visualization and the DNA sequence for design. Both are difficult for debugging large programs, whereas formal grammars give an intermediate level of abstraction for writing programs and the rules enable automated troubleshooting (Cai et al., 2009+). Rules also allow for the permutation of parts in order to create multiple designs (Densmore et al., 2010b). The high-level language is also very modular and creates and separates the rules from the construction of parts and devices. This allows the complexity of a genetic program to be in-lined by a compiler/interpreter and never seen by the user after the parts are specified.

An emerging group of tools (GenoCAD, Eugene, and GEC) propose formalisms for vocabularies and provide a means to embed domain expertise in software allowing less experienced users to benefit from it (Czar et al., 2009; Cai et al., 2010; Bilitchenko et al., 2010+). Domain experts can express problem-specific design strategies as formal languages using a controlled vocabulary to represent the types of genetic parts usable in a particular organism or even for a particular application (Cai et al., 2010). Formal languages provide a means to develop a knowledge base on a collection of defined terms organized with respect to their structural relationships to each other in the DNA sequence (Cai et al., 2007). The advantage of this approach is that the relationship of the individual parts to each other are defined in the software and can provide a means of indexing and re-



```

/* Property definitions */
Property Sequence(txt);
Property Neg35StartEnd(txt);
Property Neg10StartEnd(txt);
Property OperatorSites(txt[]);
Property OperatorSiteLocations(txt[]);
Property CorrespondingProtein(txt);
/* Part definitions */
Part Promoter(Sequence, Neg35StartEnd, Neg10StartEnd, OperatorSites,
OperatorSiteLocations);
Part RBS(Sequence);
Part CodingDNA(Sequence, CorrespondingProtein);
Part Terminator(Sequence);
/* Part declarations */
Promoter araP(.Sequence("TTTACATAGCATTTTTATCCATAATATGTTAGCGGATCCTAAGC"),
.Neg35StartEnd("[1,4]", .Neg10StartEnd("[25,29]"),
.OperatorSites(["araI1", "araI2"]), .OperatorSiteLocations(["[5,24]",
"[30,44]"]));
Promoter lacP(.Sequence("TTGACATTGTGAGCGGATAACAGTACT"),
.Neg35StartEnd("[3,6]", .Neg10StartEnd("[24,29]"), .OperatorSites(["lacO1"]),
.OperatorSiteLocations(["[7,23]"]));
Promoter tetP(.Sequence("TTGACATCCCATCATCAGTGATAGACT"),
.Neg35StartEnd("[1,6]", .Neg10StartEnd("[26,29]"), .OperatorSites(["tetO2"]),
.OperatorSiteLocations(["[7,25]"]));
RBS rbs1();
RBS rbs2();
RBS rbs3();
CodingDNA DNAlac(.CorrespondingProtein("LacI"));
CodingDNA DNAtet(.CorrespondingProtein("TetR"));
CodingDNA DNAara(.CorrespondingProtein("araC"));
Terminator term1();
Terminator term2();
Terminator term3();
/* Rule declarations and assertions */
// Each promoter appears before an RBS.
Rule promoterBeforeRBS1(araP BEFORE rbs1);
Rule promoterBeforeRBS2(lacP BEFORE rbs2);
Rule promoterBeforeRBS3(tetP BEFORE rbs3);
Assert(promoterBeforeRBS1 AND promoterBeforeRBS2 AND promoterBeforeRBS3);
// Each RBS appears immediately before the coding region.
Rule rbsBeforeCoding1(rbs1 BEFORE DNAlac);
Rule rbsNextToCoding1(rbs1 NEXTTO DNAlac);
Rule rbsBeforeCoding2(rbs2 BEFORE DNAtet);
Rule rbsNextToCoding2(rbs2 NEXTTO DNAtet);
Rule rbsBeforeCoding3(rbs3 BEFORE DNAara);
Rule rbsNextToCoding3(rbs3 NEXTTO DNAara);
Assert(rbsBeforeCoding1 AND rbsNextToCoding1);
Assert(rbsBeforeCoding2 AND rbsNextToCoding2);
Assert(rbsBeforeCoding3 AND rbsNextToCoding3);
// A terminator appears immediately after the coding region.
Rule termAfterCoding1(term1 AFTER DNAlac);
Rule termNextToCoding1(term1 NEXTTO DNAlac);
Rule termAfterCoding2(term2 AFTER DNAtet);
Rule termNextToCoding2(term2 NEXTTO DNAtet);
Rule termAfterCoding3(term3 AFTER DNAara);
Rule termNextToCoding3(term3 NEXTTO DNAara);
Assert(termAfterCoding1 AND termNextToCoding1);
Assert(termAfterCoding2 AND termNextToCoding2);
Assert(termAfterCoding3 AND termNextToCoding3);
// Specific coding regions appear before the promoter.
Rule promoterToCoding1(araP BEFORE DNAlac);
Rule promoterToCoding2(lacP BEFORE DNAtet);
Rule promoterToCoding3(tetP BEFORE DNAara);
Assert(promoterToCoding1 AND promoterToCoding2 AND promoterToCoding3);
// Each promoter and gene only appears once on the device.
num x = 1;
Rule uniquePromoter1(araP NOTMORETHAN x);
Rule uniqueCoding1(DNAlac NOTMORETHAN x);
Rule uniquePromoter2(lacP NOTMORETHAN x);
Rule uniqueCoding2(DNAtet NOTMORETHAN x);
Rule uniquePromoter3(tetP NOTMORETHAN x);
Rule uniqueCoding3(DNAara NOTMORETHAN x);
Assert(uniquePromoter1 AND uniqueCoding1);
Assert(uniquePromoter2 AND uniqueCoding2);
Assert(uniquePromoter3 AND uniqueCoding3);
/* Repressilator device declaration */
Device Repressilator(araP, rbs1, DNAlac, term1, lacP, rbs2, DNAtet, term2,
tetP, rbs3, DNAara, term3);

```

FIGURE A20-3 Semantics of genetic programs. The plasmid map (a) and Eugene code (b) for a genetic oscillator (Elowitz and Leibler, 2000) is shown (b). The author of the Eugene code is Adam Liu (Bilitchenko et al., 2010+).

trieving knowledge about parts and designs associated with defined terms. When expressing design strategies with formal languages it is possible to use parsers to verify that parts used in a design are properly positioned with respect to each other (Goler et al., 2008). Thus, users have a level of *in silico* validation through the use of such systems. Beyond this syntactic or structural layer, languages can be augmented by adding a semantic layer to derive dynamics encoded in complex DNA sequences composed of interacting parts using algorithms originally developed to compile the source code of computer programs (Cai et al., 2009+).

Instead of simulating the function of a DNA sequence, it is possible to perform a semantic analysis of DNA sequences using expert systems capable of reasoning using the relationships between defined terms to infer many aspects of their use, assembly based on data present in the part or associated with the biology from which the part was derived. An expert system would scan *in silico* designs for logical errors in construction, identify constraints that would preclude use of particular combinations of parts. Such an expert system can be constructed using ontologies—a rigorous organization of knowledge concerning a particular domain of knowledge (Arp and Smith, 2008). Ontologies are particularly good examples of expert systems in that they permit the development of a model of the entities and their relationships within the domain (Shah et al., 2009). Such definitions are logically consistent and can be used by reasoner softwares to verify known information on entities tagged by the ontology and to infer new information based upon these definitions. Softwares developed to use ontologies have the advantage that as data models change in the ontology, the software simply needs to adapt to the ontology, simplifying many aspects of software data model development and design (Shah et al., 2009). The synthetic biology community has started the Synthetic Biology Ontology Language to represent concepts tied to synthetic biology (Jiang and Hui, 2008).

A larger model that the synthetic biology community can take advantage of is the National Center for Biomedical Ontologies (Clevers, 2006). The NCBO supports researchers by providing resources to access, review, and integrate many biomedical ontologies. The Ontology for Biomedical Research (OBI) is especially interesting as it has been developed to describe the disparate elements and their relations that are necessary and sufficient to describe an experiment (Smith et al., 2007). Many of the concepts from OBI are very applicable to the description of synthetic biology workflows, protocols, parts, and device usage. NCBO also promotes standards for sharing data across ontologies, most notably the Minimum Information to Reference an External Ontology (Xiang et al., 2010). Use of MIREOT guidelines during the development of an ontology will support the consistent inclusion of terms from ontologies as diverse as Gene, Chemical Information and Pathway Information and Phenotypic Information.

Logic minimization

Several programs developed in electrical engineering have the ability to take a truth table as an input and then output a wiring diagram from simpler circuits (Figure A20-4). The ESPRESSO program, developed in the early 1980s, has been used extensively for logic minimization in VLSI design (Rudell, 1986) and it is embedded along with other tools in the abc program that is currently maintained by UC-Berkeley (Mishchenko, 2010). The output of the logic minimization tools feeds into programs, such as Logic Friday (Wu et al., 2009+), which both act as a visualization tool and enable constraints to be applied to the construction of a circuit diagram. The minimum identified by these programs is not guaranteed to be the global minimum. The logic minimization programs will have to be modi-

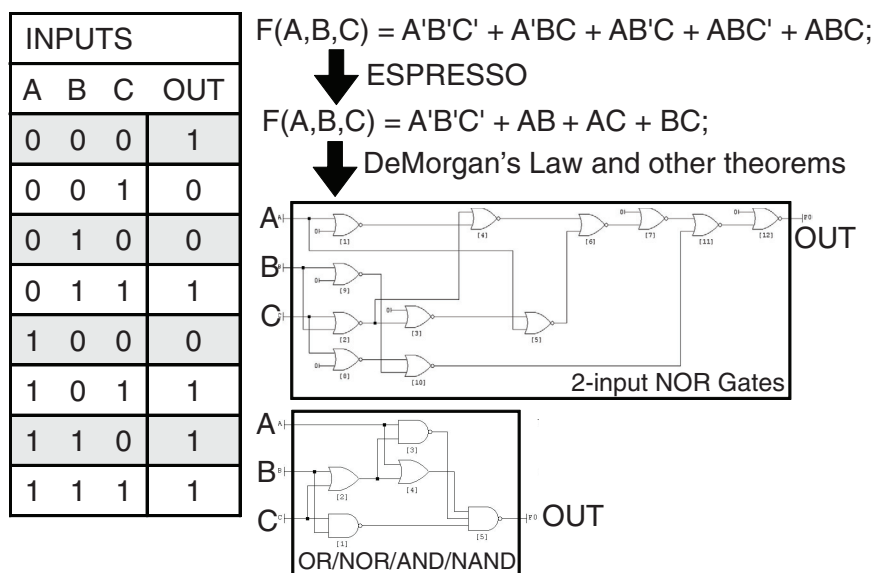


FIGURE A20-4 Automated program design using logic minimization algorithms. An example of a multi-input single-output truth table is shown. The truth table is converted to an equation F , which is a function of the four inputs (a,b,c,d). Each term corresponds to each row of the truth table where the output is 1 and the prime (') is shorthand for the NOT function. Logic minimization algorithms, such as ESPRESSO (Rudell, 1986), can be used to simplify the full equation to its minimal form (simplest sum of products). This equation is then converted to circuit diagrams using programs such as Logic Friday (Wu et al., 2009+), which can also implement constraints. For example, the large wiring diagram consists of only 2-input NOR gates (top), whereas the smaller wiring diagram was built allowing for multiple-input OR/NOR/AND/NAND gates. Biological constraints, such as gate availability and DNA size can then be applied to search for the optimal diagram.

fied to include biological constraints, including the size of the DNA, and availability and orthogonality of the gates. Two additional considerations in choosing a wiring diagram are:

- Fault tolerance with respect to asynchronous computing. Genetic circuits are asynchronous because there is no clock controlling when each layer of the computation is performed (Seitz, 1979). Delays in the progression of the signal can lead to faults in the calculation. One approach to this problem is to build a genetic clock, and progress towards a robust oscillator (Stricker et al., 2008; Danino et al., 2010++) and counter (Friedland et al., 2009++) indicate that this may be possible. The design of asynchronous logic blocks that are robust to faults is also an active area of research, especially with respect to minimizing power requirements (Cortadella et al., 2006; Manohar, 2006; Rahbaran and Steininger, 2009). The principles from this work could be applied to the construction of integrated genetic logic, although unlike simple logic minimization, there has been historically a lack of CAD tools for asynchronous circuit design (Gibson et al., 2009++; Josephs and Furey, 2002). Recently, a system for asynchronous system design (RALA) has been developed and this framework is particularly applicable for biological systems (Gershenfeld et al., 2010+).
- Robustness to perturbations. The wiring diagrams need to be robust with respect to perturbations due to fluctuations in molecules and environmental conditions (Rodrigo and Jaramillo, 2008). In the design of electronic circuits, it has been noted that asynchronous circuits are also more robust (Gibson et al., 2009++; Rahbaran and Steininger, 2009). Macia and Sole enumerated digital systems composed of layered NAND gates and found that fault tolerance resulted from designs that contained a high degree of degeneracy, defined as occurring when multiple subsets of a circuit diagram that are structurally different perform the same function (Macia and Sole, 2009+).

Biophysical models of part function

The automated selection of genetic parts is one of the most difficult aspects of synthetic biology CAD (Kazenes, 2007). Biophysical models can map the DNA sequence of a genetic part to its function. This enables the impact of an individual mutation to calculate for a part and then coupled to a dynamical model to determine the effect on circuit function. In other words, they facilitate linking a simulation of reaction kinetics to physical DNA. Biophysical models are also useful in screening parts selected from a registry for context effects and can scan the DNA sequence of programs to identify unintended functional sequences (e.g. internal promoters or terminators) and correct potential errors.

Cellular regulation is highly redundant and there are often many ways to control the same parameter. For example, there are many options in controlling the concentration of a protein, from plasmid copy number to protein stability. This gives flexibility to the designer to take whatever route is convenient. In terms of design automation, it will be important to choose those routes that are the most reliable when modeled on the computer. For example, the interactions between RNA basepairs is relatively straightforward to calculate and efficient algorithms have been developed to calculate the folding of RNA secondary structure (Dirks et al., 2007; Markham and Zuker, 2008). This has formed the core of predictive methods to capture how changes in the sequence affect the strength of ribosome binding sites (Salis et al., 2009; De Smit and van Duin, 1990; Na et al., 2010), the efficiency of terminators (Carafa et al., 1990; Yager and von Hippel, 1991; Kingsford et al., 2007+), the shape of the transfer function of an shRNA switch controlled by a small molecule (Beisel et al., 2008), and to create orthogonal rRNA–mRNA binding sequences (Chubiz and Rao, 2008). In each of these models, the effect of arbitrary mutations on part function can be quantitatively calculated.

The activity of promoters has been successfully modeled using position weight matrixes (PWMs) (Rhodius and Mutalik, 2010++; Li et al., 2002). These models predict the free energy by which a transcription factor binds to its operator sequence in a promoter. They make an additive assumption, where each base in the operator is assumed to be independent and a relatively small data set is used to parameterize a model. PWMs have suffered from high false positive rates, which has been a challenge in using them to scan genomes for promoters. However, they have been very successful at modeling how mutations affect the strength of a given promoter (Rhodius and Mutalik, 2010++). In this sense, they are ideal for applications where it is necessary to link how mutations will affect circuit dynamics by impacting promoter function.

Biophysical models are particularly applicable to a common problem that occurs when connecting genetic circuits in series (Figure A20-5). If the output of the first circuit is not in the correct dynamic range to trigger the second circuit, then the combined circuits will not function properly. One way to fix this is to vary the ribosome binding site (RBS) linking the circuits, typically by substituting different sites from a part library (Tabor et al., 2009; Temme et al., 2008) or through random mutagenesis (Anderson et al., 2006, 2007). More recently, a biophysical model of RBS function was developed and shown to be able to identify *de novo* RBS sequences that can connect two previously characterized circuits (Salis et al., 2009).

This approach could accelerate the automated connection and optimization of many genetic circuits.

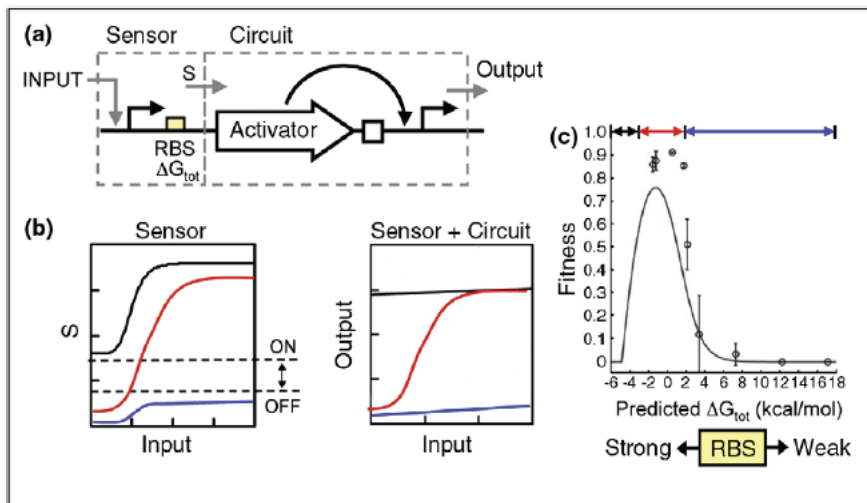


FIGURE A20-5 Connecting genetic circuits. (a) A simple program is shown consisting of a sensor and circuit. The sensor turns on a promoter in response to an INPUT. A RBS controls a translation initiation rate S that then serves as an input to the circuit. The circuit consists of an activator that turns on a promoter that serves as the output. (b) If the transfer function of the sensor is too low (blue) or the basal level is too high (black), then it will not cross the dynamic range to turn on the circuit (dotted lines). When the sensor output crosses that which is required to trigger the circuit, then the program is functional (red). (c) Experimental data is shown where the RBS is modified in the connection of a sensor to an AND gate. The solid line is the predicted fitness curve derived from the transfer functions of the sensor and circuit measured in isolation of the program.

Simulation and design environments

Kinetic simulations are commonly used to study the dynamics of molecules in the cells and there are a number of packages from systems biology that aid the modeling of signaling and regulatory networks (Andrews et al., 2010; Tomita et al., 1999; Casanova et al., 2004; Ander et al., 2004). A limitation in applying these tools to synthetic biology is the need to connect the output of a simulation to a specific design choice that is on the level of the DNA sequence, whether it be a mutation or the choice of a part. In the context of a compiler, the role of simulations would be to predict the dynamics of the assembled genetic circuits and serve as a debugging tool that would aid the user in writing the higher-level language (Figure A20-2).

A number of software tools have been developed to facilitate the selection and combination of parts gleaned from the Registry of Standard Biological Parts. For example, BioJade, SynBioSS, and TinkerCell enable the import of BioBricks

along with descriptive parameters sets that can form the basis for simulations or algorithms for part selection (Hill et al., 2008; Goler, 2004; Chandran and Bergmann, 2009; Cooling et al., 2010). Several algorithms enable the selection of parts to match an objective function that describes a desired circuit, such as an oscillator, toggle switch or logic gate (Rodrigo and Jaramillo, 2008; Dasika and Maranas, 2008). All of these simulation packages suffer from a lack of parts and circuits whose kinetics are well-documented and easily imported.

An underlying assumption of this approach is that parts will identical kinetic parameters when measured individually or in the context of different genetic programs. There are several elegant examples where this assumption has been shown to hold, including in the construction of AND gates (Salis and Kaznessis, 2006), toggle switches, and feedforward loops (Ellis et al., 2009++) through the assembly of different permutations of underlying parts. In our work with the edge detector, we found that the behavior of the program could be explained based on the component circuits (Salis and Kaznessis, 2006). However, it is not clear how often this is true and there are many published (as well as unpublished!) reports where the final behavior could not be predicted based on the components (Tan et al., 2009; Guet et al., 2002; Murphy et al., 2007). How to quantitatively measure and report this information is still an open question.

Genetic circuits usually consist of small numbers of molecules, and stochastic effects can dominate. There has been much work performed in the area of stochastic modeling and this has been applied to understanding natural and synthetic genetic networks (Salis and Kaznessis, 2006). A challenge is to convert the results of a stochastic simulation to a design choice; for example, the selection of a promoter. Noise can be controlled through the position of an operator in a promoter (Murphy et al., 2007), and it is influenced by the number of layers in a genetic program (Pedraza and van Oudenaarden, 2005). In a theoretical study, Hasty and co-workers demonstrated that the variability of the circuit response can be tuned by independently controlling the transcription and translation rates and DNA copy number (Lu et al., 2008). Cytometry data is the most common source of data in the characterization of genetic circuits and this contains significant and often unutilized information about the variability in the circuits. Munsky et al. have developed a method that enables the full cytometry distributions to be used to parameterize a genetic circuit, including its stochastic behavior (Munsky et al., 2009++).

Optimizing DNA assembly and experimental design

Once the DNA sequence is debugged on the computer, it needs to be constructed. This can either be by automated DNA synthesis or by the robotic assembly of parts. Despite the declining cost of DNA synthesis, the ability to automatically assemble parts using BioBricks and related cloning strategies will remain advantageous when it is desired to make many combinatorial variants of

a genetic system. This is useful when there is potential degeneracy in the design, allowing multiple constructs to be tested for function. Often, only a subset of the permutations function as expected.

Grammars have been modified to include information for assembly (Cai et al., 2010). The inclusion of rules can significantly reduce the construction cost by reducing the number of variants that need to be assembled (Densmore et al., 2010b). This can be further coupled to kinetic simulations to reduce the permutations to those that are likely to perform a given circuit function. For example, Peccoud and co-workers have applied grammars to permutations of the toggle switch in order to identify those that are likely to perform the correct function (Cai et al., 2009+). After rules and simulations constrain the set of permutations, a path to fabrication needs to be determined. Densmore and co-workers have developed algorithmic methods to reduce the path length in the number of assembly steps when constructing a genetic system (Densmore et al., 2010a+). The same approach can be applied to reducing the cloning steps when using the same set of underlying parts to assemble many systems.

Methods from the statistical design of experiments (DOE) could also be integrated into a CAD program (Bailey, 2008; Atkinson et al., 2007). Of particular interest is the design of sequential experiments, where the design of the next round of DNA constructs is influenced by the results from the previous round. This has been applied to highly dimensional problems, such as the optimization of fermentation, where there are many variables [pH, feed rate, oxygen, media, etc.] that need to be optimized. In the context of genetic programs, the rigorous application of DOE methods would randomize an initial set of constructs. After the constructs were measured for the desired function, this would be fed back to the algorithm to identify the next set of constructs. The process is iterated until the function is optimized. This is a particularly powerful approach when coupled with optimization algorithm, such as Nelder-Mead or ant colonization (Dorigo et al., 2006).

Conclusions

Genetic engineering is moving towards becoming an information science. The model of storing and distributing genetic material is slowly losing relevance. It is routine to outsource the task of constructing DNA from the designer to synthesis facilities. This has created a strong need for computer-aided design programs that are able to facilitate the organization and construction of large projects. Once the parts are experimentally characterized, it is unnecessary to distribute the DNA. Rather, the CAD program can access the sequence and function information to design a genetic program. The sequence information corresponding to the desired program is sent to a synthesis/assembly facility for construction.

Existing genetic circuits have not been designed to be sufficiently robust to be automatically connected using CAD. These circuits and the genetic parts on

which they are based need to be constructed specifically with the intent of coupling with a CAD program. One of the key problems is that there simply are not enough robust circuits. There are significant efforts underway to develop computational methodologies specifically to create the orthogonal parts that would underlie such programs (Chubiz and Rao, 2008; Mandell and Barbas, 2006; Desai et al., 2009++). What is particularly needed are sets of orthogonal transcription factors that bind to different operator sequences. It is the co-development of fundamental circuits with the computational algorithms to assemble them that will allow us to move towards the vision of being able to program living cells.

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A21

PROKARYOTIC GENE CLUSTERS: A RICH TOOLBOX FOR SYNTHETIC BIOLOGY⁹⁶

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Bacteria construct elaborate nanostructures, obtain nutrients and energy from diverse sources, synthesize complex molecules, and implement signal processing to react to their environment. These complex phenotypes require the coordinated action of multiple genes, which are often encoded in a contiguous region of the genome, referred to as a gene cluster. Gene clusters

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Abbreviations: PCB, polychlorinated biphenyls; **PHA**, poly(hydroxyalkanoate); **PHB**, poly(3-hydroxybutyrate)

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sometimes contain all of the genes necessary and sufficient for a particular function. As an evolutionary mechanism, gene clusters facilitate the horizontal transfer of the complete function between species. Here, we review recent work on a number of clusters whose functions are relevant to biotechnology. Engineering these clusters has been hindered by their regulatory complexity, the need to balance the expression of many genes, and a lack of tools to design and manipulate DNA at this scale. Advances in synthetic biology will enable the large-scale bottom-up engineering of the clusters to optimize their functions, wake up cryptic clusters, or to transfer them between organisms. Understanding and manipulating gene clusters will move towards an era of genome engineering, where multiple functions can be “mixed-and-matched” to create a designer organism.

1. Introduction

Gene clusters are the genetic building blocks of bacteria and archaea. Prokaryotic genomes are highly organized and the genes associated with a particular function often occur near each other (Fischbach et al., 2008). Occasionally, all of the genes that are necessary for a discrete function form a cluster in the genome. These clusters encode functions that affect all aspects of the life style of bacteria, including nutrient scavenging, energy production, chemical synthesis, and environmental sensing. Large protein scaffolds, nanomachines, and cytoplasmic organelles are also encoded within clusters. These functions could play a central role for many applications in biotechnology; however, their complexity makes them difficult to engineer. Here, we survey the wide range of cellular functions that are known to be encoded in these genetically compact units with an eye on their potential ability to be transferable modules in multiple host species for engineering applications.

The organization of genes into clusters may facilitate the transfer of complete functions during evolution (Fischbach et al., 2008; Lawrence and Roth, 1996). All of the gene clusters presented in this review have some evidence for horizontal transfer, including phylogenetic trees disparate from ribosomal RNA, differing G+C content, and the presence of flanking transposon/integron genes (Ochman et al., 2000; Karlin, 2001). Phage genomes and conjugative plasmids also contain bacterial gene clusters, implying that a mobile element can confer a fitness advantage on its host by adding a novel function. For example, the photosynthetic apparatus (Lindell et al., 2004) and type IV pili (Karaolis et al., 1999) have been observed in phage genomes. Because gene clusters appeared and were shaped by interspecies transfer, it is intriguing that they could be fodder for genome building, where they provide a convenient unit of DNA that could be utilized to introduce a novel function into a synthetic organism. To date, such transfers are sometimes successful and sometimes fail for unknown reasons (Dixon and Postgate, 1972; Hansen-Wester et al., 2004). Potential problems include that the

cluster may rely on regulatory interactions that are not present in the new host, the genes do not express or express at the wrong ratios, or there are auxiliary interactions with, or dependencies on, the host (Fischbach et al., 2008).

Within gene clusters, there can also be sub-gene clusters that evolve separately. This modular organization of clusters within clusters enables rapid diversification and can replicate a useful function in multiple contexts. Two examples of such sub-clusters are microcompartments that can sequester toxic metabolic intermediates (Section 2.2) and the stressosome that can integrate signals and control different signaling mechanisms (Section 6.1). Sub-clusters also occur within metabolic pathways, where particular conversion (*e.g.*, the modification of a sugar moiety) can occur in different contexts. Examples of such sub-clusters are present in the erythromycin pathway (Section 4.3). The useful functions encoded by these sub-clusters have propagated into different metabolic and signaling pathways.

As the number of complete sequenced genomes grows, it has become clear that many gene clusters are “cryptic;” in other words, there are no known conditions under which the genes are expressed (Challis, 2008). Homology analysis can be useful (albeit inexact) in predicting the general classes of molecules produced. For example, there may be many novel antibiotics and other pharmaceuticals that are encoded by such clusters. Sometimes, it is possible to “wake up” a cluster by engineering its regulatory circuitry (Scherlach and Hertweck, 2009). This could either be through the deletion of a repressor or the addition of an inducible system. However, many clusters remain intransigent to these approaches. As the sequence databases grow, it is going to be increasingly tempting to access the functions encoded therein.

Genetic engineering is moving towards the era of the genome. Automated DNA synthesis has continued to advance, with the size of routine orders increasing to >50 000 bp, and declining cost and turnaround time (Czar et al., 2008). Recently, the entire wild-type genome of *Mycoplasma* was synthesized and transferred into a new cell, producing a living organism (Gibson et al., 2010). However, no design was implemented in this tour-de-force project; essentially, a natural genome was replicated. In an attempt to improve our design capacity, synthetic biology has been contributing a growing toolbox of genetic parts (*e.g.*, ribosome binding sites, promoters, terminators) and devices (*e.g.*, genetic circuits, sensors) that enable programmable control over transcription and translation (Voigt, 2006). In addition, methods have been developed to rapidly assemble these parts into intermediate 10-kb fragments (Gibson et al., 2009), which can then be further assembled to multi-100-kb pieces (Benders et al., 2010).

As synthetic biology moves towards genome design, gene clusters are an appropriate intermediate stepping stone. On one hand, they are themselves composed of genetic parts and devices. On the other, they could be hierarchically combined to add functions to a genome. Indeed, this type of construction occurs frequently in nature, where large plasmids have been discovered that contain

multiple gene clusters (e.g., the pRSB107 plasmid combines nine antibiotic resistance gene clusters and one that scavenges iron) (Szczepanowski et al., 2005).

This review is organized to describe and compare clusters that encode a variety of functions from different species. The clusters are loosely organized into five classes according to the type of function they encode: structural, scavenging, synthesis, energy, and sensing. Each example focuses on a well-studied instance of that cluster. Many variations of each cluster exist. We also describe the current and potential applications in biotechnology for each of the gene clusters.

2. Nano-Machines, Organelles, and Large Protein Structures

2.1 Molecular Hypodermic Needle: Type III Secretion System *Salmonella typhimurium* (*sprB* to *invH*)

The type III secretion system (T3SS) is a molecular machine that exports proteins from the cytoplasm to the extracellular environment (Fig. A21-1) (Cornelis, 2006). Many Gram-negative pathogens have such systems, where it forms a syringe-like structure (Schraidt et al., 2010) that injects proteins into animal or plant cells to hijack a variety of host processes. The length of the needle varies depending on the function, from 60 nm in *Yersinia* to 2 μ m in *Pseudomonas syringae*, where it needs to penetrate a thick plant cell wall. Effector proteins that are exported have an N-terminal secretion tag and a chaperone-binding domain that direct it to the needle. The proteins are actively unfolded before being transported through a 2-nm pore. All of the genes that are required to form the needle, as well as chaperones and effectors, are encoded within a single gene cluster (Fig. A21-2). Additional effector proteins can be scattered throughout the genome. A complex regulatory network encoded within the cluster integrates environmental signals and controls the dynamics of gene expression (Temme et al., 2008). Pathogens often have multiple clusters encoding needles that are responsible for different phases of an infection. The flagellum, involved in propulsion when bacteria swim, is a distantly related T3SS, but its genes are usually not organized into a single cluster.

Salmonella and other pathogenic bacteria have been explored for therapeutic uses as a mechanism to deliver vaccines and to block the growth of tumors. As a live vaccine, *Salmonella* has been used to deliver heterologous antigens from diverse sources to the immune system, including through the integration of peptides into effectors that are secreted from the T3SS encoded in *Salmonella* pathogenicity island 1 (SPI-1) (Galen et al., 2009). *Salmonella* naturally localizes to tumors after infection and it has been explored as an anticancer therapeutic, and has gone through clinical trials. The growth suppression of tumors has been shown to be dependent on the T3SS encoded within *Salmonella* pathogenicity island 2 (SPI-2) (Pawelek et al., 2002).

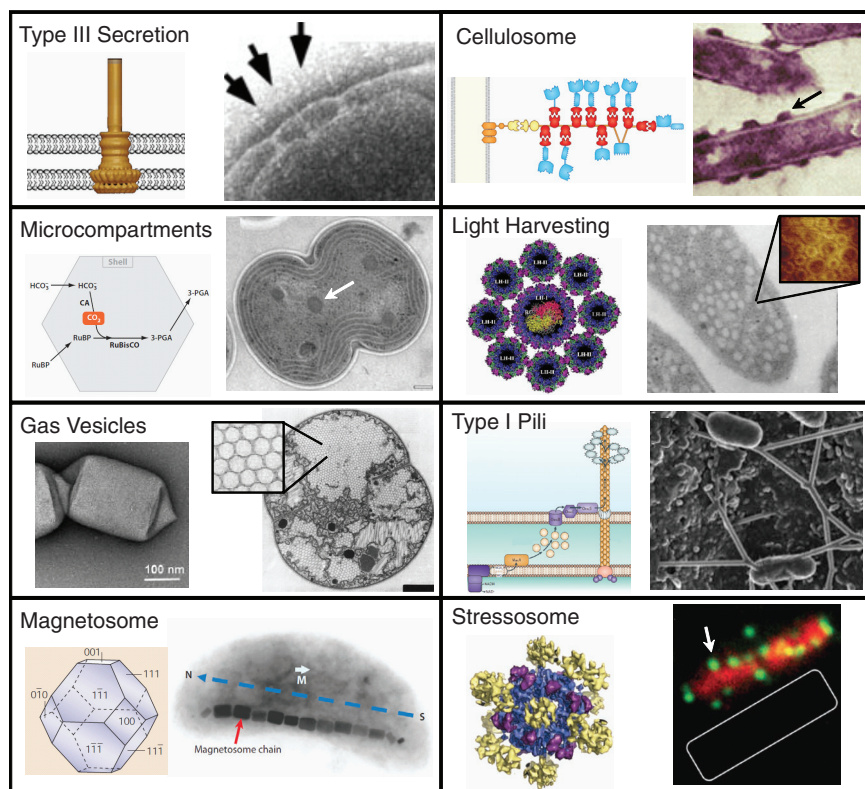


FIGURE A21-1 Gene clusters encode organelles and molecular machines. A schematic (left) and image (right) is shown for each system that appears in this review. Clockwise from top left: A reconstruction of the cryo-EM structure of the *Salmonella* type III secretion system is shown along with an image of multiple needle complexes spanning the inner and outer membranes (Kubori et al., 1998, 2000). A schematic of the *C. thermocellum* cellulosome is shown with their surface localization (Fontes and Gilbert, 2010). The crystal structure of the *R. sphaerodes* Reaction center and LH1 (center) and LH2 (outer eight rings) are shown next to a high-resolution AFM of the photosynthetic membrane of *Rsp. photometricum* (inset) (Scheuring et al., 2004). A cartoon of the type I pili from *G. sulfurreducens* (Fe^{3+} oxide is shown localized at the tip) (Lovley, 2006) is compared to a SEM of the pilus-like appendages from *S. oneidensis* MR-1 (Ntarlagiannis et al., 2007). The cryo-EM structure of the *B. subtilis* stressosome and their localization using RsbR-specific antibodies are shown (Marles-Wright et al., 2008). An idealized $\{100\} + \{111\}$ Fe_3O_4 crystal is shown with an image of a magnetosome chain (Jogler and Schuler, 2009). An electron micrograph of a gas vesicle from *Hfx. Mediterranei* (Pfeifer, 2006) and the packing of multiple vesicles in *Microcystis* sp. (Walsby, 1994) are shown. Carboxysomes are shown from *Synechocystis* sp. PCC 6803 along with the pathway for carbon dioxide fixation (Yeats et al., 2010). All images reproduced with permission.

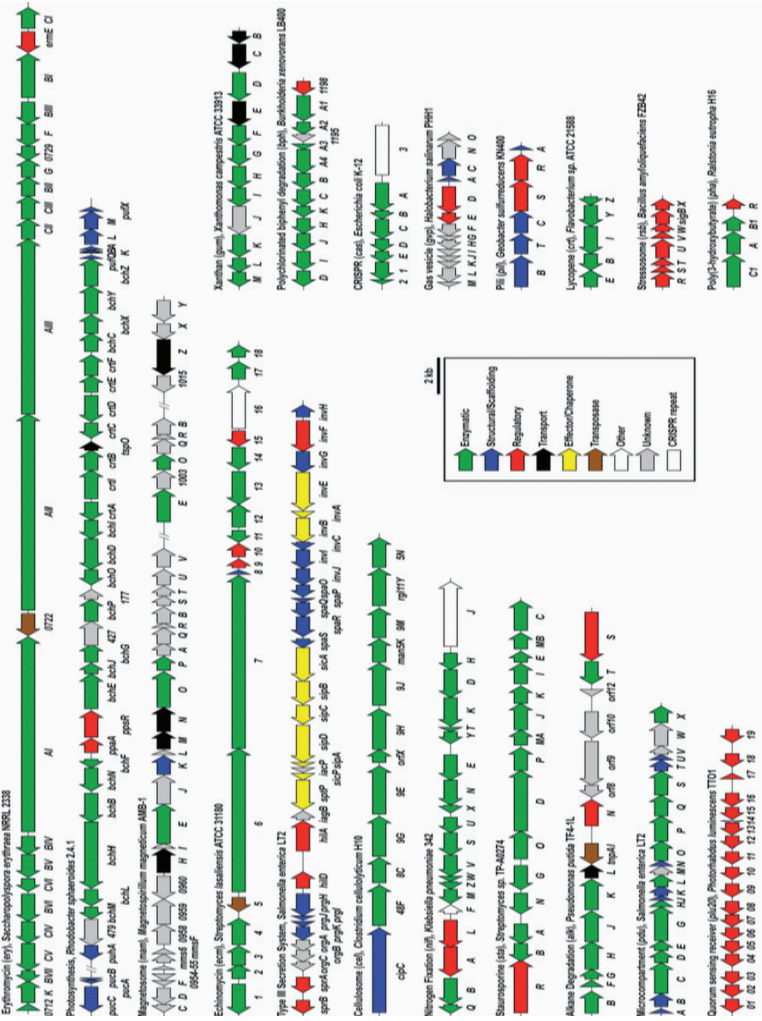


FIGURE A21-2 Gene clusters described in this review are compared. The colors of the genes loosely classify their functions. Many genes contain multiple functions. A classification of being “structural” includes genes that associate with a large complex and are necessary for function; for example, ATPases in type I pili and type III secretion.

2.2 Chemical Reactors Within Bacteria: Microcompartments *Salmonella typhimurium* (*pduA* to *pduX*)

Many bacteria build geometrically regular polyhedral organelles with a diameter of 80–200 nm (Fig. A21-1) (Yeats et al., 2010; Kerfeld et al., 2010). These act as microcompartments to encapsulate enzymes that participate in metabolic pathways where an intermediate is toxic or requires concentration. The canonical example is the carboxysome, which co-localizes carbonic anhydrase, which increases the concentration of CO₂ with the CO₂-fixing enzyme RuBisCo. There are several examples where a pathway produces an intermediate molecule that causes toxicity when it is produced in the cytoplasm. Examples include pathways for the utilization of propanediol and ethanolamine, whose toxic intermediates are aldehyde and acetaldehyde, respectively. An example of the former is the *pdu* gene cluster in *Salmonella typhimurium*. The proteins that form the microcompartments produce a sixfold symmetry with a 2–3-Å pore that can potentially expand to 11–13 Å. The pore controls the transport of molecules in and out of the microcompartment and has a variable amino acid sequence. The core structural components of the microcompartment are conserved and appear in gene clusters with the enzymes that make up the metabolic pathway (Fig. A21-2). For example the ethanolamine utilization gene cluster has 17 genes, 4 of which are the compartment shell proteins.

Microcompartments exist in natural pathways to solve many of the problems that emerge in metabolic engineering. Frequently, an intermediate metabolite is toxic and this is detrimental to the cell if the flux in and out are unbalanced, causing the intermediate to accumulate (Mukhopadhyay et al., 2008). Further, some enzymes have an intrinsically high K_m, requiring that the substrate be concentrated. Other functions require an anaerobic environment, and it has been shown that microcompartments can exclude oxygen molecules. Effectively harnessing microcompartments will require that targeting sequences be available to direct enzymes to the organelle (Fan et al., 2010) and protein engineering methods to alter the pores to allow desired substrates to enter (Yeats et al., 2010). Several microcompartments have been predicted to be involved in pathways of direct relevance to biotechnology, including in the production of ethanol in *Vibrio* (Wackett et al., 2007) and butyrate in *Clostridium* (Seedorf et al., 2008).

2.3 Balloons in Bacteria: Gas Vesicles *Halobacterium salinarum* (*gvpA* to *gvpM*)

A variety of species of archaea and bacteria control their buoyancy by forming gas-filled balloons in their cytoplasm (Fig. A21-1) (Pfeifer, 2006; Walsby, 1994). They function to maintain a desired depth in an aquatic environment, and their inflation and deflation are regulated by environmental signals that are used to identify the correct depth, including UV, light intensity, salinity, and oxygen. To have an effect on buoyancy, at least 10% of the cell volume needs to be composed of gas vesicles; thus, some bacteria have >10 000 per cell depend-

ing on their size. These vesicles are large, ranging in size from 33 to 250 nm in diameter, but they have very thin walls—about 2 nm—made entirely of protein. The protein that is the major component of the wall is GpvA, which forms a hydrophobic inner surface that blocks the formation of water droplets via surface tension. Gas enters the vesicles via diffusion and it is not stored there, rather it is in equilibrium with the surrounding concentration of dissolved gas (Walsby, 1994). The filling time is 0.4 μ s. The vesicles can be filled by small and large gas molecules, including O₂, N₂, H₂, CO₂, CO, CH₄, Ar, and C₄F₈. All of the genes necessary for gas vesicle formation occur together in a gene cluster, including the core structural proteins, accessory proteins that control the size and shape, chaperones, seeding proteins, and regulators (Fig. A21-2). Vesicles are produced when the gene cluster from *Bacillus megaterium* is transferred into *Escherichia coli* (Li and Cannon, 1998).

Recombinant proteins can be inserted to an accessory protein (GvpC) that participates in the formation of the shell of the gas vesicle. Antibodies have been produced and purified in this way, where the buoyancy of the vesicle aids purification and delivery (Sremac and Stuart, 2008). Gas vesicles isolated from cyanobacteria have been shown to improve oxygen transport in a mammalian cell fermentation (Sundararajan and Ju, 2000). Very interestingly, vesicles have been discovered in terrestrial bacteria that have been studied for their diverse secondary metabolisms, including antibiotic production (such as *Streptomyces avermitilis*), although their role in these organisms has yet to be determined (van Keulen et al., 2005). Perhaps the most interesting aspect of blowing up balloons in bacteria is that it is fun (Endy et al., 2005).

2.4 Metal Nanoparticles: Magnetosomes *Magnetospirillum magneticum* AMB-1 (*mamC* to *mamF*)

Biomining is a process by which bacteria build intricate 3-D nanostructures from dissolved metal. One of the most dramatic structures is the magnetosome, which is used by bacteria that can orient their swimming to align with the geomagnetic field (magnetotaxis) (Fig. A21-1) (Jogler and Schuler, 2009). Spherical nanocrystals (diameter ~30–50 nm) of iron oxide magnetite (Fe₃O₄) are contained within lipid organelles (Komeili, 2007). A string of these crystals are held in a chain orientation by proteins that resemble those involved in the cytoskeleton. All of the genes that encode the proteins involved in the biosynthesis, organization, and regulation of the magnetosome are encoded in a conserved Magnetosome Island (MAI) region of the genome that spans 80–150 kb (Fig. A21-2). This region has been noted to be unstable, and is frequently lost in lab culture. MAIs are phylogenetically widespread and there is diversity in the size, shape, and mineral composition of the crystals (Komeili, 2007).

The process by which magnetosome crystals are grown produces remarkably uniform and highly ordered structures (Jogler and Schuler, 2009). The bacteria also have the ability to concentrate low environmental abundances of metals and

form crystals under mild conditions compared to chemical routes (Schuler and Frankel, 1999). Further, their synthesis in a lipid membrane makes them easily dispersed in an aqueous environment. Thus, magnetosome-producing cells have the potential to be harnessed for the industrial production of magnetic nanoparticles, with diverse applications in medicine, imaging, and commercial uses in magnetic tape and ink (Schuler and Frankel, 1999; Matsunaga et al., 2007). Beyond Fe-based magnetosomes, many bacteria are able to build structures of diverse size (1–6000 nm), complexity (spherical, triangular, octahedral, decahedral, cubic, plates), and out of a variety of materials (gold, silver, cadmium, palladium, selenium, titanium, lanthanum, zinc, uranium, lead) (Korbekandi et al., 2009).

3. Scavenging for Nutrients

3.1 Molecular Chainsaws: The Cellulosome *Clostridium cellulolyticum* (*cipC* to *cel5N*)

Cellulose is a polymer of sugar molecules and is an abundant component of plant cell walls. It is the most abundant polymer in biomass and many organisms have the ability to degrade this material to obtain carbon and energy. They do this by secreting cellulases and other enzymes that are able to break down complex cell walls to release simpler sugars that can diffuse into the cell. In clostridia and rumen organisms, these enzymes frequently cluster to form a large cellulosome that protrudes from the cell surface (Fig. A21-1) (Fontes and Gilbert, 2010).

The cellulosome consists of a scaffolding protein (scaffoldin) that contains a series of cohesin domains. These domains bind to dockarin domains at the N termini of the cellulases that are involved in the assembly of the cellulosome. The scaffoldin is tethered to the cell surface (often to the S layer) and it and many of the cellulases contain cellulose-binding domains so that they bind to the cellulose in the plant cell wall (Desvaux, 2005). The advantage of having a cellulosome has been postulated to be that there is a higher likelihood that the released sugars will be consumed by the organism (Fontes and Gilbert, 2010). There is much diversity in the size and composition of cellulosomes and additional enzyme activities are commonly present, including hemicellulases and pectins to aid the decomposition of the plant cell wall. The cellulosomes can be very large, with up to 200 enzymes, and can be up to 16 MDa (Desvaux, 2005; Ding et al., 2001). Within species, the cohesin-dockarin interactions are not specific and individual enzymes are not discriminated at each position in the scaffoldin. Remarkably, the enzyme composition of the cellulosome reflects the substrate on which the cells were grown; for example, on grass clippings, pectins are expressed, and on sewage soils consisting of insect biomass, chitinases are expressed (Desvaux, 2005). The genetic regulation that controls this adaptation is unknown.

The scaffolding protein and many of the enzymes are often organized into a gene cluster (Fig. A21-2). The cluster in *C. cellulolyticum* is a model system with 12 genes encoded in a 26-kb region, and the pattern of phylogenetic distribution

implies horizontal transfer (Desvaux, 2005). CipC is the scaffoldin and there are 8 cellulases, 1 hemicellulase (Man5K), and 1 pectinase (Cc-Rgl11Y) present in the cluster. The breakdown of crystalline cellulose is complex and requires synergistic activities between multiple enzymes. Some enzymes cut the polymer at random locations (exoglucanase), whereas others start from either free reducing or non-reducing ends and progressively cleave the polymer (endoglucanase). The most prevalent enzymes of this cellulosome represent one from each category: Cell9E and Cell48F (Desvaux, 2005). Cell9E randomly cuts the cellulose strands. In contrast, Cell48F forms a long hydrophobic tunnel, through which cellulose strands are threaded as it progressively cuts and releases simpler sugars. *C. cellulolyticum* contains 62 enzymes that contain dockarin domains and the enzyme composition has been shown to vary based on the substrate on which bacteria grow (Blouzard et al., 2010).

Biofuels and renewable chemicals require a source of carbon. Currently, this is frequently obtained in the form of sugar from crops such as corn and sugarcane. Particularly for fuels, this poses sustainability problems as agricultural crops would be diverted from food. In contrast, the amount of carbon that could be extracted from biomass is on the same scale as the fuels industry (and microbes have been estimated to naturally release the equivalent of 640 billion barrels of crude per year!) (Fontes and Gilbert, 2010). The problem is in the efficient liberation of carbon from cellulosic feedstocks, and this is currently a very active area of research. Significant effort has been put into the engineering of the cellulosome for this purpose (Bayer et al., 1994). One approach is to metabolically engineer natural cellulosic microbes to produce valuable products. A problem with this approach is that organisms containing cellulosomes are often not adapted for high carbon fluxes (Desvaux, 2005). A variety of metabolic engineering approaches have been used to increase the catabolism of these organisms. Another approach is to move the cellulosome into a noncellulolytic organism. This has been achieved for moving a minimal cellulosome to *C. acetobutylicum* (Sabathe and Soucaille, 2003) and the yeast *S. cerevisiae* (Lilly et al., 2009). Finally, the adhesin-dockarin domains have been harnessed as modules that control protein-protein interactions for a variety of applications outside of bioenergy, including protein purification and display (Nordon et al., 2009).

3.2 Eating Oil: Alkane Degradation Pathways *Pseudomonas putida* GPO1 (*alkB* to *alkS*)

Numerous marine and terrestrial bacteria have the ability to utilize hydrocarbons as a carbon and energy source (van Hamme et al., 2003). When oil leaks into seawater, this leads to the growth of a bloom of bacteria that are obligate consumers of alkanes (Yakimov et al., 2007). Many of the genes involved in the utilization of hydrocarbons occur together in a gene cluster (van Beilen et al., 2001). Petroleum is a chemically diverse substance and there are a range of en-

zymes and related pathways that break down different classes of molecules (van Beilen et al., 2001; van Beilen and Funhoff, 2007). The gene cluster in *P. putida* is one of the most well-studied systems and is able to degrade medium-length alkanes (Fig. A21-2) (van Beilen et al., 2001). The metabolic pathway begins with an alkane hydroxylase (AlkB—a membrane-associated non-heme diiron monooxygenase), which converts the alkane to an alcohol (Fig. A21-3) (van Beilen and Funhoff, 2007). Often, strains contain multiple alkane hydroxylases to broaden the range of substrates that can be consumed (van Hamme et al., 2003). Electrons are delivered to AlkB by two rubredoxins (AlkF and AlkG). The alcohol is converted to acyl-CoA in three steps (AlkHJK), at which point it can enter metabolism. Two additional proteins, AlkL and AlkN, putatively encode an importer and chemotaxis sensory protein, respectively. AlkS acts as an alkane

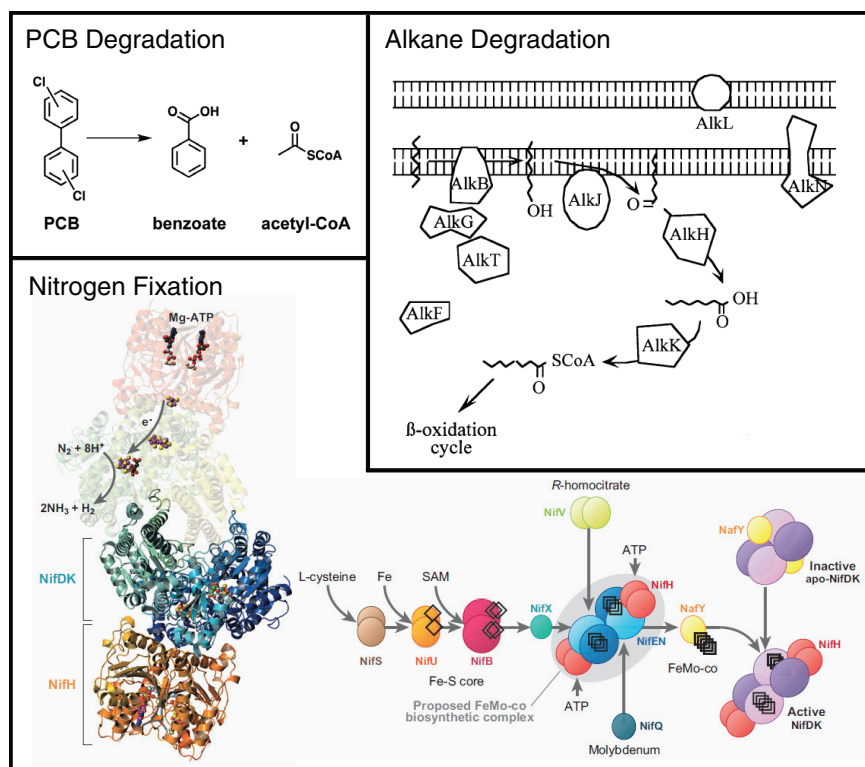


FIGURE A21-3 Utilization and breakdown pathways encoded in gene clusters are shown. The alkane degradation pathway from *P. putida* is adapted from Witholt and co-workers (van Beilen et al., 2001). Nitrogenase is shown along with the pathway for the production of FeMoCo (Rubio and Ludden, 2008). All images reproduced with permission.

sensor and up-regulates gene expression. The *alk* gene cluster occurs in many phylogenetically distinct bacteria (van Hamme et al., 2003). It has a lower G+C content than the genome and is flanked by transposon genes, which indicate frequent horizontal transfer.

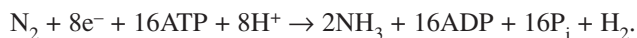
Petroleum-degrading organisms have been proposed to be used in a wide variety of industrial applications. This includes a variety of potential roles in environmental cleanup, from biosensing and site evaluation to environmental dispersal, fermenter-based waste treatment, refinery waste treatment, and tanker ballast cleaning (van Hamme et al., 2003). Organisms and related pathways have been identified that can break down nearly all of the components of petroleum, including benzene, ethylbenzene, trimethylbenzene, toluene, ethyltoluene, xylene, naphthalene, methyl naphthalene, phenanthrene, C₆–C₈ alkanes, C₁₄–C₂₀ alkanes, branched alkanes, and cymene (van Hamme et al., 2003). In addition, alkane-degrading organisms could be used as biocatalysts to add value to petroleum products (van Hamme et al., 2003). For example, *Alcanivorax* has been engineered to direct the carbon flux from alkanes to the production and export of the bioplastic precursor poly(hydroxyalkanoate) (PHA) (Sabirova et al., 2006). A particularly interesting use is for microbial enhanced oil recovery (MEOR), where bacteria are introduced into oil wells to facilitate secondary recovery (van Hamme et al., 2003). The injection of oil-degrading organisms can increase recovery by reducing viscosity or secreting surfactants. MEOR has been tested worldwide, including in the USA, and has led to increases of 15–23% for oil wells in Japan and China (van Hamme et al., 2003). Finally, the alkane-sensing transcription factor (AlkS) and the AlkB promoter have been transferred into *E. coli* to construct a genetic biosensor (Sticher et al., 1997).

3.3 Fertilizer Factories: Nitrogen Fixation *Klebsiella pneumoniae* (*nifJ* to *nifQ*)

The availability of nitrogen limits the growth of many organisms (Igarashi and Seefeldt, 2003). In agriculture, fixed nitrogen is a critical component of fertilizer and its availability has been linked to the growth of the human population. The primary source of nitrogen is from the atmosphere in the form of N₂. Converting this into a form that can enter metabolism—such as ammonia (NH₃)—is a difficult chemical reaction. The Haber-Bosch process can chemically convert N₂ to NH₃ using high temperatures and pressures using an iron catalyst. In contrast, biological nitrogen fixation uses a complex enzyme (nitrogenase) to perform this reaction (Fig. A21-3). Remarkably, the current flux of fixed nitrogen from synthetic chemical and natural biological processes is about equal (Igarashi and Seefeldt, 2003).

Only prokaryotes and some archaea have the ability to fix nitrogen (Dixon and Kahn, 2004). Often, all of the genes necessary for nitrogen fixation are encoded in a gene cluster. One of the simplest and most well-studied clusters is from *K. pneumoniae*, which consists of 20 genes encoded in 23 kb (Fig. A21-2) (Rubio and Ludden, 2008). These genes encode all of the necessary components for nitrogen fixation, including the nitrogenase, a metabolic pathway for the synthesis

of metal co-factors, e^- transport, and a regulatory network. Nitrogenase consists of two core proteins (NifH and the NifDK complex) that participate in a reaction cycle (Igarashi and Seefeldt, 2003). The reaction itself is very energy and redox intensive with the balance



Each reaction cycle consists of the transfer of 1 e^- and the consumption of 2 ATP (the energy of which is used to greatly accelerate e^- transfer). It is marked by a transient interaction between NifH, which receives an e^- from a variety of sources, and NifDK, which contains the reaction center where N_2 binds and fixation occurs. The cycle of binding, electron transfer, and dissociation needs to be repeated eight times to fix a single N_2 molecule. Nitrogenase is slow ($k_{\text{cat}} = 5 \text{ s}^{-1}$) and is thought to be limited by the dissociation step (Igarashi and Seefeldt, 2003). Three co-factors form the core of the e^- transfer and catalysis: $[\text{Fe}_4\text{-S}_4]$ in NifH, the P cluster $[\text{Fe}_8\text{-S}_7]$ in NifDK, and FeMo-co $[\text{Mo-Fe}_7\text{-S}_9\text{-X}]$ (Fig. A21-4) where the reaction occurs (Rubio and Ludden, 2008). The enzymes involved in the synthesis of these co-factors and chaperones for their incorporation to form mature nitrogenase make up the majority of the cluster (Fig. A21-3). It has been proposed that these proteins all form a macromolecular “biosynthetic factory” centered on the NifEN proteins (Rubio and Ludden, 2005). NifF and NifJ are flavodoxins that feed electrons to NifH, with pyruvate as one source (Rubio and Ludden, 2005). Nitrogenase is extremely oxygen sensitive and expensive for the cells to make and run (Fischer, 1994). A simple regulatory cascade is formed by the activator NifA and the anti-activator NifL, which integrate signals to ensure that the genes are only expressed in the absence of oxygen and fixed nitrogen (Dixon and Kahn, 2004). Since the earliest tools in genetic engineering were developed, it has been a dream of biotechnology to create cereal crops that can fix their own nitrogen. The complexity of the nitrogen fixation pathway and a lack of efficient tools for modifying non-model plants have hindered progress in this area (Dixon et al., 1997). In contrast, the complete gene cluster was functionally transferred from *Klebsiella* to *E. coli* relatively early in 1972 (Dixon and Postgate, 1972). The chloroplast may be a potentially good target for the maturation and function of nitrogenase because: (1) it is where ammonia assimilation occurs, (2) ATP is generated there, (3) there is evidence that the ancillary proteins for Fe-S formation exist, and (4) the genetic context is similar to a prokaryote, including the ability to transcribe operons (Cheng, 2008). Individual genes from the pathway have been transferred to the Tobacco genome with a chloroplast-targeting peptide and to the plastid in the algae *Chlamydomonas reinhardtii* (Dixon et al., 1997). Neither of these efforts yielded appreciable expression. Besides the difficulty of expressing *Klebsiella*-encoded genes in these contexts, there are regulatory issues around the oxygen sensitivity of nitrogenase and, thus, its inconsistency with the photosynthetic processes in the chloroplast. One way to overcome this would be to place the nitrogenase under the control of light or oxygen sensitive

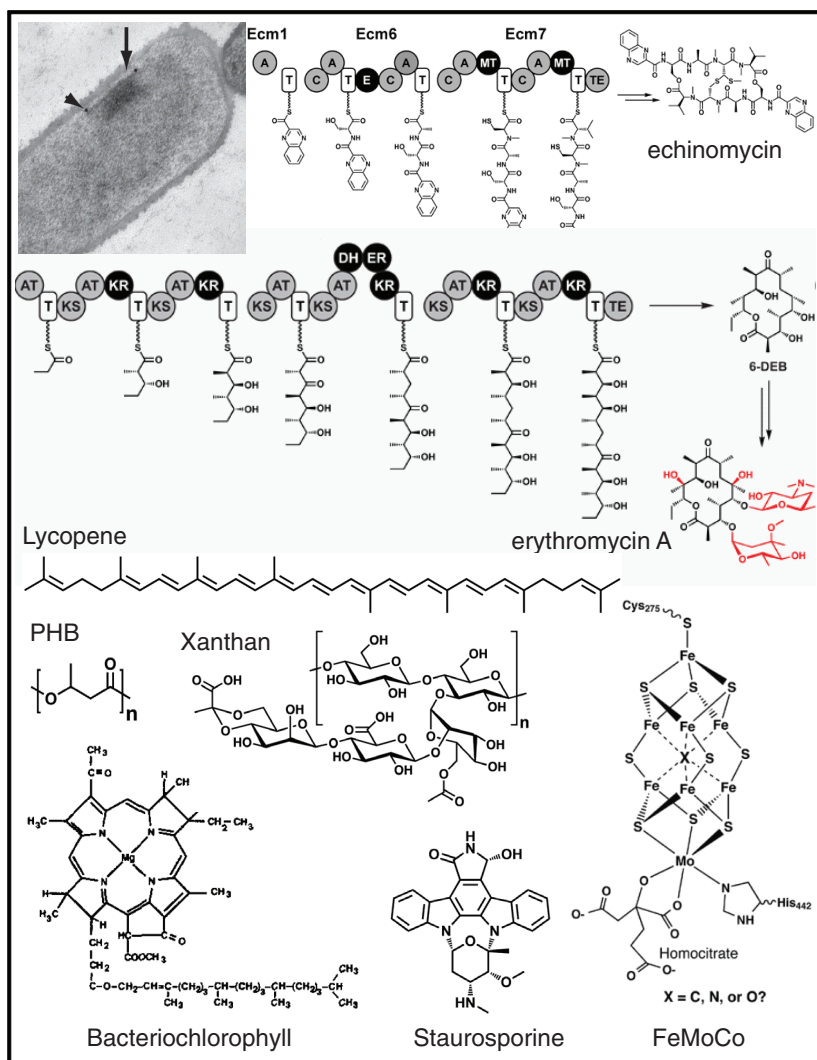


FIGURE A21-4 Chemical production pathways are often encoded within gene clusters. The image is of an organelle containing 10–100 associated 2.5 MDa NRPS-PKS mega-complexes from *B. subtilis* (Straight et al., 2007). The erythromycin pathway is shown from *Saccharopolyspora erythraea* NRRL 2338 and echinomycin pathway from *Streptomyces lasaliensis*. For erythromycin, chemical groups added by post-assembly-line tailoring enzymes (two P450s and two glycosyltransferases) are shown in red. A, adenylation; T, thiolation; C, condensation; E, epimerization; MT, methyltransferase; TE, thioesterase; AT, acyltransferase; KS, ketosynthase; KR, ketoreductase; DH, dehydratase; ER, enoylreductase. Bacteriochlorophyll (bottom left) is incorporated into light harvesting complexes. FeMoCo (bottom right) is produced by a metabolic pathway and incorporated into nitrogenase (image from Noodleman et al. [2004]). All images reproduced with permission.

transcription factors (Dixon et al., 1997), or to express oxygen-protective factors, including the “Shethna” protein from *Azotobacter vinelandii* (Moshiri et al., 1994) and some nitrogenases are intrinsically less sensitive to oxygen (Cheng, 2008). Several applications of nitrogenase have been proposed that do not involve moving the system into a plant, including for the use of N_2 as a cheap source of nitrogen during fermentation (Chen et al., 2001), cyanide detoxification (Gupta et al., 2010), and the use of bacteria as biofertilizer (Bhattacharjee et al., 2008), and for the industrial production of ammonia (Brouers and Hall, 1986).

3.4 Bioremediation: Polychlorinated Biphenyl Degradation *Burkholderia xenovorans* LB400 (*orf0* to *bphD*)

Some bacteria can use harmful organic pollutants as their sole source of carbon and energy (Pieper, 2005). For example, *Burkholderia xenovorans* LB400 can subsist on polychlorinated biphenyls (PCBs), which are used industrially as, among others, fire retardants and plasticizers (Fig. A21-4) (Pieper and Seeger, 2008). This capability has made *B. xenovorans* and other PCB-metabolizing bacteria key elements of bioremediation strategies for chemical spills. Highly chlorinated PCBs are reductively dehalogenated by organisms such as *Dehalococcoides*, which can use PCBs as a terminal electron acceptor for anaerobic respiration (Pieper and Seeger, 2008). These lower chlorinated PCBs are the substrate for the *B. xenovorans* degradation pathway, which consists of a series of enzyme-mediated oxidations culminating in the cleavage of one of the linked aromatic rings by the ring-opening dioxygenase BphC. The cleaved ring is converted to two equivalents of acetate in a three-step pathway, while the uncleaved ring is released as benzoic acid and then further processed to catechol by the protein products of the *benABCD* gene cluster (Fig. A21-2) (Pieper and Seeger, 2008).

Several strategies are being employed to increase the number of PCBs that can be degraded microbially, including directed evolution of a ring-cleaving dioxygenase (Fortin et al., 2005) and functional screening of metagenomic libraries from activated sludge (Suenaga et al., 2007). Future efforts may attempt to introduce PCB degradation gene clusters into bacterial strains that synthesize compounds of industrial value, which would allow these strains to consume feedstocks that would otherwise require expensive and environmentally unfriendly disposal.

4. Biosynthesis of Chemicals

4.1 Bioplastic Biosynthesis: Poly(3-hydroxybutyrate) *Ralstonia eutropha* H16 (*phbA* to *phbC*)

Many bacteria synthesize poly(3-hydroxybutyrate) (PHB) and other PHAs as a means of storing carbon and energy intracellularly (Fig. A21-4). The biosynthetic pathway for PHB, exemplified by the *phb* gene cluster in *Ralstonia eutropha* (Fig. A21-2) (Pohlmann et al., 2006), consists of three steps: PhbA catalyzes a

Claisen condensation to convert two molecules of acetyl-CoA to acetoacetyl-CoA, PhbB reduces acetoacetyl-CoA to 3-hydroxybutyryl-CoA, and PhbC polymerizes 3-hydroxybutyryl-CoA with release of CoA to form PHB (Madison and Huisman, 1999). PHB is hydrophobic and accumulates in cytoplasmic granules.

PHB and other PHAs are versatile bioplastics; biodegradable forms of a diverse set of products ranging from plastic bottles to golf tees are produced commercially from bacterially synthesized PHAs (Madison and Huisman, 1999). Efforts to metabolically engineer the synthesis of bioplastics are proceeding along two tracks. First, the genes for the production of PHB and other PHAs have been introduced into plants to realize the benefits of using CO₂ as a carbon source rather than fermentation feedstocks (Slater et al., 1999). However, these efforts have been only modestly successful; to date, the best PHA production titer seen in plants is only ~10% of dry weight. Second, a variety of engineering efforts including genetic engineering and the provision of unnatural substrate derivatives in the fermentation broth have led to the optimization of PHA yields in native and engineered hosts and the production of novel PHA derivatives (Aldor and Keasling, 2003).

4.2 Nonribosomal Peptide Biosynthesis: Echinomycin *Streptomyces lasaliensis* (*ecm1* to *ecm18*)

Nonribosomal peptides (NRPs) are a class of peptidic small molecules that includes the antibiotic vancomycin and the immunosuppressant cyclosporine (Fischbach and Walsh, 2006). The gene cluster for echinomycin (Fig. A21-4), a DNA-damaging NRP from the quinoxaline class, is typical in encoding four categories of gene products (Fig. A21-2): (1) Genes for miniature, self-contained metabolic pathways that provide unusual monomers. Eight *ecm*-encoded enzymes convert tryptophan into quinoxaline-2-carboxylic acid (QC), an unusual monomer that enables echinomycin to intercalate between DNA base pairs; (2) Genes for an assembly-line-like enzyme known as an NRP synthetase (NRPS) that link monomers (typically amino acids) into a peptide and then release it from covalent linkage to the assembly line, often with concomitant macrocyclization. The *ecm* gene cluster encodes two NRPS enzymes, Ecm6 (2608 amino acids) and Ecm7 (3135 amino acids), that convert QC, serine, alanine, cysteine, and valine into a cyclic, dimeric decapeptidolactone; (3) Genes for chemical 'tailoring' after release from the NRPS. Two *ecm*-encoded enzymes oxidatively fuse the two cysteine side chains into a thioacetal; and (4) Genes that encode regulatory and resistance functions. Transporters are also commonly found in NRP gene clusters (Gorby et al., 2006).

There are two ways in which synthetic biology is being used in the area of NRPS engineering. First, efforts are being made to express NRPS gene clusters in heterologous hosts, either in their native form (Penn et al., 2006) or re-engineered for *E. coli* (Gorby et al., 2006). Expression in a heterologous host can serve three purposes: making the encoded NRP accessible for structure elucidation or

biological characterization, particularly useful if the native host is unknown or unculturable; making the genes easier to manipulate, which is useful if the native host is not amenable to genetics; and improving the production titer of its small molecule product, which is helpful if the gene cluster is repressed by an external regulatory system in the native host. Second, the production of NRP derivatives has been engineered by replacing portions of NRPs genes with variants from other gene clusters that lead to the incorporation of alternative amino acid building blocks. This technique has been used most extensively to generate derivatives of the NRP antibiotic daptomycin (Baltz, 2009).

4.3 Polyketide Biosynthesis: *Erythromycin Saccharopolyspora erythraea* NRRL 2338 (*SACE_0712 to eryCI*)

Polyketides (PKs) are a class of acetate- and propionate-derived small molecules that includes the immunosuppressant FK506, the antibiotic tetracycline, the cholesterol-lowering agent lovastatin, and a number of rapamycin analogues made by genetic engineering are in clinical trials (Fischbach and Walsh, 2006). The biosynthetic pathways for PKs and fatty acids are similar in their chemical logic and use related enzymes: both involve the polymerization of acetate- or propionate-derived monomers by a series of Claisen condensations followed by reduction of the resulting β -keto thioester (Fischbach and Walsh 2006). The gene cluster for erythromycin (Fig. A21-4), an antibacterial PK from the macrolide class, encodes the following classes of gene products (Fig. A21-2):

- (1) 3 large PK synthase (PKS) enzymes—DEBS 1 (3545 amino acids), DEBS 2 (3567 amino acids), and DEBS 3 (3171 amino acids)—that convert seven equivalents of the propionate-derived monomer methylmalonyl-CoA into the intermediate 6-deoxyerythronolide B (6-DEB);
- (2) 2 P450s that hydroxylate the nascent scaffold;
- (3) 12 enzymes that synthesize the unusual sugars desosamine and mycarose from glucose and attach them to the nascent scaffold. Without these sugars, erythromycin does not have appreciable antibiotic activity; and
- (4) an erythromycin resistance gene that modifies the 50S subunit of the ribosome to prevent erythromycin from binding (Staunton and Weissman, 2001).

Many PKSs have been expressed in heterologous hosts such as *E. coli*, including the PKSs for erythromycin and the anticancer agent epothilone (Fujii, 2009). Another notable heterologous host is a variant of the *Streptomyces fradiae* strain used for the industrial production of the antibiotic tylosin; having gone through many rounds of classical strain improvement, the metabolism of this strain is well suited to the production of PKs. A variant of the strain was created in which the tylosin gene cluster was replaced by the erythromycin PKS yielding

a high titer of the non-native product (Rodriguez et al., 2003). The PKS genes have been mutated or replaced with variants from other gene clusters to generate PK derivatives (McDaniel et al., 1999), or to create custom PKSs that synthesize small PK fragments by assembling portions of several PKS genes (Menzella et al., 2005).

4.4 Terpenoid Biosynthesis: Lycopene *Rhodobacter capsulatus* (*crtE* to *crtY*)

Terpenoids are a class of molecules that include the anticancer agent taxol, the antibiotic pleuromutilin, and the carotenoid pigments. While terpenoids are more common among plants than bacteria (Walsh and Fischbach, 2010), carotenoids are produced by a range of bacteria. Lycopene and other carotenoids are generally used in one of two ways: to harvest light (either for energy or photoprotection) or as antioxidants (Fig. A21-4). As with other terpenoids, the first step in the biosynthetic pathway for lycopene is the CrtE-catalyzed polymerization of the C₅ monomer isopentenyl pyrophosphate (IPP) or its Δ² isomer dimethylallyl pyrophosphate (DMAPP), in this case to the C₂₀ polymer geranylgeranyl diphosphate (GGDP). CrtB then dimerizes two equivalents of GGDP in a tail-to-tail fashion, resulting in the formation of the linear C₄₀ polymer phytoene. CrtI catalyzes four successive desaturations to yield lycopene. Alternative products such as beta-carotene are formed by the action of CrtY, which cyclizes the termini of the linear polymer (Umeno et al., 2005). All of the genes in this pathway occur together in a cluster (Fig. A21-2).

The colored nature of carotenoids has enabled their pathways to be engineered by genetic screens with colony color phenotypes. For example, a library of shuffled phytoene desaturases was screened in an *E. coli* strain harboring the *crt* gene cluster, resulting in the identification of desaturase clones that enabled the production of two lycopene variants, 3,4,3',4'-tetrahydrolycopene and torulene (Schmidt-Dannert et al., 2000). Much synthetic biological work has been done by Keasling and coworkers on the production of plant terpenes (*e.g.*, artemisinin) in the microbial hosts *S. cerevisiae* (Cogdell et al., 2006) and *E. coli* (Martin et al., 2003). This effort has involved two key challenges. First, since biosynthetic genes are not physically clustered in plant genomes, identifying the genes involved in terpenoid biosynthesis has been difficult, although the ongoing projects to sequence the genomes of hundreds of plants should enable bioinformatic efforts to identify biosynthetic genes. Second, the metabolism of *S. cerevisiae* and *E. coli* has been optimized for the production of terpenoids by increasing the flux of carbon toward IPP and DMAPP; in *E. coli* this was accomplished by supplementing the endogenous IPP biosynthetic pathway with the one from *S. cerevisiae*.

4.5 Oligosaccharide Biosynthesis: Xanthan *Xanthomonas campestris* pv. *campestris* (*gumB* to *gumM*)

Every year, 10 000–20 000 tons of xanthan are produced for use in foods (*e.g.*, to control the crystallization of ice cream and to emulsify salad dress-

ings) and in industry (*e.g.*, to modulate the viscosity of explosives and laundry detergents) (Becker et al., 1998). Xanthan, an oligosaccharide produced by the plant pathogen *Xanthomonas campestris*, is composed of a cellulose backbone, on alternating sugars of which a mannose- β -1,4-glucuronate- β -1,2-mannose trisaccharide is appended (Fig. A21-4). A portion of the terminal mannoses have pyruvate linked as a ketal to the 4'- and 6'-hydroxyls, and some of the internal mannoses are acetylated on the 6'-hydroxyl. Owing to the glucuronate units and pyruvoyl substituents, xanthan is an acidic polymer. Xanthan biosynthesis involves the action of five glycosyltransferases (GumDMHKI), and the growing chain is anchored on undecaprenyl pyrophosphate, similarly to peptidoglycan biosynthesis (Fig. A21-2). Three tailoring enzymes (GumFGL) add the aforementioned pyruvoyl and acetyl substituents, and GumBCE are required for xanthan export (Becker et al., 1998).

Future efforts are likely to proceed along two tracks. First, while substrate to xanthan conversion rates of 60–70% have been achieved (Becker et al., 1998), *X. campestris* could be engineered to grow on cheaper feedstocks or to make the separation of the cells from the xanthan less costly; alternatively, the *gum* gene cluster could be moved to an alternative host. Second, changes to the structure of xanthan have important effects on its rheological properties. Efforts to use genetic engineering to alter the structure (and therefore the rheological properties) of xanthan—or of other microbial exopolysaccharides such as alginate or gellan (Sa-Correia et al., 2002)—have the potential to create new polymers, *e.g.*, with altered viscosity and shear stability.

4.6 Indolocarbazole Biosynthesis: *Staurosporine Streptomyces sp. TP-A0274 (staR to staMB)*

Indolocarbazoles are natural products formed by the oxidative fusion of primary metabolic monomers (Walsh and Fischbach 2010). Staurosporine, an indolocarbazole, is a promiscuous, nanomolar inhibitor of serine/threonine protein kinases that binds in an ATP-competitive manner to these enzymes (Fig. A21-4) (Sanchez et al., 2006; Nakano and Omura, 2009). The staurosporine gene cluster encodes three categories of gene products (Fig. A21-2): (1) Four oxidoreductases (two P450s and two flavoenzymes) that catalyze a net 10-electron oxidation to fuse two molecules of tryptophan into the indolocarbazole aglycone (Howard-Jones and Walsh, 2006); (2) nine enzymes to synthesize and attach an unusual hexose to the indolocarbazole scaffold at the indole nitrogens; and (3) a transcriptional activator that regulates the expression of the gene cluster. Other naturally occurring indolocarbazoles differ in the oxidation state of the indolocarbazole scaffold, the derivatization of the indole ring by chlorination, and the sugar substituent appended to the indolocarbazole aglycone.

More than 50 unnatural indolocarbazole derivatives have been made by assembling artificial gene clusters in a non-native host (Salas and Mendez, 2009). These molecules harbor chemical modifications that would be difficult to introduce by semisynthetic derivatization of naturally occurring indolocarbazoles

or by total synthesis. The majority of these efforts have used genes from the gene clusters for indolocarbazoles as the building blocks for the artificial gene clusters. Future efforts to explore the activities of completely unrelated enzymes (*e.g.*, ring-opening dioxygenases) may enable the modification of portions of the indolocarbazole scaffold—such as the external six-membered rings—that would be difficult to access using synthetic organic chemistry or enzymes from indolocarbazole gene clusters.

5. Energy Generation and Transfer

5.1 Solar Powerpacks: Photosynthetic Light Harvesting *Rhodobacter sphaeroides* (*pufH* to *pufX*—and *puc* genes)

Sunlight is converted into power by the light harvesting system of anaerobic photosynthetic bacteria (Fig. A21-1) (Jones, 2009). Light energy is captured by two light harvesting complexes (LH1 and LH2) and is funneled to a reaction center (RC) (Cogdell et al., 2006). The RC uses the energy to produce a transmembrane charge separation mediated by the reduction of a quinone. This ultimately causes a proton to move from the cytoplasm to periplasm, which powers the production of ATP via the protonmotive force. The *R. sphaeroides* membrane has spherical invaginations that increase the surface area and number of RCs. Each RC occupies a hole in a ring formed by LH1. LH2 also forms rings that surround the RC:LH1 complex in the membrane. The LH complexes use carotenoids and bacteriochlorophyll (Fig. A21-4) to absorb green and near-infrared light, respectively (Cogdell et al., 2006). The photosynthetic genes are frequently found in a single cluster in purple bacteria (Naylor et al., 1999; Alberti et al., 1995). In *R. sphaeroides*, the photosynthesis gene cluster is 40.7 kb long and contains all of the necessary genes for the formation of the RC/LH1 (*puf* genes), and LH2 (*puc* genes) (Fig. A21-2). Two biosynthetic pathways make up the bulk of the cluster, where bacteriochlorophyll is produced from heme in a 16-gene pathway (*bch* genes) and the carotenoid sphaeroidine is produced from isopentenyl pyrophosphate in a 7-gene pathway (*crt* genes). The gene cluster is regulated by oxygen concentration, as well as the light intensity and color (Cogdell et al., 2006).

The *Rhodobacter* light harvesting system has been a model system for studying photosynthesis. It is relatively simple, there is only one photosystem, and the organism is genetically tractable. This has enabled detailed quantum mechanical measurements to be made on light absorption and electron transfer (Cogdell et al., 2006), which may enable the design of next-generation “biologically inspired” photovoltaic cells. Going one step further, the light harvesting complex can be functionally reconstituted *in vitro* and this has led to the construction of various hybrid systems, where the electrons are shuttled to inorganic materials (Lu et al., 2007). A particularly interesting approach is the development of a self-assembled monolayer, where the RC is tethered to a metal (Pt, Hg or Au) surface by an organic molecule that ends with a quinone. Multi-layer films have been constructed

and shown to efficiently capture electrons. *Rhodobacter* has also been harnessed for producing H₂ from light for use in fuel cells or microbial fuel cells (Logan and Regan, 2006).

5.2 Nanowires: Conductive Surface Pili *Geobacter sulfurreducens* (*pilB* to *pilA*)

Metal-reducing bacteria are able to discharge electrons to solid surfaces through “wires” formed by pili that extrude from the cell surface (Fig. A21-1) (Reguera et al., 2005). This gives these bacteria the required terminal electron acceptor for oxidative phosphorylation in the absence of other dissolved acceptors (oxygen, nitrate, sulfate, etc) (El-Naggar et al., 2008). *Geobacter* is able to form pili that attach to Fe(III) oxide surfaces. The genes that form the pilus are encoded in a gene cluster, with PilA being the pilin subunit that is homologous with the Type IV pili from other organisms (Fig. A21-2) (Reguera et al., 2005). The pili have a diameter of 50 nm and can extend up to 20 μm from the cell surface (Revil et al., 2010). High electron transfer rates of 10¹¹/s through the pili have been observed (El-Naggar et al., 2008). The nanowires can also connect multiple bacteria, implying that a community of cells can be wired for rapid electron transfer (Fig. A21-1) (Gorby et al., 2006). Beyond *Geobacter*, many other species have been shown to produce conductive pili in response to electron-acceptor limitation (Gorby et al., 2006).

Microbial fuel cells have emerged as a potential source of alternative energy (Gorby et al., 2006). A microbial fuel cell involves bacteria that are sequestered such that the only mechanism of electron transfer during respiration is to a graphite or gold anodes (Richter et al., 2008). The closest applications for microbial fuel cells are for long-term sensors deployed in the ocean and in wastewater treatment. Ocean sensors make use of the natural generation of electrical currents in the sea floor for power (Revil et al., 2010). Electricity can also be recovered from wastewater treatment as a byproduct of the breakdown of biomass. To deliver electricity to the anode, either a dissolved electron carrier or pili nanowires are required. *Geobacter* is one of the most efficient electron donors where it forms 50 μm thick biofilms on the surface (Revil et al., 2010). Pili are critical for electron transfer through the biofilm (Richter et al., 2008).

6. Environmental Sensing and Signal Processing

6.1 Physical Integrated Circuits: The Stressosome *Bacillus subtilis* (*rsbR* to *rsbX*)

One goal of synthetic biology is to build genetic circuits that can integrate information from environmental sensors or produce a dynamic response. To date, connecting circuits to form a program involves building a cascade at the level of transcription or translation. Each layer of the cascade requires about 20 min to complete (Hooshangi et al., 2005). More complex operations that require multiple layers can be particularly slow in the propagation of the signal. It would be much

faster if the circuitry could be built as a molecular machine, where signals are directly received and signal integration occurs due to conformational changes or signal propagation (*e.g.*, via a phosphorelay).

Bacteria have such a machine, known as the stressosome (Marles-Wright et al., 2008). In *B. subtilis*, it is in the gene cluster that contains many regulatory factors that converge on the anti-sigma factor σ_B , which controls the general stress response. This pathway integrates energy and environmental stresses through a complex partner-switching mechanism (Fig. A21-5) involving anti-sigma factors, anti-anti-sigma factors, kinases, and phosphatases, most of which occur together in a gene cluster (Fig. A21-2). Three of these proteins (RsbRST) form

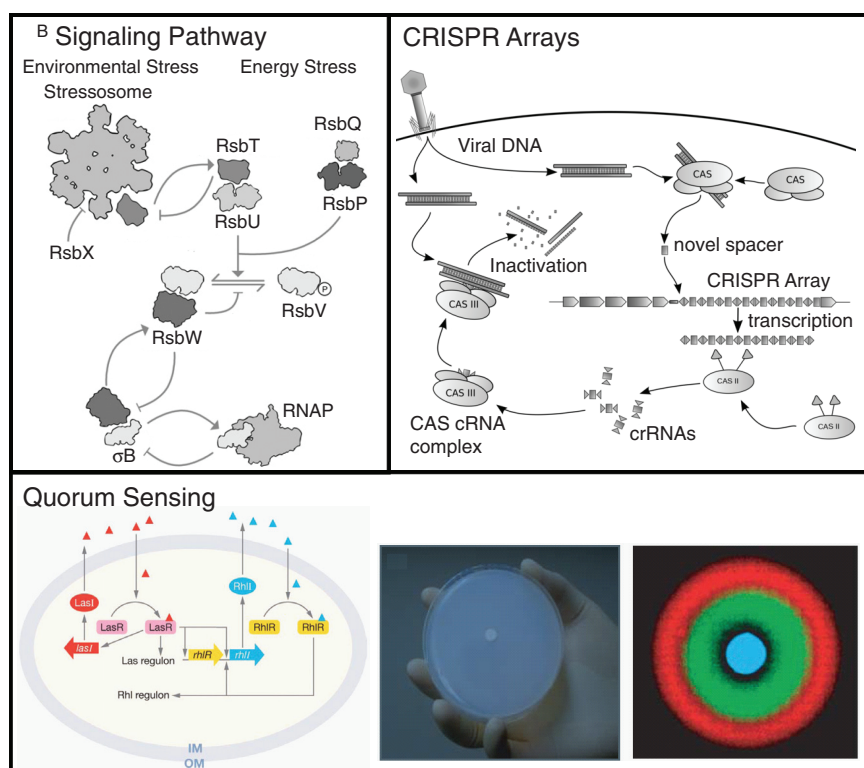


FIGURE A21-5 Complex regulatory pathways can be encoded by gene clusters. The signaling network formed by the σ_B gene cluster is shown (Marles-Wright et al., 2008). Environmental stress is received by the stressosome, whereas energy stress is sensed by a different branch of the pathway. Quorum sensing pathways from *Pseudomonas aeruginosa* (Waters and Bassler, 2005) are shown with their synthetic use to build pattern-forming programs in *E. coli* (Basu et al., 2005). All images reproduced with permission. The CRISPR image adapted from one drawn by James Atmos.

the structure of the stressosome, which is as large as a ribosome (1.8 MDa, 300 Å diameter) and appears as a spiked ball, with a core and protrusions (Fig. A21-1) (Marles-Wright and Lewis, 2010). The protrusions are composed of RsbR as well as four RsbR paralogs (Marles-Wright et al., 2008). The N-terminal domains of these proteins are variable, leading to the hypothesis that they act like sensors to receive diverse signals, including small molecules, protein-protein interactions, and even light (Marles-Wright and Lewis, 2010; Hecker et al., 2007). These signals are integrated by the core of the stressosome through conformational changes or other biochemical mechanisms. The RbsT protein interacts with the stressosome and transmits the signal to the σ_B pathway. The stressosome is induced by environmental stress and the release of RsbT is highly cooperative (a Hill factor of $n=8$). About 20 stressosomes are present in a single *B. subtilis* cell and they are closely associated with nucleoids and exhibit little diffusion. The σ_B gene cluster contains a number of other regulatory proteins that participate in partner-swapping and kinase/phosphatase interactions and there are internal transcriptional positive and negative feedback loops (Fig. A21-5). Together, this regulation ensures that the response is: (1) transient with a 30-min pulse of activity, (2) fast, (3) graded, and (4) the magnitude matches the degree of stress (Hecker et al., 2007; Igoshin et al., 2007).

The stressosome and the σ_B stress response pathway have several applications in biotechnology. The core proteins of the stressosome (RsbRST) are present in many species, including Gram negatives, and are associated with a variety of regulatory mechanisms, including aerotaxis, two-component sensors, and the biosynthesis of signaling molecules (Marles-Wright et al., 2008; Hecker et al., 2007). It may be that this structure is a common mechanism by which signals are integrated and understanding how to “reprogram” this structure would potentially enable much faster signal integration than transcriptional circuitry. In *B. subtilis*, there is evidence that five signals are integrated, but given the size of the structure, many more may be possible. The complete σ_B gene cluster is much less distributed than the stressosome. Several industrially relevant strains contain σ_B and related gene clusters, including *Bacillus* and *Streptomyces* (Hecker et al., 2007). The general stress response mediated by σ_B is involved with a number of stresses that are relevant to biotechnology, including response to shifts in salt concentration, pH, ethanol, ATP, cell wall stress, and UV light (Marles-Wright and Lewis, 2010). These types of stresses are common as the result of product accumulation, shifts in growth phase, and occur in different microenvironments in a bioreactor. Understanding how to rapidly integrate these signals would enable the construction of cell controllers that could regulate metabolic flux based on the changing environmental conditions of a fermentation.

6.2 An Immune System Against Phage: CRISPR Arrays *Escherichia coli* (*cas3* to CRISPR4)

Many bacteria and archaea contain an “immune system” that recognizes and intercepts foreign DNA based on previous exposure (Horvath and Barrangou,

2010; Marraffini and Sontheimer, 2010). This improves resistance against phage and the conjugative transfer of plasmid (Fig. A21-5). This function is encoded by a clustered, regularly spaced short palindromic repeat (CRISPR) region that occurs next to a gene cluster (Fig. A21-2). Each repeat spacer in the CRISPR region represents a DNA sequence of a phage or plasmid to which the bacteria have been exposed. The region is actively reprogrammed to respond to new challenges, which lead to the extension of the CRISPR region. Each repeat consists of a ~31-bp region of the targeted DNA and up to 374 repeats in a sinster (Marraffini and Sontheimer, 2010). Several genes are encoded in the cluster that form the Cas complex, which performs the tasks for the insertion of new spacer repeats and for the destruction of foreign DNA. The whole Cas-CRISPR cluster has undergone frequent horizontal transfer and some organisms have multiple clusters. An extreme example is *Methanocaldococcus jannaschii*, which contains 18 complete clusters with a total of 1188 repeat elements (Marraffini and Sontheimer, 2010).

CRISPR operates as an immune system by incorporating foreign DNA as a new repeat spacer and then recognizing this sequence in foreign DNA and destroying it (Fig. A21-5) (Horvath and Barrangou, 2010). The Cas complex cleaves foreign DNA and integrates it into the CRISPR region. The spacers are then transcribed together and processed into individual CRISPR RNAs (crRNAs), which associate with the Cas complex. The Cas-crRNA complex then recognizes the sequences in foreign DNA based on the crRNA sequence and the DNA is directed for degradation.

Bacteriophages are relevant in biotechnology as they are notorious for disrupting fermentations involving bacteria (Jones et al., 2000; Los et al., 2004). Traditionally, this is done through a process of “phage immunization” where resistant bacteria are identified through serial dilution of surviving cells (Jones et al., 2000). In one industrial example, dairy starter cultures of *S. thermophilus* were isolated and are now in use where phage resistance is conferred by CRISPR (Marraffini and Sontheimer, 2010). Bacteriophages have also been harnessed in biotechnology as agents for self-organization in the construction of materials (Flynn et al., 2003) and to be used as antibiotics (Sulakvelidze et al., 2001; Lu and Collins, 2007). For the latter, one of the issues that arises is rapid resistance that arises in the bacteria. In a study where bacteriophages were used to treat tooth decay, resistance in *Streptococcus mutans* occurred due to CRISPR immunity (van der Ploeg, 2009). The use of CRISPR elements has also been proposed as a mechanism to block the transfer of plasmids that confer antibiotic resistance and the horizontal transfer of pathogenicity islands that confer virulence (Horvath and Barrangou, 2010; Marraffini and Sontheimer, 2010).

6.3 Smelling Bacteria: *Quorum Sensing Receiver Clusters Photorhabdus luminescens* (*yhfS* to *rafZ*)

A nematode (*Heterorhabditis bacteriophora*) implements biological warfare on its insect targets (Clarke and Photorhabdus, 2008). It has developed a symbiotic relationship with the bacterium *P. luminescens* to attack and digest a

wide range of insects. The genome of the bacterium is filled with more toxins and virulence factors than any known organism (Duchaud et al., 2003). After the nematode invades, the bacteria are released into the bloodstream where they kill and breakdown the host. *P. luminescens* is filled with gene clusters that encode multiple type I pili for adhesion, a type III secretion system, many toxin and virulence factor pathways, and 22 clusters that encode PKSs and NRPSs. These produce a variety of small molecules, including antibiotics that kill other bacteria that may compete for the dead insect's nutritional resources.

Interestingly, the genome contains clusters of homologues to LuxR homologues—32 divided into two major clusters (Fig. A21-2) (Duchaud et al., 2003). In *Vibrio fischeri*, LuxR and LuxI participate in a quorum sensing circuit that enables bacteria to communicate (Waters and Bassler, 2005). LuxI is an enzyme that produces a small molecule (AI-1) that freely diffuses through the membrane. AI-1 accumulates and, when a threshold is crossed, it activates the response regulator LuxR. Many bacteria contain multiple orthogonal pairs of LuxI/LuxR homologues, which forms a sort of “language” by which cells can communicate (Fig. A21-5). Oddly, *P. luminescens* has many LuxR homologues, but no corresponding LuxI homologues (Duchaud et al., 2003). It has been postulated that the cluster of LuxR proteins may be there to sense many of the bacteria that would be competing for the nutrients available in the diseased insect. In addition, some of these sensors may have evolved to respond to host hormones, such as insect juvenile hormone, to determine the identity and developmental state of the insect (Wilkinson et al., 2009).

It is useful to be able to program communication between cells for a variety of applications biotechnology (Brenner et al., 2008). Quorum sensing provides a language by which this can be achieved, where each chemical signal represents a channel for communication (Waters and Bassler, 2005). The LuxI enzyme is a “sender device” that produces the signal and LuxR is a “receiver device” that responds to the signal (Basu et al., 2005). When these devices are separated between cells, this enables cells to communicate. This has been used to program cells to form patterns, including bull's eyes (Fig. A21-5) (Basu et al., 2005) and to implement an edge detection algorithm (Tabor et al., 2009). More direct applications in biotechnology have been proposed for quorum sensing, including controlling the density of a population of bacteria in a fermentor and killing cancer cells once an invading population crosses a threshold density (Brenner et al., 2008; Anderson et al., 2006).

7. Conclusions

In this review, we have attempted to capture the diversity of functions that are encoded in gene clusters. All of these have many potential applications in biotechnology. Achieving this potential will require methods that enable the reliable re-engineering of clusters. To date, this has been challenging because of the size of clusters, the number of genetic parts that are involved, and the complexity of the genetic regulation. Recently, progress in genetic engineering has increased

the scale of projects that are achievable. Synthetic biology has emerged as a field and libraries have been populated with genetic “parts” that can carefully control transcription and translation and “devices” that encode regulatory sensors and circuits. Methods have been developed for the rapid assembly of these parts on the scale of gene clusters, and methods exist to ultimately combine multiple clusters into a host. Together, these advances will enable the bottom-up assembly of synthetic gene clusters to simplify and optimize their function. Beyond functions that are naturally organized in clusters, it will be interesting if the design principles from studying these systems could be applied to other functions—such as the flagellum—to create similarly transferable units. This may move genetic engineering to an era of genome design where simplified gene clusters are combined in order to pull together functions from many diverse organisms to build a synthetic one.



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THE SILICON TRYPANOSOME⁹⁹

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Summary

African trypanosomes have emerged as promising unicellular model organisms for the next generation of systems biology. They offer unique advan-

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tages, due to their relative simplicity, the availability of all standard genomics techniques and a long history of quantitative research. Reproducible cultivation methods exist for morphologically and physiologically distinct life-cycle stages. The genome has been sequenced, and microarrays, RNA-interference and high-accuracy metabolomics are available. Furthermore, the availability of extensive kinetic data on all glycolytic enzymes has led to the early development of a complete, experiment-based dynamic model of an important biochemical pathway. Here we describe the achievements of trypanosome systems biology so far and outline the necessary steps towards the ambitious aim of creating a 'Silicon Trypanosome', a comprehensive, experiment-based, multi-scale mathematical model of trypanosome physiology. We expect that, in the long run, the quantitative modelling enabled by the Silicon Trypanosome will play a key role in selecting the most suitable targets for developing new anti-parasite drugs.

The Ambition of Systems Biology

Systems biology seeks to understand how functional properties of living systems, such as biological rhythms, cellular differentiation or the adaptation of organisms to changes in their environment, emerge from interactions between the components in the underlying molecular networks (Bruggeman and Westerhoff, 2007). In the case of parasites with multiple hosts, differentiation and adaptation to drugs may be particularly relevant. Current systems biology is to a large extent (but not exclusively) focused on single-cell systems. These are more amenable to global molecular analysis than multicellular organisms. This is partly because high-throughput post-genomic technologies (transcriptomics, proteomics and metabolomics) make it relatively easy to measure many components of a homogeneous population of cells simultaneously. Furthermore, dynamic measurements of the response of a cell population to a shared stimulus allow insight into the functional connectivity between components (Richard et al., 1996; Hynne et al., 2001; Nikerel et al., 2006, 2009; Schmitz et al., 2009).

Mathematical methods that enable quantitative descriptions of the dynamic interplay between the molecules in living cells are being developed and, for the first time, it is possible to envisage a comprehensive molecular description of the functional circuitry of cellular systems. The Silicon Cell project (Snoep et al., 2006; Westerhoff et al., 2009) involves an international consortium of researchers aiming at a mathematical description of life at the cellular level on the basis of complete and quantitative genomic, transcriptomic, proteomic, metabolomic and phenotypic information. So far, the most ambitious whole-cell modelling efforts have targeted the model organisms *Escherichia coli* and *Saccharomyces cerevisiae*. The advanced state of understanding and the enormous amount of data relating to these organisms have made them obvious candidates for such a comprehensive description. Yet, the variety of organisms in the 'JWS Online' model repository (www.jjj.bio.vu.nl) demonstrates that the silicon cell initiative is not limited to these organisms.

The Unique Advantages of Trypanosome Systems Biology

The African trypanosome, *Trypanosoma brucei*, the causative agent of human African trypanosomiasis and Nagana cattle disease (Barrett et al., 2003), has emerged as a front runner in systems biology analysis. The relative simplicity of the energy metabolism of its bloodstream form and the early availability of a comprehensive and uniform set of kinetic data of the enzymes involved, were crucial factors for the successful construction of a detailed computer model of trypanosome glycolysis (Bakker et al., 1997). The obvious questions for this model were initially in the realm of drug-target selection; the first studies analysed in depth how sensitive the pathway overall would be to varying extents of inhibition of each enzyme (Bakker et al., 1999a). Another important factor stimulating further development of trypanosome systems biology was the possibility of reproducible *in vitro* cultivation, first of the procyclic insect stage, but later also of the long slender bloodstream form (Hirumi and Hirumi, 1989; Haanstra, 2009). Transitions between distinct life-cycle stages can be studied in a tractable and synchronous differentiation system (Fenn and Matthews, 2007). Moreover, the complete genome of *T. brucei* has been sequenced and annotated, and a metabolic pathway database has been developed (Berriman et al., 2005; Chukualim et al., 2008).

An extension of the scope of trypanosome systems biology to include gene expression is facilitated by the absence of transcriptional regulation in trypanosomes. This implies that, unlike in most other organisms, the gene-expression cascade is regulated only post-transcriptionally. The genes of African trypanosomes – as well as those of the other kinetoplastids – are arranged in polycistronic transcription units which can be hundreds of kilobases long (Berriman et al., 2005; Siegel et al., 2009). All evidence so far indicates that RNA polymerase II transcribes constitutively, without intervention of regulatory factors (Lee et al., 2009; Palenchar and Bellofatto, 2006). Individual mRNAs are excised by a *trans*-splicing complex which places identically capped 39 nt leaders at the 5k end of every mRNA (Liang et al. 2003); this splicing is co-ordinated with polyadenylation of the RNA located immediately upstream. Indeed, regulation of mRNA biogenesis may well be restricted to the processing steps (Lustig et al., 2007; Stern et al., 2009), while steady-state levels are further influenced by the rate of mRNA degradation (Clayton and Shapira, 2007). In fact, the majority of evidence concerning regulation of gene expression has implicated mRNA decay as the dominant factor (Clayton and Shapira, 2007; Haanstra et al., 2008b), and this is the only step for which mechanistic details of the regulation are available.

After the complete sequencing of the trypanosome genome (Berriman et al., 2005), mRNA microarray analyses of the differentiation from the bloodstream to the procyclic form have demonstrated that the expression of whole sets of mRNAs is coordinately regulated (Queiroz et al., 2009). When gene-expression is studied during synchronous differentiation, accurate time profiles of extremely homogenous cell populations can be obtained (Kabani et al., 2009). Most results

so far suggest that such regulation is mediated by RNA-binding proteins that bind to specific sequences in the 3k-untranslated regions of mRNAs (Archer et al., 2009; Clayton and Shapira, 2007; Estévez, 2008). The rate of mRNA translation and protein turnover are other factors influencing the steady-state protein levels. Here too, key regulatory proteins have been identified (Paterou et al., 2006; Walrad et al., 2009).

Milestones of Trypanosome Systems Biology

A quantitative mathematical model of energy metabolism in the long slender form of the trypanosome (i.e. the form that replicates in the mammalian bloodstream) has been developed (Bakker et al., 1997) and iteratively updated after experimental testing (Bakker et al., 1999a,b; Albert et al., 2005; Haanstra et al., 2008a). This model yields quantitative predictions of the flux through glycolysis, the concomitant ATP production flux, and the concentrations of glycolytic metabolites, at steady state as well as following a perturbation. Input data for the model are kinetic equations and parameters of enzymes and their concentrations. Through this model, the effects of drugs on the glycolytic pathway can be assessed quantitatively starting from their effects on the individual enzymes.

Free-energy metabolism in the bloodstream form of *T. brucei* has been a logical starting point for the ‘bottom-up’ construction of a ‘Silicon Trypanosome’. Bloodstream forms of the parasite depend exclusively on substrate-level phosphorylation for ATP production through glycolysis, which proceeds as far as pyruvate (Flynn and Bowman, 1973) (Fig. A22-1). Pyruvate is the end product and is secreted from the cell. Many of the glycolytic enzymes differ in terms of allosteric regulation from their mammalian counterparts, and this probably relates to the fact that in *T. brucei* the first seven enzymes of the pathway reside within membrane-bounded, peroxisome-like organelles called glycosomes (Oppendoes and Borst, 1977; Parsons, 2004; Michels et al. 2006; Haanstra et al. 2008), which isolate most of the glycolytic pathway from the rest of the metabolic network involved in consumption of ATP and NAD(H). Even in growing and dividing trypanosomes, virtually all glucose is converted to pyruvate, as the amount required for biosynthesis is quantitatively negligible (Haanstra, 2009). This finding supports the initial choice to model the glycolytic pathway without any branches other than the one to glycerol. Glycerol production is crucial under anaerobic conditions (Fairlamb et al., 1977).

Since the publication of the first version of the glycolysis model (Bakker et al., 1997), there have been two major updates (Helfert et al., 2001; Albert et al., 2005). Both of these involved updates and extensions of the enzyme kinetic dataset, e.g. the explicit inclusion of individual enzymes that were previously grouped into a net multi-step conversion. In the second update (Albert et al., 2005) the enzyme expression levels (V_{\max}) were adapted to reflect the concentrations observed in trypanosomes obtained from controlled state-of-the-art *in vitro*

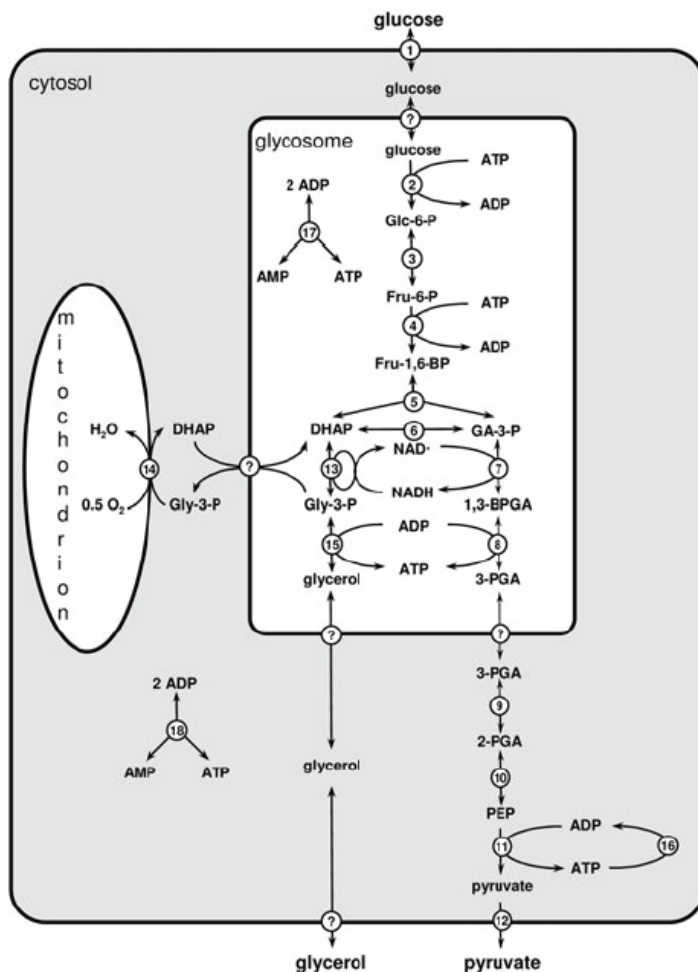


FIGURE A22-1 The glycolytic pathway in *Trypanosoma brucei*. Reaction numbers indicate:

1. glucose transport; 2. hexokinase; 3. phosphoglucose isomerase; 4. phosphofructokinase; 5. aldolase; 6. triose-phosphate isomerase; 7. glyceraldehyde-3-phosphate dehydrogenase; 8. phosphoglycerate kinase; 9. phosphoglycerate mutase; 10. enolase; 11. pyruvate kinase; 12. pyruvate transport; 13. glycerol-3-phosphate dehydrogenase; 14. glycerol-3-phosphate oxidase (a combined process of mitochondrial glycerol-3-phosphate dehydrogenase and trypanosome alternative oxidase); 15. glycerol kinase; 16. combined ATP utilization; 17. glycosomal adenylate kinase; 18. cytosolic adenylate kinase. Question marks indicate uncharacterized transport processes. Abbreviations of metabolite names: Glc-6-P: glucose 6-phosphate; Fru-6-P: fructose 6-phosphate; Fru-1,6-BP: fructose 1,6-bisphosphate; DHAP: dihydroxyacetone phosphate; Gly-3-P: glycerol 3-phosphate; GA-3-P: glyceraldehyde 3-phosphate; 1,3-BPGA: 1,3-bisphosphoglycerate; 3-PGA: 3-phosphoglycerate; 2-PGA: 2-phosphoglycerate; PEP: phospho-*enol*pyruvate.

cultivation. Key missing pieces of information remain the mechanism and kinetics of the transport of glycolytic metabolites across the glycosomal membrane. The identification of semi-selective pores in peroxisomal membranes suggests that the smaller metabolites equilibrate across the glycosomal membrane, while bulkier molecules like ATP or NADH require specific transporters (Grunau et al., 2009; Rokka et al., 2009). This idea justifies, with hindsight, the choice to model the transport of a number of small intermediates as rapid-equilibrium processes.

A number of basic and applied biological questions have been addressed using the glycolysis model. For example, it was predicted and then experimentally confirmed (Bakker et al., 1999a,b) that the uptake of glucose across the plasma membrane was a major flux controlling step and therefore an interesting drug target. Enzymes that have been suggested to control glycolysis in mammalian cells, like hexokinase, phosphofructokinase and pyruvate kinase (Schuster and Holzhütter, 1995), exerted little control in trypanosomes, according to the model (Bakker et al., 1999a; Albert et al., 2005). Experiments, in which the expression of these enzymes was knocked down, confirmed this prediction qualitatively. However, the enormous overcapacity of some enzymes, which was predicted by the model, was shown to be exaggerated (Albert et al., 2005). This suggests that there are *in vivo* regulation mechanisms affecting these enzymes in a currently unknown fashion. Protein phosphorylation may contribute, since a glycosomal phosphatase has been identified in developmental signalling (Szoor and Matthews, unpublished data). The inhibition of anaerobic glycolysis by glycerol was also reproduced by the model, first qualitatively and then quantitatively (Bakker et al., 1997; Albert et al., 2005).

An interesting biological feature that was revealed by the model was the relationship between compartmentation of glycolysis in glycosomes and the virtual absence of allosteric regulation of the glycolytic enzymes. Glycolysis models predict that glycolytic intermediates accumulate readily due to the investment of ATP at the beginning of the pathway (Teusink et al., 1998; Bakker et al., 2000). This risky 'turbo' effect can be avoided either by allosteric feedback regulation of hexokinase or by compartmentation of the pathway in glycosomes (Fig. A22-2). Compartmentation prevents the accumulation of intermediates, because the net ATP production occurs outside the glycosome and this excess of ATP cannot activate the first enzymes of glycolysis. This model prediction was recently confirmed experimentally (Haanstra et al., 2008a), providing a clear example of model-driven experimental design and hypothesis-driven systems biology. According to model predictions the glycolytic intermediates glucose 6-phosphate, fructose 6-phosphate and fructose-1,6-bisphosphate should accumulate on addition of glucose if the glycolytic enzymes are not properly located in the glycosome. Indeed, accumulation of glucose 6-phosphate could be measured in a *PEX14-RNAi* mutant in which protein import into the glycosomes is disturbed. A similar phenotype was observed on glycerol addition, which led to accumulation of glycerol 3-phosphate, both in the model and in the *PEX14-RNAi* cells.

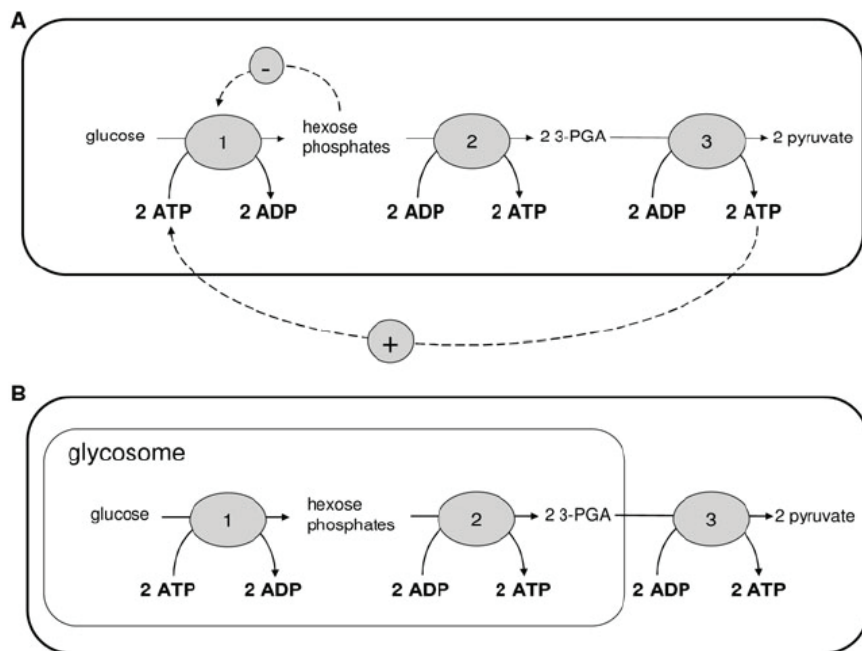


FIGURE A22-2 A. The positive feedback from the ATP produced by glycolysis to the initial kinase reactions can lead to toxic accumulation of hexose phosphates. In many organisms this is prevented by a negative feedback from the hexose phosphates to hexokinase. B. In trypanosomes, the compartmentation of glycolysis prevents the positive feedback. This renders the negative feedback unnecessary, and indeed there is no evidence for such feedback in trypanosomes.

Also in accordance with model predictions, a down-regulation of the expression of the genes encoding hexokinase and glycerol kinase rescues the *PEX14-RNAi* cells on glucose and glycerol, respectively (Kessler and Parsons, 2005; Haanstra et al., 2008a).

More recently, a model of the gene-expression cascade, based on quantitative knowledge of transcription, RNA precursor degradation, *trans*-splicing and mRNA degradation for phosphoglycerate kinase (PGK) has been generated (Haanstra et al. 2008b). The model allowed a quantitative analysis of the control and regulation of the expression of the PGK isoenzymes. It was shown that regulation of mRNA degradation explains 80–90% of the regulation of mature mRNA levels, while precursor degradation and *trans*-splicing make only minor contributions.

In spite of the success of the model, it covers to date only a small part of trypanosome metabolism. This relates, for instance, to the fact that even the

compartmentalised glycolysis does branch into other pathways, for example towards the biosynthesis of glycoconjugates and the pentose phosphate pathway. Although the fluxes into these branches may be small, they are vital for trypanosomes. Sufficient kinetic data have become available to enable extension of the model to include the pentose phosphate pathway which provides NADPH for reductive biosyntheses and also reducing equivalents to sustain cellular redox balance. Since redox balance is intimately related to the biosynthesis of trypanothione (from polyamine and glutathione precursors), a natural next step in a bottom-up systems biology approach to trypanosome metabolism would be the inclusion of the trypanothione–pentose phosphate pathway and related areas of redox metabolism (Fig. A22-3).

Growth Stages of Building a Silicon Trypanosome

Our current level of knowledge of trypanosome redox metabolism, as well as its biological importance (Krauth-Siegel and Comini, 2008), render it a natural choice for a next model extension (Fig. A22-3). The inclusion of redox metabolism is particularly interesting as trypanosome redox metabolism is sufficiently different from its human counterpart to offer perspectives for drug discovery. The unusual polyamine–glutathione conjugate trypanothione or bis(glutathionyl) spermidine (Fairlamb and Cerami, 1992) takes on the majority of roles served by glutathione in most other cell types. In addition, work in the last few years revealed that the enzymes involved in the synthesis and reduction of trypanothione are essential for the parasite (Krauth-Siegel and Comini, 2008).

The trypanocidal drug eflornithine exerts its trypanocidal activity as an irreversible inhibitor of the enzyme ornithine decarboxylase (Bacchi et al., 1980), an enzyme involved in trypanothione biosynthesis (enzyme 10 in Fig. A22-3). A significant amount of information is available on kinetic parameters of that pathway, too. Preliminary attempts to model trypanothione metabolism have been made (Xu Gu, University of Glasgow PhD thesis, unpublished). Information available on the abundance of key metabolites measured in bloodstream form *T. brucei* grown *in vitro* (Fairlamb et al., 1987) and *in vivo* (Xiao et al., 2009), before and after exposure to eflornithine, was used to determine whether predicted behaviour under those perturbed conditions emulated the measured behaviour. The scarcity of kinetic data describing the whole pathway, however, has presented many challenges to constructing a model that captures observed behaviour. The acquisition of new kinetic data and the implementation of new mathematical tools to fill gaps in the data (Nikerel et al., 2006; Smallbone et al., 2007; Resendis-Antonio, 2009) should improve this.

An extension of the glycolysis model to include the pentose phosphate pathway (Hanau et al., 1996; Barrett, 1997; Duffieux et al., 2000) and trypanothione metabolism should be a suitable next step in the modular approach that we envisage towards a complete Silicon Trypanosome. Initial efforts in this direction (not

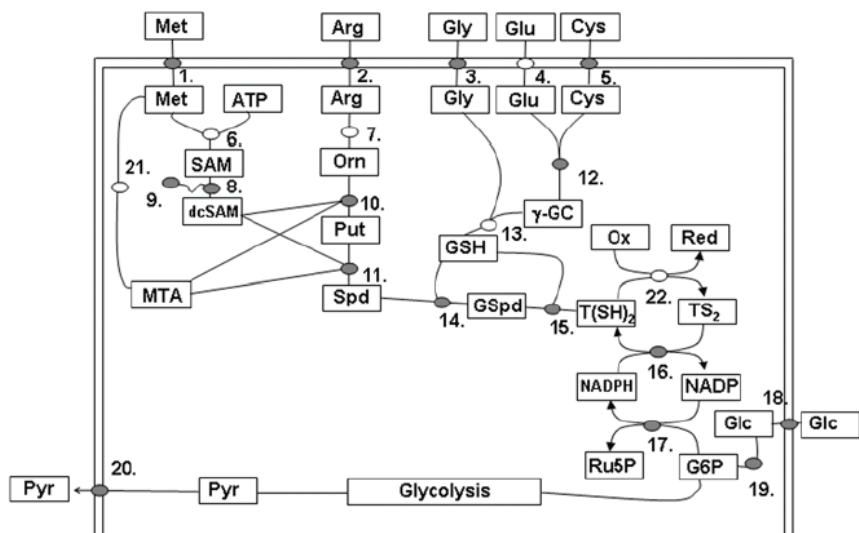


FIGURE A22-3 The glycolytic and trypanothione pathways are linked through the oxidative pentose phosphate pathway. Metabolites are presented in abbreviated form within rectangles. Enzymes and transporters are circles. Kinetic data is available for those shaded grey. Met = methionine; Arg = arginine; Gly = glycine; Glu = glutamate; Cys = cysteine; ATP = adenosine triphosphate; SAM = S-adenosylmethionine; dcSAM = decarboxylated S-adenosylmethionine; MTA = methylthioadenosine; Orn = ornithine; Put = putrescine; Spd = spermidine; c-GC = c-glutamylcysteine; GSH = glutathione; GSpd = glutathionylspermidine; T(SH)₂ = reduced trypanothione; TS₂ = oxidised trypanothione; NADP = nicotinamide adenine dinucleotide phosphate; NADPH = reduced nicotinamide adenine dinucleotide phosphate; Glc = glucose; G6P = glucose 6-phosphate; Ru5P = ribulose 5-phosphate; Ox = oxidised cellular metabolites; Red = reduced cellular metabolites. 1. = methionine transport; 2. = arginine transport; 3. = glycine transport; 4. = glutamate transport; 5. = cysteine transport; 6. = methionine adenosyltransferase; 7. = arginase (N.B., a robust arginase gene orthologue has not been annotated in the *T. brucei* genome project, raising the possibility that arginine does not serve as a source of ornithine in these cells); 8. = S-adenosylmethionine decarboxylase; 9. = prozyme; 10. = ornithine decarboxylase; 11. = spermidine synthase; 12. = c-glutamylcysteine synthetase; 13. = glutathione synthetase; 14. = c-glutathionylspermidine synthetase; 15. = trypanothione synthetase/amidase (in *T. brucei* 14. & 15. are catalysed by a single protein); 16. = trypanothione reductase; 17. oxidative pentose phosphate pathway (glucose 6-phosphate dehydrogenase, 6-phosphogluconolactonase & 6-phosphogluconate dehydrogenase); 18. = glucose transporter; 19. = hexokinase (this enzyme links the redox pathway to glycolysis); 20. = pyruvate transporter; 21. = methionine cycle enzymes; 22. The pathway of electrons from reduced trypanothione for final acceptance on oxidised cellular metabolites or macromolecules is complex, involving transfers via other redox active intermediates including tryparedoxin (thioredoxin-like proteins) and peroxidoxin.

published) have indicated the importance of the compartmentation of the pentose phosphate pathway. Although most of the enzymes of the pathway have a peroxisome targeting sequence (PTS1), a significant fraction of their activity is often found in the cytosol (Michels et al., 2006; Heise and Opperdoes, 1999; Duffieux et al., 2000). A correct localisation of the enzymes as well as good estimates of the transport of intermediates across the glycosomal membrane will be key to a good model of the pentose phosphate pathway.

Challenges of Trypanosome Systems Biology

The first initiatives in systems biology of trypanosomes as well as of other organisms dealt with enzymatic sub-systems, such as glycolysis. The models have depended largely on kinetic data for isolated enzymes. However, the abundance of these enzymes can, in principle, be controlled by the rates of transcription, RNA processing, translation, protein modification and turnover. These processes themselves may be regulated through complex signalling networks in response to both internal and external conditions (Westerhoff et al. 1990).

To include gene expression in a Silicon Trypanosome requires a dramatic increase in model complexity – as well as the acquisition of new types of data on a large scale. Fortunately, the absence of transcriptional control of most individual open reading frames makes trypanosome gene expression simpler than that of yeast or even *E. coli*, rendering it uniquely amenable to mathematical modelling.

It may well be possible to describe much of trypanosome mRNA metabolism using the following parameters: the rate constant of processing of the precursor RNA, i.e. of *trans*-splicing; the rate constant of degradation of the precursor (which competes with its *trans*-splicing); the rate constant of polyadenylation; and the rate constant of mRNA degradation. The rates of degradation of the precursor and the mature mRNA can be measured by inhibiting splicing and transcription. To measure the rate of mRNA processing two approaches are possible. First, one can inhibit transcription alone, and assay precursor decay; this approach is, however, compromised by practical constraints since splicing is very rapid. Second, the splicing rate can be calculated based on the steady-state abundance of the precursor mRNA, and the half-life and abundance of the products. This methodology has already been applied to the mRNA encoding PGK and it was demonstrated that splicing occurred within less than one minute; mRNA decay was the primary determinant of mRNA abundance (Haanstra et al., 2008b).

Previous microarray studies with yeast have yielded estimates of the half-lives and polysomal loading of many mRNAs (e.g. Grigull et al., 2004). Deep sequencing technology – being more sensitive – should allow measurement of the abundances of all mRNAs and precursors on a genome-wide scale and to the accuracy required for the modelling; from these data, it should be possible to derive the steady-state abundances and half-lives of all RNAs, revealing regulated or inefficient processing. This – combined with global polysome profiling

– will provide quantitative data which allow quantifying the regulation of the processing, degradation and translation of each mRNA (Daran-Lapujade et al. 2007). The next challenge would then be to integrate such measurements with metabolic modelling in order to provide a complete model of pathways, from DNA to metabolic end-products.

Anticipated Outcomes from a Silicon Trypanosome

So far, the systems biology approach to trypanosomes has contributed to a fundamental understanding of cellular regulation (Bakker et al., 1999a; Haanstra et al., 2008a), as well as to improvements in the drug-target selection process (Bakker et al., 1999a,b; Albert et al., 2005; Hornberg et al., 2007). Since the initial systems biology analysis only addressed processes associated with less than 1% of the organism's genome, we would expect many more new insights to lie ahead.

Drugs currently used against human African trypanosomiasis are unsatisfactory for a number of reasons, including their extreme adverse effects in the patient and the emergence of resistant parasites. New drugs are urgently needed and there is hope that a better understanding of the control points of the metabolic network can guide the selection of optimal drug targets. This has already been achieved for enzymes of the glycolytic pathway (Hornberg et al., 2007). This information can be used alongside enhanced chemoinformatics (Frearson et al., 2007) in order to determine those components of the trypanosome that are most amenable to drug targeting.

As a consortium we have embarked on the construction of a Silicon Trypanosome. In this review we have discussed the current status and future directions of trypanosome systems biology that form the context of this endeavour. Our ambition is to achieve a comprehensive, quantitative description of the flow of information from gene, through transcript and protein, to metabolism and back. This will allow prediction of how the parasite responds to changes in its environment, with respect to nutrients, temperature and/or chemical inhibitors. It will also assist the deciphering of complex phenotypes generated by genetic perturbations in the laboratory or in the field. Thus, model predictions will improve our biological understanding of the differentiation and adaptation of the parasite as well as stimulate the discovery of inhibitors that attack processes which control trypanosome growth. The latter should contribute to the development of new optimised drugs for trypanocidal chemotherapy. Pioneering efforts have focused on energy metabolism and recently started to include adaptations of the parasite via gene expression (Haanstra, 2009).

The construction of a complete Silicon Trypanosome, which integrates metabolism, gene expression and signal transduction is an ambitious project. Clearly the route towards this objective will be long, and many challenges will emerge as the datasets required to build such a model are collected and analysed. How-

ever, the emergence of methods to allow collection of massive datasets, at every level, suggests that we may, in time, be able to generate a reasonably complete mathematical description of trypanosome cellular biology. Even if completion is not feasible, the evolving description will always represent the best conceivable dynamic representation of our knowledge of trypanosome biology. As a result, drug development programmes will have at their disposal a predictive model of the trypanosome to help identify those parts of metabolism most amenable to targeting by novel drugs and to controlling vital functions of the parasite. The project will be strengthened by parallel world-wide systems biology projects of human metabolism, in which some of us will be involved. After all, killing trypanosomes is easy. The difficulty is to kill the trypanosome without harming its host (Bakker et al., 2002). A careful comparison of the behaviour of our Silicon Trypanosome to quantitative knowledge of the control of human metabolism, will allow the identification of *selective* targets.

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Appendix B

Agenda

The Science and Applications of Synthetic and Systems Biology

March 14-15, 2011
Keck Building, Room 100
500 Fifth Street, NW
Washington, DC

DAY 1: MONDAY, MARCH 14, 2011

- | | |
|-----------------------|--|
| 8:00–8:30 am | Registration and Continental Breakfast |
| 8:30–8:45 am | Welcoming Remarks
David Relman, M.D., Chair, and Jim Hughes, M.D.,
and Lonnie King, D.V.M., Vice-Chairs, Forum on
Microbial Threats |
| 8:45–9:30 am | Keynote Remarks
David Relman, Moderator

Synthetic Biology and Biodefense
Andrew Ellington, Ph.D. , University of Texas at Austin |
| 9:30–10:00 am | Discussion |
| 10:00–10:15 am | Break |

SESSION 1: The Promise of Synthetic and Systems Biology for the Understanding and Management of Infectious Diseases

Jesse Goodman, Moderator

- 10:15–10:45 am Synthetic biology, systems biology, and microbial threats
James J. Collins, Ph.D., Boston University
- 10:45–11:15 am A metabolic engineering platform for discovery and production of new therapeutics
Greg Stephanopoulos, Ph.D., Massachusetts Institute of Technology (MIT)
- 11:15–11:45 am Insights into pathogen-host interactions from systems biology
Bernhard Palsson, Ph.D., University of California, San Diego
- 11:45–12:15 pm Commercial applications of synthetic biology
David Berry, M.D., Ph.D., Flagship Ventures
- 12:15–12:45 pm Discussion
- 12:45–1:30 pm Lunch**

SESSION 2: “The Basics” of Synthetic Biology: Bottom-Up vs. Top-Down Approaches

George Poste, Moderator

- 1:30–2:00 pm Systems for synthetic biology
Hans Westerhoff, Ph.D., University of Manchester and the VU University Amsterdam
- 2:00–2:30 pm Design and complexity
Herbert Sauro, Ph.D., University of Washington
- 2:30–3:00 pm DNA synthesis
George Church, Ph.D., Harvard University
- 3:00–3:30 pm Synthetic biology “from scratch”
Gerald Joyce, M.D., Ph.D., The Scripps Research Institute
- 3:30–3:45 pm Break**

- 3:45–4:15pm Protein engineering and high throughput immune function analyses for the discovery of the next generation of protein therapeutics
George Georgiou, Ph.D., University of Texas at Austin
- 4: 15–4:45 pm Synthesis and installation of genomes
Clyde Hutchison III, Ph.D., J. Craig Venter Institute
- 4:45–5:15 pm Access through refactoring: Rebuilding complex functions from the ground up
Chris Voigt, Ph.D., University of California, San Francisco
- 5:15–6:00 pm Discussion
- 6:00 pm Meeting Adjourns**

DAY 2: TUESDAY, MARCH 15, 2011

- 8:45–9:15 am Continental breakfast
- 9:15–9:30 am Summary of Day One and Preview of Day Two: David Relman, M.D., Chair, Forum on Microbial Threats

SESSION 3: Using Synthetic/Systems Biology**Approaches to Manage Biofilms**

David Relman, Moderator

- 9:30–10:00 am Harnessing and controlling social activities of bacteria
E. Peter Greenberg, Ph.D., University of Washington
- 10:00–10:30 am Developing a synthetic biology device that detects biofilm formation on indwelling catheters
Paul Freemont, Prof., Imperial College London
- 10:30–10:45 am Break**
- 10:45–11:15am Novel approaches to combat biofilm drug tolerance
Kim Lewis, Ph.D., Northeastern University
- 11:15–11:45 am Engineering viruses to destroy biofilms
Timothy Lu, M.D., Ph.D., Massachusetts Institute of Technology

11:45–12:15 pm Discussion

12:15–1:00 pm Lunch

SESSION 4: Synthetic/Systems Biology Products and Platforms: Potential for Novel Preventives, Diagnostics, and Therapeutics in Infectious Diseases

Kent Kester, Moderator

1:00–1:30 pm Engineering microbial metabolism for production of anti-malarial drugs

Jay Keasling, Ph.D., University of California, Berkeley

1:30–2:00 pm Dynamics of clonal evolution: Immune system on a chip

Jim Heath, Ph.D., California Institute of Technology

2:00–2:30 pm Using systems biology to understand and develop more effective vaccines

Bali Pulendran, Ph.D., Emory University

2:30–2:45 pm Break

2:45–3:15 pm HTP screening system based on synthetic genes and proteins

Stephen Johnston, Ph.D., Arizona State University

3:15–3:45 pm Synthetic biology and the art of biosensor design

Chris French, Ph.D., University of Edinburgh

3:45–4:45 pm Open Discussion with Panelists, Forum Members, and Audience

4:45–5:00 pm Wrap-up Discussion, Jim Hughes and David Relman

5:00 pm Meeting Adjourns

Appendix C

Acronyms

ACP	acyl carrier protein
AGP	antimicrobial growth promoter
AHL	acyl homoserine lactone
AMR	antimicrobial resistance
ARDC	American Research and Development Corporation
bp	base pair
CAD	computer-aided design
CAMK4/CaMKIV	calcium-calmodulin dependent kinase 4
CF	cystic fibrosis
CFU	colony forming unit
DARPA	Defense Advanced Research Projects Agency
DNA	deoxyribonucleic acid
DspB	DispersinB [®]
EPS	extracellular polymeric substance
ESBL	extended-spectrum β -lactamase
FDA	Food and Drug Administration
GFP	green fluorescent protein

HAI	hemagglutination inhibition
HTS	high-throughput screening
ICEs	integrative conjugative elements
iGEM	International Genetically Engineered Machine competition
IOM	Institute of Medicine
IS	insertion sequence
JCVI	J. Craig Venter Institute
kb	kilobase
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
LAIV	live attenuated influenza vaccine
MART-1	melanoma antigen recognized by T-cells 1
mb	millibase
MDR	multidrug resistant
MIC	minimum inhibitory concentration
NIH	National Institutes of Health
NSABB	National Science Advisory Board for Biosecurity
OIE	World Organisation for Animal Health
PCA	principal component analysis
PCR	polymerase chain reaction
PET	positron emission tomography
R&D	research and development
rDNA	Recombinant DNA
RNA	ribonucleic acid
RNAP/RNAPol	RNA polymerase
ROS	reactive oxygen species
TIV	trivalent inactivated influenza vaccine
TLR	Toll-like receptor
WHO	World Health Organization

Appendix D

Glossary

Adhesins: Bacterial proteins that promote adherence to host-cell membranes; see http://www.nature.com/nrm/journal/v3/n10/glossary/nrm932_glossary.html (accessed August 3, 2010).

Adoptive T-cell immunotherapy: A form of transfusion therapy consisting of the infusion of various mature T-cell subsets with the goal of eliminating a tumor and preventing its recurrence (Carl, H. 2007. Principles of adoptive T cell cancer therapy. *Journal of Clinical Investigation* 117(5):1204).

Antibiotic: Class of substances that can kill or inhibit the growth of some groups of microorganisms. Originally antibiotics were derived from natural sources (e.g., penicillin from molds), but many currently used antibiotics are semisynthetic and modified with additions of man-made chemical components. *See Antimicrobial.*

Antibody: Also known as an immunoglobulin, an antibody is a large Y-shaped protein used by the immune system to identify and neutralize foreign objects like bacteria and viruses.

Antimicrobial: In this document, the term “antimicrobial” is used inclusively to refer to any agent (including an antibiotic) used to kill or inhibit the growth of microorganisms (bacteria, viruses, fungi, or parasites). This term applies whether the agent is intended for human, veterinary, or agricultural applications.

Antimicrobial resistance: Most commonly, this refers to infectious microbes that have acquired the ability to survive exposures to clinically relevant con-

centrations of antimicrobial drugs that would kill otherwise sensitive organisms of the same strain. The phrase is also used to describe any pathogen that is less susceptible than its counterparts to a specific antimicrobial compound (or combination thereof).

Apoptosis: A process of programmed cell death by which cells undergo an ordered sequence of events which lead to death of the cell, as occurs during growth and development of the organism, as a part of normal cell aging, or as a response to cellular injury.

Aptamer: An oligonucleotide or peptide molecule that binds to a specific target molecule.

Artemisinin: Also known as qinghaosu, its derivatives are a group of drugs that possess the most rapid action of all current drugs against *falciparum malaria*.

β -Lactam antibiotics: A broad class of antibiotics that include penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems, that is, any antibiotic agent that contains a β -lactam nucleus in its molecular structure. They are the most widely used group of antibiotics.

β -Lactamase: A type of enzyme produced by some bacteria that is responsible for their resistance to β -lactam antibiotics, such as penicillins, cephalosporins, cephamycins, and carbapenems.

Bacteria: Microscopic, single-celled organisms that have some biochemical and structural features different from those of animal and plant cells.

Bacteriophage: A virus that infects bacteria.

BioBricks: Standard biological parts—DNA sequences of defined structure and function—that share a common interface and are designed to be composed and incorporated into living cells such as *E. coli* to construct new biological systems. BioBrick parts represent an effort to introduce the engineering principles of abstraction and standardization into synthetic biology.

Biocontrol: Method of controlling pests (including insects, mites, weeds, and diseases) in plants that relies on predation, parasitism, herbivory, or other natural mechanisms.

Biofilms: Bacterial communities that adhere to biotic or abiotic surfaces. These microorganisms are usually encased in an extracellular polysaccharide matrix that

they themselves synthesize and may be found on essentially any environmental surface in which sufficient moisture is present.

Cephalosporins: A class of β -lactam antibiotics originally derived from *Acremonium*, which was previously known as “Cephalosporium.” They have the same mode of action as other β -lactam antibiotics (such as penicillins) but are less susceptible to enzymes that break down penicillin (penicillinases).

Chassis: In the context of synthetic biology, chassis refers to the cell or organism in which the engineered DNA or biopart is embedded in order to produce the desired device or system (Royal Academy of Engineering, 2009).

Cytokine: A category of signaling molecules that mediate and regulate immunity, inflammation and hematopoiesis, and many other cellular processes. Cytokines were initially identified as products of immune cells that act as mediators and regulators of immune processes but many cytokines are now known to be produced by cells other than immune cells and they can have effects on nonimmune cells as well.

Dendritic cells: Dendritic cells are a type of immune system cell known as antigen presenting cells; when antigens enter the body, dendritic cells communicate that information to T-cells. Toll-like receptors (TLRs) are innate receptors expressed by dendritic cells. TLRs have evolved to sense highly conserved molecular patterns within microbes and viruses.

Deoxyribonucleic acid (DNA): A nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms with the exception of some viruses. The main role of DNA molecules is the long-term storage of information.

Disease: In medicine, disease is often viewed as an observable change of the normal network structure of a system resulting in damage to the system.

DispersinB[®] (DspB): An antibiofilm enzyme, which has been shown to inhibit and disperse biofilms.

DNA synthesis: A technology that enables the de novo generation of genetic sequences that specifically program cells for the expression of a given protein.

Enterobacter: A genus of common Gram-negative, rod-shaped bacteria of the family Enterobacteriaceae. Several strains of these bacteria are pathogenic and cause opportunistic infections in immunocompromised (usually hospitalized)

hosts and in those who are on mechanical ventilation. The urinary and respiratory tracts are the most common sites of infection.

Feedback loop: Feedback is a mechanism, process, or signal that is looped back to control a system within itself. Such a loop is called a feedback loop. In systems containing an input and output, feeding back part of the output so as to increase the input is *positive feedback (regeneration)*; feeding back part of the output in such a way as to partially oppose the input is *negative feedback (degeneration)*.

Gene regulation: The process through which a cell determines—through interactions among DNA, RNA, proteins, and other substances—when and where genes will be activated and how much gene product will be produced; see <http://pubs.niaaa.nih.gov/publications/arh26-3/165-171.htm> (accessed July 19, 2010).

Genome: The complete genetic composition of an organism (e.g., human, bacterium, protozoan, helminth, fungus), contained in a chromosome or set of chromosomes or in a DNA or RNA molecule (e.g., a virus).

Genome metastructure: Organization of the genome with respect to where the various structural and functional components are located.

Gram-negative bacteria: Refers to the inability of a microorganism to accept a certain stain. This inability is related to the cell wall composition of the microorganism and has been useful in classifying bacteria.

Gram-positive bacteria: Refers to the ability of a microorganism to retain a certain stain. This ability is related to the cell wall composition of the microorganism and has been useful in classifying bacteria.

Half-life (biological): The time it takes for a substance to lose half of its pharmacologic, physiologic, or radiologic activity.

Hemagglutination inhibition (HAI): The HAI tests measure the amount of serum antibodies directed against a hemagglutinating virus, with higher levels, or titers, being associated with greater protection.

Human Genome Project: An international scientific research project with a primary goal of determining the sequence of chemical base pairs which make up DNA, and of identifying and mapping the approximately 20,000–25,000 genes of the human genome from both a physical and functional standpoint. A working draft of the genome was announced in 2000 and a complete one in 2003, with further, more detailed analysis still being published.

Immunoglobulins: A class of proteins produced in lymph tissue in vertebrates and that function as antibodies in the immune response; see <http://wordnetweb.princeton.edu/perl/webwn?s=immunoglobulin> (accessed August 2, 2010).

Integrative conjugative elements (ICEs): Chromosomally located gene clusters that encode phage-linked integrases and conjugation proteins as well as other genes associated with an observable phenotype, such as virulence or symbiosis. They can be transferred between cells and have some phage-like genes, but they do not lyse the cell or form extracellular particles; see http://www.nature.com/nrmicro/journal/v3/n9/glossary/nrmicro1235_glossary.html (accessed June 16, 2010).

Integron: A mobile DNA element that can capture and carry genes, particularly those responsible for antibiotic resistance. It does this by site-specific recombination; see <http://www.medterms.com/script/main/art.asp?articlekey=32273> (accessed June 14, 2010).

Intrinsic resistance gene: A gene that codes for traits that reduce an organism's sensitivity to antibiotics, such as efflux pumps, but is not specifically a resistance gene.

Lysogenic: The state of a bacterial cell that has an integrated phage (prophage) in its chromosome; see <http://www.everythingbio.com/glos/definition.php?word=lysogenic> (accessed June 18, 2010).

Lysogenic bacteriophage: A lysogenic phage is a "temperate" bacteriophage (such as lambda phage) that integrates its genome into the genome of the host without immediately transcribing and making new virus particles. However, at a later time, the integrated genome can be excised and begin to be actively transcribed, producing virus particles that eventually burst the cell. This is opposite to the "lytic" variety of bacteriophage (T4 phage) that immediately transcribe and make new virus after infecting the host cell, causing rapid lysis; see http://wiki.answers.com/Q/What_is_a_lysogenic_bacteriophage (accessed June, 23 2010).

Macrolides: Family of bacteriostatic antibiotics that inhibit protein synthesis by binding to the large subunit of the bacterial ribosome; includes erythromycin, clindamycin, and the newer drugs clarithromycin and azithromycin.

Metabolism: The organic processes (in a cell or organism) that are necessary for life; see <http://wordnetweb.princeton.edu/perl/webwn?s=metabolism> (accessed July 20, 2010).

Microbe: A microorganism or biologic agent that can replicate in humans (including bacteria, viruses, protozoa, fungi, and prions).

Microbiome: Term used to describe the collective genome of our indigenous microbes (microflora); see Hooper, L. V., and J. I. Gordon. 2001. Commensal host bacterial relationships in the gut. *Science* 292(5519):1115-1118.

Monoclonal antibodies: Antibodies produced against a single antigen in cells that are clones of a single parent (germ) cell.

Mutation: Genetic change that can occur either randomly or at an accelerated rate through exposure to radiation or certain chemicals (mutagens) and may lead to change in structure of the protein coded by the mutated gene.

Nomogram: A graphical calculating device, a two-dimensional diagram designed to allow the approximate graphical computation of a function; it uses a coordinate system other than Cartesian coordinates.

Pathogen: An organism capable of causing disease.

Penicillins: Any of the various antibiotics derived from *Penicillium* fungi. All penicillins are β -lactam antibiotics and are used in the treatment of bacterial infections caused by susceptible, usually Gram-positive, organisms. They are historically significant because they are the first drugs that were effective against many previously serious diseases and are still widely used today, though many types of bacteria are now resistant.

Persister cells: Cells produced by bacterial populations that neither grow nor die in the presence of antibiotics. These cells are largely responsible for the high levels of tolerance to antimicrobial agents often observed in biofilms.

Plasmid: A small cellular inclusion consisting of a ring of DNA that is not in a chromosome but is capable of autonomous replication.

Polymerase chain reaction (PCR): A scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Polypeptide antibiotics: A class of antibiotics used for eye, ear, or bladder infections in addition to aminoglycosides. They are toxic and are therefore not suitable for systemic administration. They are usually applied directly to the eye or skin

or are inhaled into the lungs. Examples include actinomycin, bacitracin, colistin, and polymyxin B.

Positron emission tomography (PET) scan: The PET scan is a nuclear medicine imaging technique that produces a three-dimensional image or picture of functional processes in the body.

Principal component analysis (PCA): A mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of uncorrelated variables called principal components.

Prodrug: A pharmaceutical substance that is inactive at the time of administration and activates once it diffuses into a cell and is modified into a reactive product by a specific enzyme.

Pseudomonas aeruginosa: A common bacterium that can cause disease in humans and animals. It is found in soil, water, skin flora, and most man-made environments throughout the world. It uses a wide range of organic material for food; in animals, this enables the organism to infect damaged tissues or people with reduced immunity. These infections cause generalized inflammation and sepsis and can be fatal if they occur in critical organs, such as the lungs, the urinary tract, and kidneys.

Quinolones: Class of purely synthetic antibiotics that inhibit the replication of bacterial DNA; includes ciprofloxacin and fluoroquinolone.

Reactive oxygen species (ROS): Chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides. They can be either inorganic or organic.

Recombinant DNA: Genetically engineered DNA prepared by transplanting or splicing genes from one species into the cells of a host organism of a different species. Such DNA becomes part of the host's genetic makeup and is replicated.

Recombination: A combining of genes or characters different from what they were in the parents; see <http://wordnetweb.princeton.edu/perl/webwn?s=recombination> (accessed July 19, 2010).

Resistome: The collection of all genes that directly or indirectly result in antimicrobial resistance.

Respiratory syncytial virus: A respiratory virus that infects the lungs and breathing passages. Most otherwise healthy people recover from a respiratory syncytial virus infection in 1 to 2 weeks; however, infection can be severe in some people, such as certain infants, young children, and older adults; see <http://www.cdc.gov/rsv/> (accessed June 18, 2010).

Ribonucleic acid (RNA): A biologically important type of molecule that consists of a long chain of nucleotide units. Each nucleotide consists of a nitrogenous base, a ribose sugar, and a phosphate. RNA is very similar to DNA, but differs in a few important structural details: in the cell, RNA is usually single-stranded, while DNA is usually double-stranded; RNA nucleotides contain ribose while DNA contains deoxyribose (a type of ribose that lacks one oxygen atom); and RNA has the base uracil rather than thymine that is present in DNA.

RNA polymerase (RNAP or RNAPol): An enzyme that produces RNA.

Sidephores: Low-molecular-weight compounds with a high binding affinity for insoluble iron-III; microorganisms release sidephores to scavenge iron-III and then transport it back into the cell.

Signal transduction: The process by which an extracellular signaling molecule activates a membrane receptor that, in turn, alters intracellular molecules, creating a response. There are two stages in this process: a signalling molecule activates a certain receptor on the cell membrane, causing a second messenger to continue the signal into the cell and elicit a physiological response. In either step, the signal can be amplified, meaning that one signalling molecule can cause many responses.

SOS response: A postreplication DNA repair system using the RecA protein that allows DNA replication to bypass lesions or errors in the DNA. It is an error-prone repair system.

Staphylococcus aureus: A Gram-positive bacteria that is the most common cause of staph infections. It is frequently part of the skin flora found in the nose and on skin. About 20 percent of the human population are long-term carriers of *S. aureus*.

Subsistome: A subset of genes in the resistome that permit microbes to degrade antibiotics and use them as an energy source.

Synthetic biology: Research that combines biology with the principles of engineering to design, construct, or adapt existing DNA or other biological structures into standardized, interchangeable building blocks. These biological “parts” have

specific functions and can be joined to create engineered cells, organisms, or biological systems that reliably behave in predictable ways to perform specific tasks.

Systems biology: The study of the behavior of complex biological organization and processes in terms of their molecular constituents.

Toll-like receptors (TLRs): A class of proteins that play a key role in the innate immune system.

Transcription: The process of creating a complementary RNA copy of a sequence of DNA. Both RNA and DNA are nucleic acids, which use base pairs of nucleotides as a complementary language that can be converted back and forth from DNA to RNA by the action of the correct enzymes. During transcription, a DNA sequence is read by RNA polymerase, which produces a complementary, antiparallel RNA strand. As opposed to DNA replication, transcription results in an RNA complement that includes uracil (U) in all instances where thymine (T) would have occurred in a DNA complement.

Transposon: A mobile piece of DNA flanked by terminal repeat sequences that can insert into a chromosome, exit, and relocate and typically bears genes coding for these functions; see <http://www.everythingbio.com/glos/definition.php?word=transposon> (accessed June 14, 2010).

Vaccine: A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe or its toxins.

Vaccinology: The science or methodology of vaccine development.

Virulence factor: Intrinsic characteristic of an infectious bacteria that facilitates its ability to cause disease; see <http://www.nature.com/scitable/definition/virulence-factor-53> (accessed August 3, 2010).

Virus: A small infectious agent that can replicate only inside the living cells of organisms.

Appendix E

Forum Member Biographies

David A. Relman, M.D. (*Chair*), is the Thomas C. and Joan M. Merigan Professor in the Departments of Medicine and of Microbiology and Immunology at Stanford University, and Chief of Infectious Diseases at the VA Palo Alto Health Care System in Palo Alto, California. He received an S.B. (biology) from Massachusetts Institute of Technology (1977), received his M.D. (*magna cum laude*) from Harvard Medical School (1982), completed his clinical training in internal medicine and infectious diseases at Massachusetts General Hospital, served as a postdoctoral fellow in microbiology at Stanford University, and joined the faculty at Stanford in 1994.

Dr. Relman's current research focus is the human indigenous microbiota (microbiome) and, in particular, the nature and mechanisms of variation in patterns of microbial diversity within the human body as a function of time (microbial succession), space (biogeography within the host landscape), and in response to perturbation, for example, antibiotics (community robustness and resilience). One of the goals of this work is to define the role of the human microbiome in health and disease. This research integrates theory and methods from ecology, population biology, environmental microbiology, genomics, and clinical medicine. During the past few decades, his research directions have also included pathogen discovery and the development of new strategies for identifying previously unrecognized microbial agents of disease. This work helped to spearhead the application of molecular methods to the diagnosis of infectious diseases in the 1990s. His research has emphasized the use of genomic approaches for exploring host-microbe relationships. Past scientific achievements include the description of a novel approach for identifying previously unknown pathogens; the identification of a number of new human microbial pathogens, including the agent of

Whipple's disease; and some of the most extensive and revealing analyses to date of the human indigenous microbial ecosystem.

Dr. Relman advises the U.S. government, as well as nongovernmental organizations, in matters pertaining to microbiology, emerging infectious diseases, and biosecurity. He is a member of the National Science Advisory Board for Biosecurity, a member of the Physical and Life Sciences Directorate Review Committee for Lawrence Livermore National Laboratory, and he advises several U.S. government departments and agencies on matters related to pathogen diversity, the future life sciences landscape, and the nature of present and future biological threats. He has served as Chair of the Board of Scientific Counselors of the National Institute of Dental and Craniofacial Research (National Institutes of Health [NIH]) and as a member of the Board of Directors, Infectious Diseases Society of America (IDSA). Dr. Relman is currently vice-chair of a National Academy of Sciences (NAS) study of the science underlying the Federal Bureau of Investigation investigation of the 2001 anthrax mailings, and he cochaired a 3-year NAS study that produced a widely cited report entitled *Globalization, Biosecurity, and the Future of the Life Sciences* (2006). He is a Fellow of the American Academy of Microbiology and a member of the Association of American Physicians. Dr. Relman received the Squibb Award from the IDSA in 2001 and was the recipient of both the NIH Director's Pioneer Award and the Distinguished Clinical Scientist Award from the Doris Duke Charitable Foundation in 2006. Dr. Relman was elected as a member of the Institute of Medicine (IOM) of the National Academies in 2011.

James M. Hughes, M.D. (*Vice-Chair*), is professor of medicine and public health at Emory University's School of Medicine and Rollins School of Public Health, serving as director of the Emory Program in Global Infectious Diseases, executive director of the Southeastern Center for Emerging Biologic Threats, and senior advisor to the Emory Center for Global Safe Water. He is the senior scientific advisor for infectious diseases to the International Association of National Public Health Institutes funded by the Bill and Melinda Gates Foundation. Prior to joining Emory in June 2005, Dr. Hughes served as director of the National Center for Infectious Diseases (NCID) at the Centers for Disease Control and Prevention (CDC). Dr. Hughes received his B.A. and M.D. degrees from Stanford University and completed postgraduate training in internal medicine at the University of Washington, infectious diseases at the University of Virginia, and preventive medicine at CDC. After joining CDC as an Epidemic Intelligence Service officer in 1973, Dr. Hughes worked initially on foodborne and water-related diseases and subsequently on infection control in health care settings. He served as director of CDC's Hospital Infections Program from 1983 to 1988, as deputy director of NCID from 1988 to 1992, and as director of NCID from 1992 to 2005. A major focus of Dr. Hughes' career is on building partnerships among the clinical, research, public health, and veterinary communities to prevent, detect, and re-

spond to infectious diseases at the local, national, and global levels. His research interests include emerging and reemerging infectious diseases, antimicrobial resistance, foodborne diseases, health care-associated infections, vectorborne and zoonotic diseases, rapid detection of and response to infectious diseases and bioterrorism, strengthening public health capacity at the local, national, and global levels, and prevention of water-related diseases in the developing world. Dr. Hughes is a fellow and Council Delegate of the American Association for the Advancement of Science, fellow of the American College of Physicians and the Infectious Diseases Society of America (IDSA), President of IDSA, Councilor of the American Society of Tropical Medicine and Hygiene, and member of the International Board of the American Society for Microbiology. He is a member of the Institute of Medicine.

Lonnie J. King, D.V.M. (*Vice-Chair*), is the 10th dean of the College of Veterinary Medicine at The Ohio State University (OSU). In addition to leading this college, Dr. King is also a professor of preventive medicine and holds the Ruth Stanton Endowed Chair in Veterinary Medicine. Before becoming dean at OSU, he was the director of CDC's new National Center for Zoonotic, Vector-Borne, and Enteric Diseases (NCZVED). In this new position, Dr. King leads the Center's activities for surveillance, diagnostics, disease investigations, epidemiology, research, public education, policy development, and disease prevention and control programs. NCZVED also focuses on waterborne, foodborne, vectorborne, and zoonotic diseases of public health concern, which also include most of CDC's select and bioterrorism agents, neglected tropical diseases, and emerging zoonoses. Before serving as director, he was the first chief of the agency's Office of Strategy and Innovation.

Dr. King served as dean of the College of Veterinary Medicine, Michigan State University, from 1996 to 2006. As at OSU, he served as the CEO for academic programs, research, the teaching hospital, the diagnostic center for population and animal health, basic and clinical science departments, and the outreach and continuing education programs. As dean and professor of large-animal clinical sciences, Dr. King was instrumental in obtaining funds for the construction of a \$60 million Diagnostic Center for Population and Animal Health; he initiated the Center for Emerging Infectious Diseases in the college, he served as the campus leader in food safety, and he had oversight for the National Food Safety and Toxicology Center.

In 1992, Dr. King was appointed administrator for the Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture (USDA), in Washington, DC. In this role, he provided executive leadership and direction for ensuring the health and care of animals and plants, to improve agricultural productivity and competitiveness, and to contribute to the national economy and public health. Dr. King also served as the country's chief veterinary officer for 5 years, worked extensively in global trade agreements within the North American Free

Trade Agreement and the World Trade Organization, and worked extensively with the World Animal Health Association. During this time he was the Deputy Administrator for Veterinary Services of APHIS, USDA, where he led national efforts in disease eradication, imports and exports, and diagnostics in both Ames, Iowa, and Plum Island. He spent 5 years in Hyattsville, Maryland, in staff assignments in Emergency Programs, as well as Animal Health Information. While in Hyattsville, Dr. King directed the development of the agency's National Animal Health Monitoring System. He left APHIS briefly to serve as the director of the Governmental Relations Division of the American Veterinary Medical Association (AVMA) in Washington, DC, and served as the lobbyist for the AVMA on Capitol Hill.

Dr. King was in private veterinary practice for 7 years in Dayton, Ohio, and Atlanta, Georgia. As a native of Wooster, Ohio, Dr. King received his bachelor of science and doctor of veterinary medicine degrees from OSU in 1966 and 1970, respectively. He earned his master of science degree in epidemiology from the University of Minnesota and received his master's degree in public administration from American University in Washington, DC, in 1991. Dr. King is a board-certified member of the American College of Veterinary Preventive Medicine and has completed the Senior Executive Fellowship program at Harvard University. He served as president of the Association of American Veterinary Medical Colleges from 1999 to 2000 and was the vice-chair for the National Commission on Veterinary Economic Issues from 2000 to 2004. He has served on four NAS committees, including chairing the National Academies' Committee on Assessing the Nation's Framework for Addressing Animal Diseases. He is also Chair of the IOM Committee on Lyme Disease and Other Tick-Borne Diseases and for State of the Science, and he is also chairing the AVMA's Commission for AVMA Vision 2020. Dr. King is currently a member of the IOM Committee on Microbial Threats to Health, is a past member of the Food and Drug Administration's (FDA's) Board of Scientific Advisors, and is past president of the American Veterinary Epidemiology Society. He served as the chair for the national One Medicine Task Force for the AVMA, which helped start the country's One Health Initiative. Dr. King was elected as a member of the IOM of the National Academies in 2004.

Kevin Anderson, Ph.D., serves as a Senior Program Manager in the Department of Homeland Security's Science and Technology Directorate, providing oversight and requirements for science programs focused on rapid detection and characterization of biological agents. Since joining DHS in 2003, Dr. Anderson has provided leadership for science program development, laboratory design, and strategic planning, served as a subject matter expert and advisor to the Bioterrorism Risk Assessment and Biological Threat Characterization programs, and has participated in interagency working groups and assessments which provide guidance to medical countermeasure development, a key component of the nation's biodefense strategy. Prior to joining DHS, Dr. Anderson was a Principal

Investigator at the U.S. Army Medical Research Institute of Infectious Diseases, leading research focused on understanding basic mechanisms of viral diseases causing hemorrhagic fever and development of medical countermeasures. He received postdoctoral training in molecular virology at the University of Alabama at Birmingham and the University of North Carolina at Chapel Hill, performing basic research on human respiratory syncytial viruses, and earned Ph.D. and B.S. degrees in microbiology.

Ruth L. Berkelman, M.D., is the Rollins Professor and director of the Center for Public Health Preparedness and Research at the Rollins School of Public Health, Emory University, in Atlanta. She received her A.B. from Princeton University and her M.D. from Harvard Medical School. Board certified in pediatrics and internal medicine, she began her career at CDC in 1980 and later became deputy director of NCID. She also served as a senior advisor to the director of CDC and as assistant surgeon general in the U.S. Public Health Service. In 2001 she came to her current position at Emory University, directing a center focused on emerging infectious diseases and other urgent threats to health, including terrorism. She has also consulted with the biologic program of the Nuclear Threat Initiative and is most recognized for her work in infectious diseases and disease surveillance. She was elected to the IOM in 2004. Currently a member of the Board on Life Sciences of the National Academies, she also chairs the Board of Public and Scientific Affairs at the ASM.

David L. Blazes, M.D., M.P.H.,¹ Commander David L. Blazes is Chief of the Global Emerging Infections Surveillance and Response System (GEIS) Division at the Armed Forces Health Surveillance Center in Silver Spring, Maryland. From 2004 to 2008, he was Director of the Emerging Infections Department at the Naval Medical Research Center Detachment (now NAMRU-6) in Lima, Peru. The AFHSC-GEIS network identified the first cases of the 2009 H1N1 pandemic as well as numerous other emerging infections that threaten public health around the world. He also serves on the faculty at the Uniformed Services University in Bethesda, Maryland, and in International Health at the Johns Hopkins Bloomberg School of Public Health. He graduated from the U.S. Naval Academy in 1991 and the Johns Hopkins University School of Medicine in 1995 and completed his internal medicine and infectious diseases training at the National Naval Medical Center, the President's hospital in Bethesda. His main scientific interests are infectious diseases surveillance strategies in developing settings, optimizing outbreak response, public health capacity building, and tropical medicine training. He has taught clinical tropical medicine at the Gorgas course within Universidad Peruana Cayetano Heredia, at the Johns Hopkins Summer Institute of Tropical Medicine, and at the U.S. Military Tropical Medicine course in Bethesda.

¹ Forum member since September 1, 2011.

Enriqueta C. Bond, Ph.D., is president emeritus of the Burroughs Wellcome Fund. Dr. Bond is currently a partner in QE Philanthropic Advisors, LLC, an organization that provides consulting services to foundations and non-profits on matters of program, strategic planning, and capacity development related to medical sciences, international health, and science and math K–12 education. She received her undergraduate degree from Wellesley College, her M.A. from the University of Virginia, and her Ph.D. in molecular biology and biochemical genetics from Georgetown University. She is a member of the IOM and a fellow of the AAAS. Dr. Bond chairs the Academies' Board on African Science Academy Development and serves on the NRC Committee on the Future of the Research University. She serves on the board and executive committee of the Hamner Institute, the board of the Health Effects Institute, the board of the James B. Hunt Jr. Institute for Educational Leadership and Policy, and the NIH Council of Councils. In addition Dr. Bond serves on a scientific advisory committee for the World Health Organization (WHO) Tropical Disease Research Program. Prior to being named president of the Burroughs Wellcome Fund in 1994, Dr. Bond served on the staff of the IOM beginning in 1979, becoming its executive officer in 1989.

Roger G. Breeze, BVMS, Ph.D., MRCVS, is currently Bio-Security Deputy Program Director, Global Security Directorate, Office of Strategic Outcomes, Lawrence Livermore National Laboratory and serves on the senior management team of the Defense Threat Reduction Agency's Chemical and Biological Defense Directorate. He received his veterinary degree in 1968 and his Ph.D. in veterinary pathology in 1973, both from the University of Glasgow, Scotland. He was engaged in teaching, diagnostic pathology, and research on respiratory and cardiovascular diseases at the University of Glasgow Veterinary School from 1968 to 1977 and at Washington State University College of Veterinary Medicine from 1977 to 1987, where he was professor and chair of the Department of Microbiology and Pathology. From 1984 to 1987 he was deputy director of the Washington Technology Center, the state's high-technology sciences initiative, based in the College of Engineering at the University of Washington. In 1987, he was appointed director of the USDA's Plum Island Animal Disease Center, a Biosafety Level 3 (BSL-3) facility for research and diagnosis of the world's most dangerous livestock diseases. In that role he initiated research into the genomic and functional genomic basis of disease pathogenesis, diagnosis, and control of livestock RNA and DNA virus infections. This work became the basis of U.S. defense against natural and deliberate infection with these agents and led to his involvement in the early 1990s in biological weapons defense and proliferation prevention. From 1995 to 1998, he was South Atlantic area director for USDA's Agricultural Research Service before going to Washington, DC, to establish biological weapons defense programs for USDA. He received the Distinguished Executive Award from President Clinton in 1998 for his work at Plum Island and in biodefense. Since 2004 he has been CEO of Centaur Science Group where

his main commitment was to the Defense Threat Reduction Agency's Global Bioengagement Program.

Paula R. Bryant, Ph.D., is Chief of the Medical S&T Division, Chemical and Biological Defense Program at the Defense Threat Reduction Agency (DTRA) in Fort Belvoir, Virginia. As the Chief of the Medical S&T Division, Bryant interfaces with all levels of the Department of Defense and DTRA to plan, coordinate, integrate, and execute program activities necessary to provide timely and effective medical countermeasures against Chemical, Biological and Radiological (CBR) threats to U.S. interests worldwide. She also served as a Senior Scientist and Senior S&T Manager while at DTRA. Prior to joining DTRA, she was an assistant professor in the Department of Microbiology at The Ohio State University. She received her Ph.D. in microbiology and immunology from the Baylor College of Medicine.

John E. Burris, Ph.D., became president of the Burroughs Wellcome Fund in July 2008. He is the former president of Beloit College. Prior to his appointment at Beloit in 2000, Dr. Burris served for 8 years as director and CEO of the Marine Biological Laboratory in Woods Hole, Massachusetts. From 1984 to 1992 he was at the National Research Council/National Academies, where he served as the executive director of the Commission on Life Sciences. A native of Wisconsin, he received an A.B. in biology from Harvard University in 1971, attended the University of Wisconsin, Madison, in an M.D.-Ph.D. program, and received a Ph.D. in marine biology from the Scripps Institution of Oceanography at the University of California, San Diego, in 1976. A professor of biology at the Pennsylvania State University from 1976 to 1985, he held an adjunct appointment there until going to Beloit. His research interests are in the areas of marine and terrestrial plant physiology and ecology. He has served as president of the American Institute of Biological Sciences and is or has been a member of a number of distinguished scientific boards and advisory committees, including the Grass Foundation; the Stazione Zoologica "Anton Dohrn" in Naples, Italy; the AAAS; and the Radiation Effects Research Foundation in Hiroshima, Japan. He has also served as a consultant to the National Conference of Catholic Bishops' Committee on Science and Human Values.

Arturo Casadevall, M.D., Ph.D.,² is the Leo and Julia Forchheimer Professor of Microbiology & Immunology at the Albert Einstein College of Medicine of Yeshiva University in the Bronx, New York. He is Chairman of the Department of Microbiology and Immunology and served as Director of the Division of Infectious Diseases at the Montefiore Medical Center at the Albert Einstein College of Medicine from 2000 to 2006. Dr. Casadevall received both his M.D. and Ph.D.

² Forum member since September 1, 2011.

(biochemistry) degrees from New York University in New York, New York. Subsequently, he completed internship and residency in internal medicine at Bellevue Hospital in New York, New York. Later he completed subspecialty training in infectious diseases at the Montefiore Medical Center and Albert Einstein College of Medicine. Dr. Casadevall's major research interests are in fungal pathogenesis and the mechanism of antibody action. In the area of biodefense, Dr. Casadevall has an active research program to understand the mechanisms of antibody-mediated neutralization of *Bacillus anthracis* toxins. He has authored over 500 scientific papers. Dr. Casadevall was elected to membership in the American Society for Clinical Investigation, the American Academy of Physicians, and the American Academy of Microbiology. He was elected a fellow of the American Academy for the Advancement of Science and has received numerous honors including the Solomon A. Berson Medical Alumni Achievement Award in Basic Science from the NYU School of Medicine, the Maxwell L. Littman Award (mycology award), the Rhoda Benham Award from the Medical Mycology Society of America, and the Kass Lecture of the Infectious Disease Society of America. Dr. Casadevall is the Editor in Chief of *mBio*, the first open-access general journal of the American Society of Microbiology. He serves on the editorial board of the *Journal of Clinical Investigation*, the *Journal of Experimental Medicine*, and the *Journal of Infectious Diseases*. Previously he served as Editor of *Infection and Immunity*. He has served on numerous NIH committees including those that drafted the NIAID Strategic Plan and the Blue Ribbon Panel on Biodefense Research. Dr. Casadevall served on the NAS committee that reviewed the science behind the FBI investigation of the anthrax attacks in 2001. He is currently a member of the National Science Advisory Board for Biosecurity and co-chaired the NIAID Board of Scientific Counselors.

Since he joined the Einstein faculty in 1992 Dr. Casadevall has mentored dozens of graduate students, postdoctoral fellows, and junior faculty. Many of his trainees have gone on to have highly successful careers in science and several are currently AECOM faculty. From 2000 to 2006 Dr. Casadevall was director of the Division of Infectious Diseases at AECOM-Montefiore and oversaw the expansion of its research program. In 2001 Dr. Casadevall received the Samuel M. Rosen outstanding teacher award and in 2008 he was recognized by the American Society of Microbiology with the William Hinton Award for mentoring scientists from underrepresented groups.

Peter Daszak, Ph.D., is President of EcoHealth Alliance (formerly Wildlife Trust), a U.S.-based organization which conducts research and field programs on global health and conservation. At Wildlife Trust, Dr. Daszak manages a headquarters staff of 35 and a global staff of more than 700 which conducts research and manages initiatives to prevent emerging pandemics and conserve wildlife biodiversity. This includes research on zoonoses that spill over from wildlife in emerging disease "hotspots," including influenza, Nipah virus, SARS, West Nile

virus, and others. Dr. Daszak's work includes identifying the first case of a species extinction due to disease, the discovery of chytridiomycosis, the major cause of global amphibian declines, publishing the first paper to highlight emerging diseases of wildlife, coining the term "pathogen pollution," discovery of the bat origin of SARS-like coronaviruses, identifying the drivers of Nipah and Hendra virus emergence, and producing the first ever emerging disease "hotspots" map.

Dr. Daszak is a member of the Council of Advisors of the One Health Commission, Treasurer of DIVERSITAS (ICSU), past member of the International Standing Advisory Board of the Australian Biosecurity CRC, and past member of the IOM Committee on Global Surveillance for Emerging Zoonoses and the National Research Council (NRC) committee on the future of veterinary research. He is Editor-in-Chief of the Springer journal *Ecohealth*, and past treasurer and a founding director of the International Ecohealth Association. In 2000, he won the Commonwealth Scientific and Industrial Research Organisation medal for collaborative research in the discovery of amphibian chytridiomycosis. He has published over 130 scientific papers and book chapters, including papers in *Science*, *Nature*, *PNAS*, *The Lancet*, *PLoS Biology*, and other leading journals. His work has been the focus of articles in the *New York Times*, *The Wall Street Journal*, *The Economist*, *The Washington Post*, *US News & World Report*, *CBS 60 Minutes*, *CNN*, *ABC*, *NPR's Talk of the Nation*, and *Morning Edition & Fresh Air with Terri Gross*. He is a former guest worker at the CDC, where he assisted in the pathology activity during the 1999 Nipah virus outbreak. His work is funded by the John E. Fogarty International Center of NIH, the National Institute of Allergy and Infectious Diseases (NIAID), the National Science Foundation (NSF), the U.S. Agency for International Development (USAID), Google.org, Rockefeller, and other foundations. To date, his group is one of the few to have been awarded three prestigious NIH/NSF Ecology of Infectious Disease awards and is one of four partners to share a recent multi-million-dollar award from USAID ("PREDICT") with the goal of predicting and preventing the next emerging zoonotic disease.

Jeffrey Scott Duchin, M.D., is Chief of the Communicable Disease Epidemiology & Immunization Section for Public Health–Seattle & King County, Washington, and Professor of Medicine, Division of Infectious Diseases and Adjunct Professor in the School of Public Health and Community Medicine at the University of Washington.

Dr. Duchin trained in internal medicine at Thomas Jefferson University Hospital. He completed a fellowship in general internal medicine and emergency medicine at the Hospital of the University of Pennsylvania and infectious disease subspecialty training at the University of Washington. After several years on the faculty at the University of Pennsylvania, he joined the CDC's Epidemic Intelligence Service program where he was assigned to the National Center for Infectious Diseases, and the CDC's Preventive Medicine Residency program. He worked for CDC as a medical epidemiologist in the Divisions of Tuberculosis

Elimination and HIV/AIDS Special Studies Branch before assuming his current position.

Dr. Duchin is a member of the IOM's Forum on Medical and Public Health Preparedness for Catastrophic Events and a current member of the CDC's Advisory Committee on Immunization Practices (ACIP). He is a Fellow of the IDSA and of the American College of Physicians; a member of the IDSA's National and Global Public Health Committee and Pandemic Influenza Task Force; and is past chair of the IDSA's Bioemergencies Task Force.

Dr. Duchin serves on the Editorial Board and Technical Advisory Group for Communicable Disease Alert and Response to Mass Gatherings for the WHO and previously served as a member of the Department of Health and Human Services 2004 Tiger Team consulting with the Government of Greece on health preparations for the 2004 Olympics, in Athens, Greece.

Dr. Duchin's peer-review publications and research interests focus on communicable diseases of public health significance, and he has authored textbook chapters on the epidemiology of HIV/AIDS, bioterrorism, and outbreak investigations.

Jonathan Eisen, Ph.D., is a professor at the Genome Center at the University of California (UC), Davis, and holds appointments in the Department of Evolution and Ecology in the College of Biological Sciences and Medical Microbiology and Immunology in the School of Medicine.

His research focuses on the mechanisms underlying the origin of novelty (how new processes and functions originate). Most of his work involves the use of high-throughput DNA sequencing methods to characterize microbes and then the use and development of computational methods to analyze this type of data. In particular, his computational work has focused on integrating evolutionary analysis with genome analysis—so-called phylogenomics. Previously, he applied this phylogenomic approach to cultured organisms, such as those from extreme environments and those with key properties as they relate to evolution or global climate cycles. Currently he is using sequencing and phylogenomic methods to study microbes directly in their natural habitats (i.e., without culturing). In particular he focuses on how communities of microbes interact with each other or with plant and animal hosts to create new functions. Dr. Eisen is also coordinating one of the largest microbial genome sequencing projects to date—the “Genomic Encyclopedia of Bacteria and Archaea” being done at the Department of Energy (DOE) Joint Genome Institute, where he holds an adjunct appointment.

In addition to his research, Dr. Eisen is also a vocal advocate for “open access” to scientific publications and is the Academic Editor-in-Chief of *PLoS Biology*. He is also an active and award-winning blogger/microblogger (e.g., <http://phylogenomics.blogspot.com>, <http://twitter.com/phylogenomics>). Prior to moving to UC Davis he was on the faculty of The Institute for Genomic Research (TIGR) in Rockville, Maryland. He earned his Ph.D. in biological sciences from

Stanford University, where he worked on the evolution of DNA repair processes in the lab of Philip C. Hanawalt, and his undergraduate degree in biology from Harvard College.

Ralph L. Erickson, M.D., Dr.P.H., M.P.H., M.S., COL MC,³ is currently the new Commander of The Walter Reed Army Institute of Research. He was most recently the Command Surgeon of the United States Central Command from 2009 to 2011. Colonel Erickson has enjoyed a diverse mix of assignments in operational medicine, teaching, research, and public health surveillance. As a junior officer he spent 5 years in Special Operations, frequently deploying overseas to include participation in Operations Ernest Will, Desert Shield, and Provide Comfort. His assignments have included Preventive Medicine Residency Program Director, Walter Reed Army Institute of Research (WRAIR); Director of Epidemiology and Disease Surveillance, U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM); Preventive Medicine Consultant to The Surgeon General; Chief of Preventive Medicine (Landstuhl Regional Medical Center); and Commander, USACHPPM-Europe. During Operation Iraqi Freedom (OIF-1) Colonel Erickson served in Baghdad and Balad as the civil-military (G-5 Main) staff officer for the 30th Medical Brigade. Upon his return stateside, he served as Science Director (WRAIR), Liaison Officer to the U.S. Department of Health and Human Services, and Director of the U.S. Department of Defense-Global Emerging Infections Surveillance and Response System (DOD-GEIS). Colonel Erickson holds degrees from the University of Washington (B.S.-Honors), the Uniformed Services University of the Health Sciences (M.D.), Harvard University (M.P.H.), Johns Hopkins University (Dr.P.H.-Delta Omega Alpha), and the National War College (M.S. Distinguished Graduate). He is the recipient of The Surgeon General's "A" Proficiency Designator (2000), The Army Surgeon General's Physician Recognition Award (2002), the Society of the Federal Health Agencies (AMSUS) Gorgas Medal (2007), and the Naval War College Association of the United States Army Writing Award (2009). His military awards include the Defense Superior Service Medal (1 OLC), the Legion of Merit, and the Bronze Star.

Mark B. Feinberg, M.D., Ph.D., is vice president for medical affairs and policy in global vaccine and infectious diseases at Merck & Co., Inc., and is responsible for global efforts to implement vaccines to achieve the greatest health benefits, including efforts to expand access to new vaccines in the developing world. Dr. Feinberg received a bachelor's degree magna cum laude from the University of Pennsylvania in 1978 and his M.D. and Ph.D. degrees from Stanford University School of Medicine in 1987. His Ph.D. research at Stanford was supervised by Dr. Irving Weissman and included time spent studying the molecular biology of

³ Forum member since September 1, 2011.

the human retroviruses—human T-cell lymphotropic virus, type I (HTLV-I) and HIV—as a visiting scientist in the laboratory of Dr. Robert Gallo at the National Cancer Institute. From 1985 to 1986, Dr. Feinberg served as a project officer for the IOM Committee on a National Strategy for AIDS. After receiving his M.D. and Ph.D. degrees, Dr. Feinberg pursued postgraduate residency training in internal medicine at the Brigham and Women's Hospital of Harvard Medical School and postdoctoral fellowship research in the laboratory of Dr. David Baltimore at the Whitehead Institute for Biomedical Research. From 1991 to 1995, Dr. Feinberg was an assistant professor of medicine and microbiology and immunology at the University of California, San Francisco (UCSF), where he also served as an attending physician in the AIDS-oncology division and as director of the virology research laboratory at San Francisco General Hospital. From 1995 to 1997, Dr. Feinberg was a medical officer in the Office of AIDS Research in the Office of the Director of the NIH, the chair of the NIH Coordinating Committee on AIDS Etiology and Pathogenesis Research, and an attending physician at the NIH Clinical Center. During this period, he also served as executive secretary of the NIH Panel to Define Principles of Therapy of HIV Infection. Prior to joining Merck in 2004, Dr. Feinberg served as professor of medicine and microbiology and immunology at the Emory University School of Medicine, as an investigator at the Emory Vaccine Center, and as an attending physician at Grady Memorial Hospital. At UCSF and Emory, Dr. Feinberg and colleagues were engaged in the preclinical development and evaluation of novel vaccines for HIV and other infectious diseases and in basic research studies focused on revealing fundamental aspects of the pathogenesis of AIDS. Dr. Feinberg also founded and served as the medical director of the Hope Clinic of the Emory Vaccine Center—a clinical research facility devoted to the clinical evaluation of novel vaccines and to translational research studies of human immune system biology. In addition to his other professional roles, Dr. Feinberg has also served as a consultant to, and a member of, several IOM and NAS committees. Dr. Feinberg currently serves as a member of the National Vaccine Advisory Committee and is a member of the Board of Trustees of the National Foundation for Infectious Diseases. Dr. Feinberg has earned board certification in internal medicine; he is a fellow of the American College of Physicians, a member of the Association of American Physicians, and the recipient of an Elizabeth Glaser Scientist Award from the Pediatric AIDS Foundation and an Innovation in Clinical Research Award from the Doris Duke Charitable Foundation.

Jacqueline Fletcher, Ph.D., Regents Professor of Plant Pathology at Oklahoma State University, received a B.S. in biology from Emory University, Atlanta, Georgia, an M.S. in botany from the University of Montana, and a Ph.D. in plant pathology from Texas A&M University. She served as a postdoctoral associate at the University of Illinois before joining OSU in 1984, where she was appointed Sarkeys Distinguished Professor in 2001 and Regents Professor in 2008. She was

named a Fellow of the American Phytopathological Society (APS) in 2005 and a Fellow of AAAS in 2007.

Dr. Fletcher is Director of the National Institute for Microbial Forensics and Food and Agricultural Biosecurity (NIMFFAB), a multidisciplinary OSU initiative that addresses high-priority national issues in research, teaching/education, and outreach with emphases in microbial forensics applications in plant pathology and produce safety. The NIMFFAB serves as a spoke laboratory for the DHS-affiliated National Bioforensic Analysis Center, in the area of plant pathogen forensics. Dr. Fletcher's research focuses on mechanisms of virulence and insect transmission of plant pathogenic bacteria; on the relationships between human pathogens, such as *Salmonella* and *Escherichia coli*, and plants; and on the emerging disciplines of microbial forensics and agricultural biosecurity.

Dr. Fletcher served on the APS Council for 10 years, including the 4-year APS presidential sequence. In the months following September 11, 2001, Dr. Fletcher led APS responses and input to new national biosecurity initiatives. She has served for 9 years on the APS Public Policy Board (4 years as chair) and is currently on the APS Threatening Pathogens Advisory Committee. She also serves on several federal biosecurity advisory panels.

Jesse L. Goodman, M.D., M.P.H., became chief scientist and deputy commissioner for science and public health of FDA in 2009. He has broad responsibility for and engagement in leadership and coordination of FDA's crosscutting scientific and public health efforts. From 2003 to 2009, Dr. Goodman was director of FDA's Center for Biologics Evaluation and Research, which oversees medical and public health activities critical to U.S. and global preparedness and the development, evaluation, safety, quality, and availability of biologics. A graduate of Harvard, Dr. Goodman received his M.D. from the Albert Einstein College of Medicine and did residency and fellowship training at the Hospital of the University of Pennsylvania and at the University of California, Los Angeles (UCLA), where he was also Chief Medical Resident. Prior to joining FDA, he was professor of medicine and chief of infectious diseases at the University of Minnesota, where he directed the multihospital infectious diseases research, training, and clinical programs, and where his NIH-funded laboratory first isolated and characterized *Anaplasma phagocytophilum*, the infectious agent causing a new tickborne disease, human granulocytic ehrlichiosis. Dr. Goodman has authored numerous scientific papers and edited the book *Tick Borne Diseases of Humans* (ASM Press, 2005). Dr. Goodman has been elected to the American Society for Clinical Investigation and to the IOM of the NAS, where he is a longstanding member of the Forum on Microbial Threats. He is an active clinician and teacher who is board certified in internal medicine, oncology, and infectious diseases and is staff physician and infectious diseases consultant at the National Naval and Walter Reed Army Medical Centers. Dr. Goodman is adjunct professor of medicine at the University of Minnesota.

Eduardo Gotuzzo, M.D., is principal professor of the Department of Medicine and director of the “Alexander von Humboldt” Institute of Tropical Medicine and Infectious Diseases, Peruvian University Cayetano Heredia in Lima, Peru, and head of the Department of Transmissible Diseases at the Cayetano Heredia Hospital. He is also an adjunct professor of medicine at the University of Alabama, Birmingham, School of Medicine. He is director of the International Gorgas Course in Clinical Tropical Medicine, Universidad Peruana Cayetano Heredia, taught jointly with the University of Alabama, Birmingham. He is an adjunct faculty member of the William J. Harrington Training Programs for Latin America, University of Miami School of Medicine (since 1983); was associate to the International Health Department of the Johns Hopkins University (1986–1998); and was fellow of the Center for the Americas at Vanderbilt, Vanderbilt University. Dr. Gotuzzo is an active member in numerous international societies and has been president of the Latin American Society of Tropical Disease (2000–2003); the IDSA Scientific Program (2000–2003); the International Organizing Committee of the International Congress of Infectious Diseases (1994 to present); the International Society for Infectious Diseases (1996–1998); the PanAmerican Infectious Diseases Association; the International Federation for Tropical Medicine (2005–2008); and president of the Peruvian Society of Internal Medicine (1991–1992). He works on several research areas and teaches on subjects including emerging diseases, TB, HTLV-1, free-living amoebas, brucellosis, and parasites. He has published more than 290 articles and chapters as well as six manuals and one book. Recent honors and awards include being named an honorary member of the American Society of Tropical Medicine and Hygiene in 2002; an honorary member of the Society of Internal Medicine in 2000; and a distinguished visitor at the Faculty of Medical Sciences, University of Cordoba, Argentina (1999). In 1988, Dr. Gotuzzo received the Golden Medal for Outstanding Contribution in the Field of Infectious Diseases awarded by Trnava University, Slovakia. In 2007, Dr. Gotuzzo received the Society Citation Award from the IDSA. He was an honorary member of the Australian Society for Infectious Diseases (2008), the American Society of Tropical Medicine and Hygiene (2002), Academia de Medicina de México, Sociedad Nenzolana de Infectología, Sociedad Paraguaya de Infectología, and the National Academy of Medicine of Mexico (2010).

Carole A. Heilman, Ph.D., serves as director of the Division of Microbiology and Infectious Diseases (DMID) of NIAID, a component of NIH. DMID supports research to prevent and control diseases caused by virtually all human infectious agents (except HIV), including bacterial, viral, parasitic, and prion diseases. DMID supports a wide variety of projects spanning the spectrum from basic biology of human pathogens and their interaction with human hosts, through translational and clinical research, toward the development of new and improved diagnostics, drugs, and vaccines for infectious diseases. As director, Dr. Heilman provides scientific direction, oversight, and management for an extramural research portfolio that encompasses 300 different organisms.

DMID supports the nation's biodefense as well as a solid research infrastructure that readily responds to public health challenges, such as emerging diseases. These resources were recently mobilized to respond to the emergence of 2009 H1N1 influenza by providing the first in-depth characterization of the H1N1 pandemic virus and conducting nine clinical trials that provided safety and efficacy data to inform public health practice.

Dr. Heilman has a Ph.D. in microbiology from Rutgers University. She did her postdoctoral work in molecular virology at the National Cancer Institute (NCI) and continued at the NCI as a senior staff fellow in molecular oncology. She later moved into health science administration, where she focused on respiratory pathogens, particularly vaccine development. Dr. Heilman has received numerous awards for scientific management and leadership, including three Department of Health and Human Services (HHS) Secretary's Awards for Distinguished Service recognizing her efforts on development of acellular pertussis vaccines, AIDS vaccines, and on accelerating biodefense research and development (R&D). Dr. Heilman serves as an infectious disease expert on the Board of Scientific Counselors for CDC. She also serves on the scientific board of the Fondation Mérieux's annual Advanced Course of Vaccinology and is a lecturer in this highly selective training program for decision makers in vaccinology. Throughout her career, Dr. Heilman has been a pioneer supporting the advancement of women in biomedical careers and serves as a mentor to a number of women within and outside of NIAID.

David L. Heymann, M.D., is currently chair of the Health Protection Agency, United Kingdom; professor and chair, infectious disease epidemiology, at the London School of Hygiene and Tropical Medicine; and head of the Global Health Security Programme at Chatham House, London. Until April 2009, he was assistant director-general for Health Security Environment and Representative of the director-general for Polio Eradication at WHO. Prior to that, from July 1998 until July 2003, he was executive director of the WHO Communicable Diseases Cluster, which included WHO's programs on infectious and tropical diseases, and from which the public health response to severe acute respiratory syndrome (SARS) was mounted in 2003. From October 1995 to July 1998, he was director of the WHO Programme on Emerging and Other Communicable Diseases, and prior to that he was the chief of research activities in the WHO Global Programme on AIDS. Dr. Heymann has worked in the area of public health for the past 35 years, 25 of which were on various assignments from CDC, and 10 of which have been with WHO. Before joining WHO, Dr. Heymann worked for 13 years as a medical epidemiologist in sub-Saharan Africa (Cameroon, Côte d'Ivoire, Malawi, and the Democratic Republic of Congo, formerly Zaire) on assignment from CDC in CDC-supported activities. These activities aimed at strengthening capacity in surveillance of infectious diseases and their control, with special emphasis on the childhood immunizable diseases, including measles and polio, African hemorrhagic fevers, poxviruses, and malaria. While based in Africa, Dr.

Heymann participated in the investigation of the first outbreak of Ebola in Yam-buku (former Zaire) in 1976, then again investigated the second outbreak of Ebola in 1977 in Tandala, and in 1995 directed the international response to the Ebola outbreak in Kikwit for WHO. Prior to assignments in Africa, he was assigned for 2 years to India as a medical epidemiologist in the WHO Smallpox Eradication Programme. Dr. Heymann's educational qualifications include a B.A. from the Pennsylvania State University, an M.D. from Wake Forest University, a Diploma in Tropical Medicine and Hygiene from the London School of Hygiene and Tropical Medicine, and practical epidemiology training in the 2-year Epidemic Intelligence Service of CDC. He is a member of the IOM; he was awarded the 2004 Award for Excellence of the American Public Health Association, the 2005 Donald Mackay Award from the American Society for Tropical Medicine and Hygiene, and the 2007 Heinz Award on the Human Condition. In 2009 he was appointed an honorary Commander of the Most Excellent Order of the British Empire for services to global public health, and he was recently elected a Fellow of the Academy of Medical Sciences in the United Kingdom. Dr. Heymann has been visiting professor at Stanford University, the University of Southern California, and the George Washington University School of Public Health; has published over 145 scientific articles on infectious diseases and related issues in peer-reviewed medical and scientific journals; and has authored several chapters on infectious diseases in medical textbooks. He is currently the editor of the 19th edition of the *Control of Communicable Diseases Manual*, a joint publication of the American Public Health Association and WHO.

Zhi Hong, Ph.D.,⁴ is Senior Vice President of the Infectious Diseases Therapy Area Unit Within Medicines Discovery and Development, GlaxoSmithKline (GSK), where he is accountable for designing, consolidating, developing, and delivering the infectious diseases research and development strategy and pipeline, from target identification through to post-launch development. Dr. Hong is also a Board Member of ViiV Healthcare, a specialist HIV company established by GSK and Pfizer.

Previously, Dr. Hong was Head of the Infectious Diseases CEDD. In this role, he led the company's effort in the virtualization of drug discovery through strategic alliances with a number of external biotech companies, including Anacor, Galapagos, Isis, Regulus, Santaris, and Mpex. He was instrumental in GSK's acquisition of Genelabs Technologies, Inc., a California-based biotech firm with an extensive Hepatitis C Virus portfolio. Dr. Hong also played a key role in the execution of a license agreement granting GSK exclusive worldwide rights to a Phase II compound from Idenix Pharmaceuticals for the treatment of HIV/AIDS.

Prior to joining GSK in 2007, Dr. Hong was the Chief Scientific Officer and Executive Vice President of Research at Ardea Biosciences, a publicly traded

⁴ Forum member since November 1, 2011.

biotech company in California. Before that, he was the Vice President of Research at Valeant Pharmaceuticals International. During his drug discovery career in the pharmaceutical industries, Dr. Hong and his teams successfully delivered many drug candidates into the clinics. As a scientist, he is well respected as an opinion leader in the field of antiviral research.

Dr. Hong has many years of experience in drug discovery and product development. He received a bachelor of science degree from Fudan University at Shanghai, China, and his Ph.D. from the State University of New York at Buffalo. He has authored/co-authored more than 100 research publications and given numerous lectures/speeches at various meetings. He has more than 40 issued and/or published patents.

Philip Hosbach currently holds the position of vice president of immunization policy and government relations at sanofi pasteur. The departments under his supervision are state government affairs, federal government affairs, medical communications, strategic advocacy, and immunization initiatives. His responsibilities include oversight of both public policy and immunization policy development. Mr. Hosbach acts as sanofi pasteur's principal liaison with CDC. He is currently coordinating sanofi pasteur's global efforts in responding to the emerging H1N1 pandemic. He is a graduate of Lafayette College (1984); shortly after that he began his professional career in the pharmaceutical industry with American Home Products. That career has now spanned 25 years, including the last 22 years focused solely on vaccines. Mr. Hosbach joined sanofi pasteur (then Connaught Labs) in 1987 in Clinical Research and held positions of increasing responsibility, including Director of Clinical Operations. While in Clinical Research, he also served as project manager for the development and licensure of Tripedia, the first diphtheria, tetanus, and acellular pertussis vaccine approved by FDA for use in U.S. infants. During his clinical research career at sanofi pasteur, he contributed to the development and licensure of seven vaccines. Following his work in clinical research, Mr. Hosbach took a position in the commercial operations area of sanofi pasteur and quickly moved through the ranks on the business administration side of the vaccine division. During that time, Mr. Hosbach led a number of departments within sanofi pasteur, gaining valuable business experience within U.S. Commercial Operations. The departments he led during that time included Public Health Sales and Marketing, Public Relations, Public Affairs, New Product Marketing, and Business Intelligence. He has been a member of the IOM Forum on Microbial Threats since 2005 and has been a Steering Committee member of the Influenza Summit, which is jointly sponsored by the CDC and the American Medical Association, since its inception. Since 2000 Mr. Hosbach has served on the Board of Directors for Pocono Medical Center and Pocono Health Systems, located in East Stroudsburg, Pennsylvania. He also serves as chairman of the Compensation Committee.

Stephen Albert Johnston, Ph.D., is currently director of the Center for Innovations in Medicine in the Biodesign Institute at Arizona State University. His center focuses on formulating and implementing disruptive technologies for basic problems in health care. The center has three divisions: Genomes to Vaccines, Cancer Eradication, and DocInBox. Genomes to Vaccines has developed high-throughput systems to screen for vaccine candidates and is applying them to predict and produce chemical vaccines. The Cancer Eradication group is working on formulating a universal prophylactic vaccine for cancer. DocInBox is developing technologies to facilitate presymptomatic diagnosis. Dr. Johnston founded the Center for Biomedical Inventions (also known as the Center for Translation Research) at the University of Texas, Southwestern, the first center of its kind in the medical arena. He and his colleagues have developed numerous inventions and innovations, including the gene gun, genetic immunization, the tobacco etch virus protease system, organelle transformation, digital optical chemistry arrays, expression library immunization, linear expression elements, synbodies, immunosignaturing diagnosis, and others. He also was involved in transcription research for years, first cloning *Gal4* and later discovering functional domains in transcription factors and the connection of the proteasome to transcription. He has been professor at the University of Texas Southwestern Medical Center at Dallas and associate and assistant professor at Duke University. He has been involved in several capacities as an adviser on biosecurity since 1996 and is a founding member of BioChem 20/20.

Kent Kester, M.D.,⁵ is currently the commander of the Walter Reed Army Institute of Research (WRAIR) in Silver Spring, Maryland. Dr. Kester holds an undergraduate biology degree from Bucknell University (1982) and an M.D. from Jefferson Medical College (1986). He completed his internship and residency in internal medicine at the University of Maryland Hospital/Baltimore VA Medical Center (1989) and a fellowship in infectious diseases at the Walter Reed Army Medical Center (1995). A malaria vaccine researcher with over 50 authored or coauthored scientific manuscripts and book chapters, Dr. Kester has played a major role in the development of the candidate falciparum malaria vaccine known as RTS,S, having safely conducted the largest number of experimental malaria challenge studies ever attempted to date. Dr. Kester's previous military medical research assignments have included director of the WRAIR Malaria Serology Reference Laboratory; chief, Clinical Malaria Vaccine Development Program; chief of the WRAIR Clinical Trials Center; and director of the WRAIR Division of Regulated Activities. He currently is a member of the Steering Committee of the NIAID/Uniformed Services University of the Health Sciences Infectious Disease Clinical Research Program, as well as multiple NIAID Safety Monitoring Committees. He also serves as the consultant to the U.S. Army Surgeon

⁵ Forum member until August 31, 2011.

General in Medical Research and Development. Board certified in both internal medicine and infectious diseases, Dr. Kester is also a fellow of both the American College of Physicians and the IDSA. He holds faculty appointments at both the Uniformed Services University of the Health Sciences and the University of Maryland School of Medicine.

Gerald T. Keusch, M.D., is associate provost and associate dean for global health at Boston University and Boston University School of Public Health. He is a graduate of Columbia College (1958) and Harvard Medical School (1963). After completing a residency in internal medicine, fellowship training in infectious diseases, and 2 years as an NIH research associate at the Southeast Asia Treaty Organization Medical Research Laboratory in Bangkok, Thailand, Dr. Keusch joined the faculty of the Mt. Sinai School of Medicine in 1970, where he established a laboratory to study the pathogenesis of bacillary dysentery and the biology and biochemistry of Shiga toxin. In 1979 he moved to Tufts Medical School and New England Medical Center in Boston to found the Division of Geographic Medicine, which focused on the molecular and cellular biology of tropical infectious diseases. In 1986 he integrated the clinical infectious diseases program into the Division of Geographic Medicine and Infectious Diseases, continuing as division chief until 1998. He has worked in the laboratory and in the field in Latin America, Africa, and Asia on basic and clinical infectious diseases and HIV/AIDS research. From 1998 to 2003, he was associate director for international research and director of the Fogarty International Center at NIH. Dr. Keusch is a member of the American Society for Clinical Investigation, the Association of American Physicians, the ASM, and the IDSA. He has received the Squibb (1981), Finland (1997), and Bristol (2002) awards of the IDSA. In 2002 he was elected to the IOM.

Rima F. Khabbaz, M.D., is deputy director for infectious diseases at CDC. Prior to her current position, she served as director of CDC's National Center for Preparedness, Detection, and Control of Infectious Diseases and held other leadership positions across the agency's infectious disease national centers. She is a graduate of the American University of Beirut, Lebanon, where she obtained both her bachelor's degree in science and her medical doctorate degree. She trained in internal medicine and completed a fellowship in infectious diseases at the University of Maryland, Baltimore. She joined CDC in 1980 as an epidemic intelligence service officer, working in the Hospital Infections Program. During her CDC career, she has made major contributions to advance infectious disease prevention, including leadership in defining the epidemiology of non-HIV retroviruses (HTLV-I and II) in the United States and developing guidance for counseling HTLV-infected persons, establishing national surveillance for hantavirus pulmonary syndrome following the 1993 U.S. outbreak, and developing CDC's blood safety and food safety programs related to viral diseases. She has

also played key roles in CDC's responses to outbreaks of new and/or reemerging viral infections, including Nipah, Ebola, West Nile, SARS, and monkeypox, as well as the 2001 anthrax attacks. She is a fellow of the IDSA and member of the American Epidemiologic Society, the ASM, and the Council of State and Territorial Epidemiologists. She served on IDSA's Annual Meeting Scientific Program Committee and currently serves on the society's National and Global Public Health Committee. In addition to her CDC position, she serves as clinical associate professor of medicine (infectious diseases) at Emory University. She is a graduate of the National Preparedness Leadership Initiative at Harvard University and of the Public Health Leadership Institute at the University of North Carolina.

Stanley M. Lemon, M.D., is professor of medicine at the University of North Carolina, School of Medicine, Chapel Hill, North Carolina. He received his undergraduate A.B. degree in biochemical sciences from Princeton University summa cum laude and his M.D. with honors from the University of Rochester. He completed postgraduate training in internal medicine and infectious diseases at the University of North Carolina, Chapel Hill, and is board certified in both. From 1977 to 1983 he served with the U.S. Army Medical Research and Development Command, followed by a 14-year period on the faculty of the University of North Carolina, School of Medicine. He moved to the University of Texas Medical Branch in 1997, serving first as chair of the Department of Microbiology and Immunology, then as dean of the School of Medicine from 1999 to 2004. Dr. Lemon's research interests relate to the molecular virology and pathogenesis of the positive-stranded RNA viruses responsible for hepatitis. He has had a long-standing interest in antiviral and vaccine development and has served as chair of FDA's Anti-Infective Drugs Advisory Committee. He is the past chair of the Steering Committee on Hepatitis and Poliomyelitis of the WHO Programme on Vaccine Development. He is past chair of the NCID-CDC Board of Scientific Counselors and currently serves as a member of the U.S. Delegation to the U.S.–Japan Cooperative Medical Sciences Program. He was co-chair of the NAS Committee on Advances in Technology and the Prevention of Their Application to Next Generation Biowarfare Threats, and he recently chaired an IOM study committee related to vaccines for the protection of the military against naturally occurring infectious disease threats.

Edward McSweegan, Ph.D., is a program officer at NIAID. He graduated from Boston College with a B.S. in biology in 1978. He has an M.S. in microbiology from the University of New Hampshire and a Ph.D. in microbiology from the University of Rhode Island. He was an NRC associate from 1984 to 1986 and did postdoctoral research at the Naval Medical Research Institute in Bethesda, Maryland. Dr. McSweegan served as an AAAS diplomacy fellow in the U.S. State Department from 1986 to 1988, where he helped to negotiate science and technology agreements with Poland, Hungary, and the former Soviet Union.

After moving to NIH, he continued to work on international health and infectious disease projects in Egypt, Israel, India, and Russia. Currently, he manages NIAID's bilateral program with India, the Indo-U.S. Vaccine Action Program, and he represents NIAID in the HHS Biotechnology Engagement Program with Russia and related countries. He is a member of AAAS, the ASM, and the National Association of Science Writers. He is the author of numerous journal and freelance articles.

Mark A. Miller, M.D., is currently the Director of the Division of International Epidemiology and Population Studies for the Fogarty International Center at the NIH in Bethesda, Maryland. He is also a physician at the Yukon-Kuskokwim Delta Regional Hospital in Bethel, Alaska, which primarily serves Native Americans. He previously served as a medical officer on the Children's Vaccine Initiative for WHO and the CDC, and medical epidemiologist for the CDC National Immunizations Program and Epidemiology Program Office, Office of the Director. He also conducted research at the Armed Forces Research Institute for Medical Studies in Bangkok, Thailand, the Yale Arbovirus Research Unit, and Cornell University Medical College.

Dr. Miller received his B.A., magna cum laude, in neuroscience, biology, and human ecology from Amherst College in 1983, and his M.D. from Yale University School of Medicine in 1990. He completed his internal medicine residency at Yale New Haven Hospital/Hospital of St. Raphael and became board certified in 1994. He has served as a member of many professional societies and steering committees, including the Secretary's Advisory Council on Public Health Preparedness Smallpox Modeling and several NSF, HHS, and NIH task forces. He has presented and consulted nationally and internationally for organizations including USAID, the Pan American Health Organization, and the World Bank. Dr. Miller is a reviewer for nine journals, including the *Journal of Infectious Diseases*, *The Lancet*, and the *Journal of the American Public Health Association*. He has won many awards, including the Distinguished Service Medal, from the U.S. Public Health Service and the CDC. He has published more than 50 scientific articles in the peer-reviewed literature, nine books and/or book chapters, and more than 50 letters and abstracts.

Paul F. Miller, Ph.D.,⁶ is chief scientific officer for antibacterials research. He received his undergraduate degree in biology from LeMoyne College, and subsequently earned a Ph.D. in microbiology and immunology from the Albany Medical College in 1987. Following 4 years of postdoctoral studies on yeast molecular genetics at NIH in Bethesda, Maryland, Dr. Miller joined the Parke-Davis Pharmaceutical Research Division of Warner-Lambert Company in Ann Arbor, Michigan, in 1990 as a senior scientist in the Infectious Diseases Depart-

⁶ Forum member until July 31, 2011.

ment, where he developed a number of novel screens and mechanism-of-action tools. He then moved to Pfizer in 1997 as manager of the Antibacterials Biology Research group within the Antibacterials, Immunology, and Cancer Zone at the Groton, Connecticut, research labs, and has taken on increasing responsibility since that time. In his current role, he is responsible for all antibacterial research activities through early clinical development, as well as collaboratively establishing R&D strategies in this disease area. His specific research interests and expertise include genetic mechanisms of intrinsic antibiotic resistance in bacteria as well as the use of novel genetic technologies for the elucidation of antibiotic mechanisms of action.

George Poste, Ph.D., D.V.M., is chief scientist, Complex Adaptive Systems Initiative, and Del E. Webb Professor of Health Innovation at Arizona State University (ASU). He assumed this post in 2009. From 2003 to 2009 he directed and built the Biodesign Institute at ASU. In addition to his academic post, he serves on the Board of Directors of Monsanto, Exelixis, Caris Life Sciences, LGC, and the Scientific Advisory Board of Synthetic Genomics. From 1992 to 1999 he was Chief Science and Technology Officer and President, R&D, of SmithKline Beecham (SB). During his tenure at SB he was associated with the successful registration of 31 drug, vaccine, and diagnostic products. In 2004 he was named “R&D Scientist of the Year” by *R&D Magazine*, in 2006 he received the Einstein award from the Global Business Leadership Council, and in 2009 he received the Scrip Lifetime Achievement award voted by the leadership of the global pharmaceutical industry.

He has published over 350 research papers and edited 14 books on pharmaceutical technologies and oncology. He has received honorary degrees in science, law, and medicine for his research contributions and was honored in 1999 by Her Majesty Queen Elizabeth II as a Commander of the British Empire for his contributions to international security. He is a Fellow of the Royal Society, the Royal College of Pathologists, and the U.K. Academy of Medicine; a Distinguished Fellow at the Hoover Institution, Stanford University; and a member of the Council on Foreign Relations. He has served on numerous government panels related to biosecurity and national competitiveness.

John C. Pottage, Jr., M.D.,⁷ has been vice president for Global Clinical Development in the Infectious Disease Medicine Development Center at GlaxoSmithKline since 2007. Previously he was senior vice president and chief medical officer at Achillion Pharmaceuticals in New Haven, Connecticut. Achillion is a small biotechnology company devoted to the discovery and development of medicines for HIV, hepatitis C virus, and resistant antibiotics. Dr. Pottage initially joined Achillion in May 2002. Prior to Achillion, Dr. Pottage was medical

⁷ Forum member until October 31, 2011.

director of Antivirals at Vertex Pharmaceuticals. During this time he also served as an associate attending physician at the Tufts New England Medical Center in Boston. From 1984 to 1998, Dr. Pottage was a faculty member at Rush Medical College in Chicago, where he held the position of associate professor, and also served as the medical director of the Outpatient HIV Clinic at Rush-Presbyterian-St. Luke's Medical Center. While at Rush, Dr. Pottage was the recipient of several teaching awards and is a member of the Mark Lepper Society. Dr. Pottage is a graduate of St. Louis University School of Medicine and Colgate University.

David Rizzo, Ph.D.,⁸ received his Ph.D. in plant pathology from the University of Minnesota and joined the faculty of the University of California-Davis, Department of Plant Pathology and the Graduate Group in Ecology in 1995. Research in his lab focuses on the ecology and management of forest tree diseases, including diseases caused by both native and introduced pathogens. Research in the lab takes a multiscale approach ranging from experimental studies on the basic biology of organisms to field studies across forest landscapes. Active collaborations include projects with landscape ecologists, epidemiologists, molecular biologists, entomologists, and forest managers. The primary research effort in the lab is currently *Phytophthora* species in California coastal forests, with an emphasis on Sudden Oak Death. As part of his research on Sudden Oak Death, Dr. Rizzo also serves as the scientific advisor for the California Oak Mortality Task Force. In conifer forests of the Sierra Nevada Mountains, the lab studies a variety of diseases and their relationship to past and present forest management and conservation issues. In addition to research, Dr. Rizzo teaches undergraduate and graduate courses in mycology as well as introductory biology. Since 2004, he has been director of the Science and Society program in the College of Agricultural and Environmental Sciences. Science and Society is an academic program designed to offer students the opportunity to discover the interdisciplinary connections that link the biological, physical, and social sciences with societal issues and cultural discourses.

Gary A. Roselle, M.D., is program director for infectious diseases for the VA Central Office in Washington, DC, as well as the chief of the medical service at the Cincinnati VA Medical Center. He is a professor of medicine in the Department of Internal Medicine, Division of Infectious Diseases, at the University of Cincinnati College Of Medicine. Dr. Roselle serves on several national advisory committees. In addition, he is currently heading the Emerging Pathogens Initiative for the VA. He has received commendations from the undersecretary for health for the VA and the secretary of VA for his work in the Infectious Diseases Program for the VA. He has been an invited speaker at several national and international meetings and has published more than 90 papers and several book

⁸ Forum member since September 1, 2011.

chapters. Dr. Roselle received his medical degree from the OSU School of Medicine in 1973. He served his residency at the Northwestern University School of Medicine and his infectious diseases fellowship at the University of Cincinnati School of Medicine.

Alan S. Rudolph, Ph.D., M.B.A., has led an active career in translating interdisciplinary life sciences into useful applications for biotechnology development. His experience spans basic research to advanced development in academia, government laboratories, and most recently in the nonprofit and private sectors. He has published more than 100 technical publications in areas including molecular biophysics, lipid self-assembly, drug delivery, blood substitutes, medical imaging, tissue engineering, neuroscience, and diagnostics. As a National Research Council Post-Doctoral Fellow, his earliest work at the U.S. Naval Research Laboratory (NRL) demonstrated the translational value of strategies used by organisms that survive environmental extremes to preserve Defense products such as biosensors and blood products for field deployment. After a decade at NRL he was recruited to join the Defense Advanced Research Projects Agency, to lead new strategic efforts to extract and exploit useful principles and practices in life sciences and technology and establish an agency-wide strategy for investments in biosciences and biotechnology. As Chief of Biological Sciences and Technology, Dr. Rudolph established a framework for investments that continue today. These include new programs in broad areas of bioscience and technology such as sensors, diagnostics, materials, robotics, biomolecular, cell and tissue engineering, medical devices, and neuroscience and technology, including the current efforts in revolutionizing prosthetics. He received a meritorious civil service citation from the Office of the Secretary of Defense for his contributions to defining and implementing a new generation of life sciences and national security investments.

In 2003, he left civil service for the private sector and starting new corporate biotechnology efforts. As Chief Executive Officer of Adlyfe Inc., a diagnostic platform company, and Board Chairman of Cellphire Inc., focused on development of novel hemostatic biologics for bleeding injury, he took nascent technology demonstrations and secured venture capital funding and pharmaceutical partnerships while managing all aspects of development toward first human use. These efforts included managing early manufacturing and regulatory strategies required for FDA approval of diagnostics and therapeutics. Most recently, he started a new international nonprofit foundation and, as Director of the International Neuroscience Network Foundation, he has secured corporate and private philanthropic donors to fulfill the mission of the organization focused on brain STEM efforts and clinical trial management in underserved populations. He has a doctorate degree in zoology from the University of California at Davis and an M.B.A. from the George Washington University.

Kevin Russell, M.D., M.T.M.&H., F.I.D.S.A. CAPT MC USN, is the Director, Department of Defense Global Emerging Infections Surveillance and Response System, and Deputy Director, Armed Forces Health Surveillance Center, in the U.S. Department of Defense. In this position, his priorities have been standardization, greater affiliations with world militaries, continuing to introduce scientific rigor into the network, and synchronization with other U.S. government global surveillance programs. He graduated from the University of Texas Health Science Center San Antonio Medical School in 1990; after a family practice internship, he was accepted into the Navy Undersea Medicine program. He was stationed in Panama City, Florida, at the Experimental Diving Unit where he worked in diving medicine research from 1991 to 1995. After a preventive medicine residency with a masters in tropical medicine and hygiene, he was transferred to Lima, Peru, where he became head of the Virology Laboratory. His portfolio included febrile illness (largely arboviral in origin) and HIV surveillance studies in eight different countries of South America, as well as prospective dengue transmission studies. In 2001, he moved back to the United States and became the director of the Respiratory Disease Laboratory at the Naval Health Research Center in San Diego, California. Febrile respiratory illness surveillance in recruits of all services was expanded into shipboard populations, Mexican border populations, support for outbreaks, and deployed settings. Validation and integration of new and emerging advanced diagnostic capabilities, utilizing the archives of specimens maintained at the laboratory, became a priority. A BSL-3-Enhanced was constructed. Projects expanded in 2006 to clinical trials support as Dr. Russell became the Principal Investigator for the Navy site in the FDA Phase III adenovirus vaccines trial, and more recently to support the Phase IV post-marketing trial of the recently FDA-approved ACAM2000 smallpox vaccine.

Janet Shoemaker is director of the ASM's Public Affairs Office, a position she has held since 1989. She is responsible for managing the legislative and regulatory affairs of this 42,000-member organization, the largest single biological science society in the world. Previously, she held positions as assistant director of public affairs for the ASM; as ASM coordinator of the U.S.–U.S.S.R. Exchange Program in Microbiology, a program sponsored and coordinated by the NSF and the U.S. Department of State; and as a freelance editor and writer. She received her baccalaureate, cum laude, from the University of Massachusetts and is a graduate of the George Washington University programs in public policy and in editing and publications. She is a member of Women in Government Relations, the American Society of Association Executives, and AAAS. She has coauthored articles on research funding, biotechnology, biodefense, and public policy issues related to microbiology.

P. Frederick Sparling, M.D., is professor of medicine, microbiology, and immunology at the University of North Carolina (UNC), Chapel Hill. He is director

of the SouthEast Sexually Transmitted Infections Cooperative Research Center and also the Southeast Regional Centers of Excellence in Biodefense and Emerging Infections. Previously he served as chair of the Department of Medicine and chair of the Department of Microbiology and Immunology at UNC. He was president of the IDSA from 1996 to 1997. He was also a member of the IOM Committee on Microbial Threats to Health (1990–1992) and the IOM Committee on Emerging Microbial Threats to Health in the 21st Century (2001–2003). Dr. Sparling's laboratory research has been on the genetics and molecular biology of bacterial outer membrane proteins, with a major emphasis on gonococci and meningococci. His work helped to define the genetics of antibiotic resistance in gonococci and the role of iron-scavenging systems in the pathogenesis of human gonorrhea. Current interests include pathogenesis of gonococcal infections and development of a vaccine for gonorrhea and managing a large multi-institution interactive research group focused on emerging infections and biodefense.

Terence Taylor is the founding president of the International Council for the Life Sciences (ICLS). The ICLS is an independent nonprofit organization registered in the United States and in the European Union. The ICLS is designed to promote best practices and codes of conduct for safety and security in relation to biological risks. Terence Taylor also served as the vice president, Global Health and Security, at the Nuclear Threat Initiative. Prior to these appointments Terence Taylor was assistant director at the International Institute for Strategic Studies (IISS) in London and was president and executive director of IISS-US in Washington, DC. At IISS, in addition to his overall program responsibilities, he led the Institute's work on life sciences and security. He has substantial experience in international security policy matters as a U.K. government official (both military and diplomatic) and for the United Nations (UN) both in the field and at UN Headquarters. He was a commissioner and one of the Chief Inspectors with the UN Special Commission on Iraq, with particular responsibilities for biological issues. His government experience is related to both military field operations and to the development and implementation of policies in relation to arms control and nonproliferation treaties and agreements for both conventional weapons and weapons of mass destruction and the law of armed conflict aspects of international humanitarian law. He has also conducted consulting work on political risk assessment and studies of the private biotechnology industry. He was a Science Fellow at Stanford University's Center for International Security and Cooperation. He was an officer in the British Army with experience in many parts of the world including UN peacekeeping, counterinsurgency, and counterterrorism operations.

Murray Trostle, Dr.P.H., is a foreign service officer with USAID, presently serving as the deputy director of the Avian and Pandemic Influenza Preparedness and Response Unit. Dr. Trostle attended Yale University, where he received

a master's in public health in 1978, focusing on health services administration. In 1990, he received his doctorate in public health from UCLA. His research involved household survival strategies during famine in Kenya. Dr. Trostle has worked in international health and development for approximately 38 years. He first worked overseas in the Malaysian national malaria eradication program in 1968 and has since focused on health development efforts in the former Soviet Union, Africa, and Southeast Asia. He began his career with USAID in 1992 as a postdoctoral fellow with AAAS. During his career he has worked with a number of development organizations, such as the American Red Cross, Project Concern International, and the Center for Development and Population Activities. With USAID, Dr. Trostle has served as director of the child immunization cluster, where he was chairman of the European Immunization Interagency Coordinating Committee and USAID representative to the Global Alliance on Vaccines and Immunization. Currently, Dr. Trostle leads the USAID Infectious Disease Surveillance Initiative as well as the Avian Influenza Unit.

Mary E. Wilson, M.D., is Associate Professor of Global Health and Population at the Harvard School of Public Health. Her academic interests include the ecology of infections and emergence of microbial threats, travel medicine, tuberculosis, and vaccines. Her undergraduate degree in French, English, and philosophy was awarded by Indiana University; she received her M.D. from the University of Wisconsin and completed an internal medicine residency and infectious disease fellowship at the Beth Israel Hospital in Boston (now Beth Israel-Deaconess Medical Center). She was Chief of Infectious Diseases at Mount Auburn Hospital, a Harvard-affiliated community teaching hospital in Cambridge, Massachusetts, for more than 20 years. She is a Fellow in the IDSA and the American College of Physicians. She has served on ACIP of the CDC, the Academic Advisory Committee for the National Institute of Public Health in Mexico, and on four committees for the IOM of the National Academies, including the Committee on Emerging Microbial Threats to Health in the 21st Century, whose report (*Microbial Threats to Health: Emergence, Detection, and Response*) was released in March 2003. She has worked in Haiti at the Albert Schweitzer Hospital and leads the Harvard-Brazil Collaborative Course on Infectious Diseases, which is taught in Brazil. In 1996 she was a resident scholar at the Bellagio Study Center, Italy, and in 2002 she was a Fellow at the Center for Advanced Study in the Behavioral Sciences in Stanford, California. She was a member of the Pew National Commission on Industrial Farm Animal Production, whose report *Putting Meat on the Table: Industrial Farm Animal Production in America* was released in the spring of 2008. A former GeoSentinel Site Director (Cambridge), she now serves as a Special Advisor to the GeoSentinel Surveillance Network, a global network. She has lectured and published widely, serves on several editorial boards, and is an associate editor for *Journal Watch Infectious Diseases*. She is the author of *A World Guide to Infections: Diseases, Distribution, Diagnosis* (Oxford University

Press, New York, 1991); senior editor, with Richard Levins and Andrew Spielman, of *Disease in Evolution: Global Changes and Emergence of Infectious Diseases* (New York Academy of Sciences, 1994); and editor of the volume *New and Emerging Infectious Diseases* (Medical Clinics of North America) published in 2008. She joined the Board of Trustees for ICDDR, B (International Centre for Diarrheal Disease Research, Bangladesh) in 2009 and is a member of the Board of Scientific Counselors for the CDC, the FXB-USA Board, and the APUA Board of Directors.

Appendix F

Speaker Biographies

David Berry, M.D., Ph.D., is a Partner at Flagship Ventures, having joined Flagship in 2005 while completing his M.D. from Harvard Medical School. David was previously awarded a Ph.D. through the Massachusetts Institute of Technology (MIT) Biological Engineering Division, where he studied the biological effects of complex sugars with advisors Professor Ram Sasisekharan and Professor Robert Langer. David also did his undergraduate work at MIT, graduating in 2000 Phi Beta Kappa and Sigma Xi, with a degree in brain and cognitive sciences. He was named as a member of the MIT Corporation—its Board of Trustees—in 2006, and to the MIT Enterprise Forum Global Board in 2010. David’s work has led to 12 peer-reviewed publications, over 40 patents and applications, as well as over 30 awards and honors including the prestigious Lemelson-MIT Student Prize in 2005 for invention and innovation. David was also named the Innovator of the Year under the age of 35 by *Technology Review* in 2007.

At Flagship, David focuses on investing in and founding early stage life science and cleantech ventures. He was a board member of Flagship portfolio company CGI Pharmaceuticals (acquired by Gilead in 2010). In 2005, as part of Flagship’s VentureLabs unit he co-founded and helped launch LS9, and more recently co-founded Joule Unlimited where he previously served as the founding CEO. In addition, David serves on the Board of Directors of Eleven Biotherapeutics and works closely with several other portfolio companies. He is currently co-founder and CEO of Theracrine, a company developing novel drugs to treat metastases.

George Church, Ph.D., is professor of genetics at Harvard Medical School and Director of the Center for Computational Genetics. With degrees from Duke

University in chemistry and zoology, he co-authored research on 3D software and RNA structure with Sung-Hou Kim. His Ph.D. from Harvard in biochemistry and molecular biology with Wally Gilbert included the first direct genomic sequencing method in 1984; initiating the Human Genome Project then as a research scientist at newly formed Biogen Inc. and a Monsanto Life Sciences Research Fellow at the University of California, San Francisco (UCSF), with Gail Martin. He invented the broadly applied concepts of molecular multiplexing and tags, homologous recombination methods, and array DNA synthesizers. Technology transfer of automated sequencing and software to Genome Therapeutics Corp. resulted in the first commercial genome sequence (the human pathogen, *H. pylori*, in 1994). This multiplex solid-phase sequencing evolved into polonies (1999), ABI-SOLiD (2005), and open-source Polonator.org (2007) and Personal Genomes.org. He has served in advisory roles for 12 journals (including *Nature Molecular Systems Biology*), 5 granting agencies, and 24 biotech companies (e.g., 23andme and recently founding Codon Devices, Knome, and LS9). Current research focuses on integrating biosystems modeling with personal genomics and synthetic biology.

James J. Collins, Ph.D., is an investigator of the Howard Hughes Medical Institute, and a William F. Warren Distinguished Professor, University Professor, Professor of Biomedical Engineering, Professor of Medicine and Co-Director of the Center for BioDynamics at Boston University. He is also a core founding faculty member of the Wyss Institute for Biologically Inspired Engineering at Harvard University. His research group works in synthetic biology and systems biology, with a particular focus on network biology approaches to antibiotic action and bacterial defense mechanisms.

Andrew Ellington, Ph.D., received his B.S. in biochemistry from Michigan State University in 1981, and his Ph.D. in biochemistry and molecular biology from Harvard in 1988. As a graduate student he worked with Dr. Steve Benner on the evolutionary optimization of dehydrogenase isozymes. His postdoctoral work was with Dr. Jack Szostak at Massachusetts General Hospital, where he developed methods for the in vitro selection of functional nucleic acids and coined the term "aptamer." Dr. Ellington began his academic career as an assistant professor of chemistry at Indiana University in 1992 and continued to develop selection methods. He has received the Office of Naval Research Young Investigator, Cottrell, and Pew Scholar awards. In 1998 he moved to the University of Texas at Austin and is now the Fraser Professor of Biochemistry. Dr. Ellington's lab works on the development of functional nucleic acids for practical applications, including aptamer biosensors, allosteric ribozyme logic gates (aptazymes), and internalizing nucleic acids that can deliver siRNAs to cells. The next leap forward will hopefully be to develop synthetic genetic circuits that can perform amorphous

computations. Dr. Ellington was a member of the Defense Science Study Group, and is active in the DIA advisory group Biochem2020.

Paul Freemont, Professor, FSB, holds the Chair of Protein Crystallography at Imperial College London and is currently the Head of the Division of Molecular Biosciences and Co-director of the new EPSRC Centre for Synthetic Biology and Innovation. Previous to this he was Head of the Imperial College Centre for Structural Biology having joined from Cancer Research UK London Research Institute where he was a principal scientist. His research interests over the last 20 years have focused on understanding the molecular basis and mechanisms of a number of human diseases including pathogenic infection, and he is the author of over 130 peer-reviewed scientific publications. He is currently co-leading an initiative at Imperial in the new field of synthetic biology based on engineering design principles aimed at manufacturing biologically based systems and devices. He is co-founder of a spinout company Equinox Pharma Ltd and has held a number of external positions including membership of Royal Academy of Engineering enquiry into Synthetic Biology (2006–2008); current member of the Cancer Research UK Biological Sciences funding panel (since 2006); member of the Wellcome Trust Genes Molecules and Cells funding panel (2002–2005); member of the Wellcome Trust fellowships panel (2002–2005); and chair of the London Structural Biology Consortium (since 2004).

Chris French, Ph.D., following a degree in biotechnology and bioprocess engineering, worked at the New Zealand Dairy Research Institute, developing processes for purification of high-value milk proteins, then undertook a Ph.D. at the Institute of Biotechnology, University of Cambridge, United Kingdom, during which he purified and cloned enzymes involved in biotransformations of morphine alkaloids and generated recombinant microorganisms for the bioconversion of morphine to higher-value opiate drugs. During his postdoctoral research at the University of Cambridge, he studied enzymes involved in degradation of explosives and used these to generate biosensors for explosives and transgenic plants able to break down explosive residues in soil. Dr. French was then appointed a lecturer in the Institute of Cell and Molecular Biology, University of Edinburgh, where he developed transgenic plants able to degrade chlorinated solvents, as well as biosensors for arsenic and other heavy metals. In 2006 he became interested in synthetic biology and began supervising the University of Edinburgh entries in the International Genetically Engineered Machine competition (iGEM). His current research is focused on use of synthetic biology for novel whole-cell biosensors and for conversion of renewable cellulosic biomass to useful products, and collaborating on development of new technologies for synthetic biology.

George Georgiou, Ph.D., is the Cockrell Endowed Professor at the University of Texas, Austin, where he has joint appointments in the Department of Chemical

Engineering, Section of Molecular Genetics and Microbiology and Biomedical Engineering. He is also a member of the Institute for Cell and Molecular Biology at the University of Texas, Austin. He received his B.Sc. degree from the University of Manchester, United Kingdom, and his Ph.D. from Cornell in 1987. He is a member of the U.S. National Academy of Engineering and a Fellow of American Society for Microbiology, the American Association for the Advancement of Sciences, and the American Institute of Medical and Biological Engineers. He has received numerous awards including the AIChE Professional Progress Award for outstanding contributions to chemical engineering by an individual under 45 (2003) and was named as “One of the Top 100 Eminent Chemical Engineers of the Modern Era” by AIChE (2008). His research is focused on the discovery and pharmacological optimization of protein therapeutics and also on the mechanisms of redox homeostasis and protein secretion in bacteria. Dr. Georgiou and his collaborators have developed one anti-infective antibody drug (Anthem™, currently in late-stage clinical development), an array of therapeutic enzymes in preclinical development, and finally, antibodies for cancer chemotherapy. Dr. Georgiou has published more than 170 research articles and is co-inventor of 38 U.S. patent applications of which 26 have been licensed to pharmaceutical and biotechnology companies.

E. Peter Greenberg, Ph.D., received his bachelor’s degree from Western Washington University, a master’s from the University of Iowa, and his Ph.D. from the University of Massachusetts. After a postdoctoral at Harvard, he joined the faculty at Cornell University, eventually moved back to the University of Iowa, and finally returned to the Pacific Northwest as a member of the University of Washington Medicine Microbiology faculty. He is an elected Fellow of the National Academy of Sciences, the American Academy of Arts and Sciences, the American Association for the Advancement of Science, and the American Academy of Microbiology. Dr. Greenberg has spent his scientific career uncovering the world of microbial social behavior. Due in part to his efforts we now understand that bacteria possess a chemical language for communication and we understand mechanisms of bacterial communication. Bacterial communication controls virulence in a variety of pathogenic bacteria and has thus become a target for development of new therapeutic strategies. Bacteria have also become models for studies of selection for and evolution of cooperative behavior.

Jim Heath, Ph.D., received his B.Sc. in chemistry from Baylor University in 1984 and his M.Sc. and Ph.D. in chemical physics in 1988 from Rice University. He was a Miller Research Postdoctoral Fellow at the University of California (UC), Berkeley, then joined the IBM Watson Labs as a research staff member in 1991. He was appointed assistant professor at the University of California, Los Angeles (UCLA), in 1994, and was promoted to professor in 1997. Heath founded the California NanoSystems Institute in 2000 and served as its director

until moving to Caltech. Heath has worked in a number of areas, including nano-materials, molecular electronics, and quantum phase transitions. More recently he has turned his efforts to addressing translational and fundamental research problems in oncology. Heath has been a recipient of several awards, including the Spiers Medal from the Royal Society, a Public Service Commendation from California Governor Gray Davis, the Sackler Prize in the Physical Sciences, and the Irvin Weinstein Prize and Lectureship from the American Association of Cancer Researchers. He has founded or co-founded several companies, including NanoSys, Momentum Biosciences, and Integrated Diagnostics. He serves as director of the National Cancer Institute–funded NSB Cancer Center.

Clyde A. Hutchison III, Ph.D., is a distinguished professor in the synthetic biology group headed by Dr. Hamilton Smith at the J. Craig Venter Institute (JCVI) in San Diego, California. He graduated from Yale University (B.S., 1960), and Caltech (Ph.D., 1968), where he studied with Robert L. Sinsheimer. He joined the faculty at the University of North Carolina (UNC), Chapel Hill (1968–2005), where he collaborated with Marchall Edgell on early applications of restriction enzymes. While on sabbatical in Fred Sanger's laboratory (1975–1976), he participated in determining the first complete sequence of a DNA molecule (ϕ X174). He developed site-directed mutagenesis with Michael Smith (1978). He also cloned and sequenced the beta-globin gene cluster in the mouse with Edgell. There they discovered L1, the major retrotransposon in the mammalian genome. In 1990, he began work with *Mycoplasma genitalium*, a model for the minimal cellular genome. This led to his collaboration with Smith and Venter and to his current work on synthetic genomics. In May of 2010 the Synthetic Biology Group at JCVI reported construction of the first synthetic cell. He is Kenan Professor Emeritas at UNC Chapel Hill, a member of the National Academy of Sciences, and a fellow of the American Academy of Arts and Sciences.

Stephen Albert Johnston, Ph.D., is currently director of the Center for Innovations in Medicine in the Biodesign Institute at Arizona State University. His center focuses on formulating and implementing disruptive technologies for basic problems in health care. The center has three divisions: Genomes to Vaccines, Cancer Eradication, and DocInBox. Genomes to Vaccines has developed high-throughput systems to screen for vaccine candidates and is applying them to predict and produce chemical vaccines. The Cancer Eradication group is working on formulating a universal prophylactic vaccine for cancer. DocInBox is developing technologies to facilitate presymptomatic diagnosis. Dr. Johnston founded the Center for Biomedical Inventions (also known as the Center for Translation Research) at the University of Texas, Southwestern, the first center of its kind in the medical arena. He and his colleagues have developed numerous inventions and innovations, including the gene gun, genetic immunization, the tobacco etch virus protease system, organelle transformation, digital optical chemistry

arrays, expression library immunization, linear expression elements, synbodies, immunosignaturing diagnosis, and others. He also was involved in transcription research for years, first cloning *Gal4* and later discovering functional domains in transcription factors and the connection of the proteasome to transcription. He has been professor at the University of Texas Southwestern Medical Center at Dallas and associate and assistant professor at Duke University. He has been involved in several capacities as an adviser on biosecurity since 1996 and is a founding member of BioChem 20/20.

Gerald F. Joyce, M.D., Ph.D., is a professor in the Departments of Chemistry and Molecular Biology, and an Investigator of the Skaggs Institute for Chemical Biology at The Scripps Research Institute in La Jolla, California. He received his B.A. from the University of Chicago in 1978 and both an M.D. and a Ph.D. from the University of California, San Diego (UCSD), in 1984. He carried out postgraduate medical training at Mercy Hospital in San Diego and postdoctoral research training at The Salk Institute before joining the faculty of The Scripps Research Institute in 1989. Dr. Joyce's research involves the test-tube evolution of nucleic acids and the application of these methods to the development of novel RNA and DNA enzymes. He also has a longstanding interest in the origins of life and the role of RNA in the early history of life on Earth. He has published over 130 scientific papers and is the inventor of 11 issued patents. He was elected to the U.S. National Academy of Sciences in 2001. In 2005, he received the H.C. Urey Award, presented every 6 years by the International Society for the Study of the Origin of Life, and in 2010 he received the U.S. National Academy of Sciences Miller Medal.

Jay Keasling, Ph.D., received his B.S. in chemistry and biology from the University of Nebraska in 1986, received his Ph.D. in chemical engineering from the University of Michigan in 1991, and did postdoctoral work in biochemistry at Stanford University from 1991 to 1992. Keasling joined the Department of Chemical Engineering at the University of California, Berkeley, as an assistant professor in 1992, where he is currently the Hubbard Howe Distinguished Professor of Biochemical Engineering. Keasling is also a professor in the Department of Bioengineering at Berkeley, a Senior Faculty Scientist and Associate Laboratory Director of the Lawrence Berkeley National Laboratory, and Chief Executive Officer of the Joint BioEnergy Institute. Dr. Keasling's research focuses on engineering microorganisms for environmentally friendly synthesis of small molecules or degradation of environmental contaminants. Keasling's laboratory has engineered bacteria and yeast to produce polymers, a precursor to the antimalarial drug artemisinin, and advanced biofuels and soil microorganisms to accumulate uranium and to degrade nerve agents.

Kim Lewis, Ph.D., is professor of biology and Director, Antimicrobial Discovery Center at Northeastern University in Boston. He obtained his Ph.D. in biochemistry from Moscow University in 1980 and was on the faculty of MIT, University of Maryland, and Tufts University prior to coming to Northeastern. Dr. Lewis has authored over 100 papers and is an inventor on several patents. These include the discovery of synergistically acting antimicrobials in medicinal plants; a general method to grow previously “unculturable” bacteria; the invention of sterile surface materials; the development of high-throughput screening for antimicrobials in a live infected animal; and the discovery of the culprit of recalcitrant biofilm infections, drug-tolerant persister cells. Dr. Lewis has presented over 70 invited lectures, including the 2005 Division A (Antimicrobial Chemotherapy) Lecture at the American Society for Microbiology General Meeting and the Harvard University Microbial Science Initiative lecture. Dr. Lewis has been a permanent member of the Drug Discovery and Drug Resistance NIH Study Section, and chair of two NIH Study Sections on Drug Discovery. Dr. Lewis is a member of Faculty 1000, a recipient of the MIT C.E. Reed Faculty Initiative Award for an innovative research project, and is a recipient of the NIH Director’s Transformative RO1 Grant.

Timothy Lu, M.D., Ph.D., is an assistant professor leading the Synthetic Biology Group in the Department of Electrical Engineering and Computer Science at MIT. He is also an associate member at the Broad Institute, a member of the MIT Computational and Systems Biology Initiative and the MIT Microbial Science and Engineering Program, and a cofounder of Novophage Therapeutics. Tim’s research focuses on engineering fundamental technologies to enable scalable biological systems and on applying synthetic biology to solve important medical and industrial problems, such as antimicrobial resistance and biofilms. Tim is a recipient of the Lemelson-MIT Student Prize, Grand Prize in the National Hall of Fame’s Collegiate Inventors Competition, and the Leon Reznick Memorial Prize from Harvard Medical School. He was elected to *Technology Review*’s TR35 for “Top Young Innovators Under 35” in 2010, named a Kavli Fellow of the National Academy of Sciences, and received the Bronze Medal (second place) and Best Paper in the Biomedical Technologies Session in the 27th Army Science Conference.

Bernhard Palsson, Ph.D., is the Galetti Professor of Bioengineering and the Principal Investigator of the Systems Biology Research Group in the Department of Bioengineering at UCSD. Dr. Palsson has co-authored more than 300 peer-reviewed research articles and has authored or co-authored two textbooks, with one more in press. His research includes the development of methods to analyze metabolic dynamics (flux-balance analysis, and modal analysis), and the formulation of complete models of selected cells (the red blood cell, *E. coli*, hybridoma, and several human pathogens). He sits on the editorial board of several leading

peer-reviewed microbiology, bioengineering, and biotechnology journals. He previously held a faculty position at the University of Michigan for 11 years and was named the G.G. Brown Associate Professor at Michigan in 1989, a Fulbright fellow in 1995, and an Ib Henriksen Fellow in 1996. He is the author of 38 U.S. patents, and is the co-founder of several biotechnology companies. He holds a Ph.D. in chemical engineering from the University of Wisconsin, Madison. Dr. Palsson is a member of the National Academy of Engineering and was recently voted in as a Fellow of both the AAAS and the AAM.

Bali Pulendran, Ph.D., is a Charles Howard Candler Professor of Pathology and Laboratory Medicine, and Director of the Innate Immunity Program at the Emory Vaccine Center, Emory University in Atlanta. He received his undergraduate degree from Cambridge University and his Ph.D from the Walter & Eliza Hall Institute in Melbourne, Australia, under the supervision of Sir Gustav Nossal. He did his postdoctoral work at Immunex Corporation in Seattle, with Eugene Maraskovsky and Charlie Maliszewski. Dr. Pulendran's work focuses on understanding the mechanisms by which the innate immune system regulates adaptive immunity and harnessing such mechanisms in the design of novel vaccines against global pandemics. More recently, he has begun to apply systems biological approaches to predicting the efficacy of vaccines and deciphering new correlates of protection against infectious diseases.

Dr. Pulendran's research is published in front line journals such as *Nature*, *Science*, *Cell*, *Nature Immunology*, and the *Journal of Experimental Medicine*. Dr. Pulendran is the recipient of numerous grants from the NIH and from the Bill & Melinda Gates Foundation. He serves on the editorial boards of the *Journal of Clinical Investigation* and the *Journal of Immunology*, the AIDS Vaccine Research Subcommittee, and is frequently invited to speak in the plenary sessions of many national and international conferences.

Herbert Sauro, Ph.D., is currently an associate professor in the Department of Bioengineering at the University of Washington in Seattle. His work at present is focused on a number of areas that include development of exchange standards in synthetic biology, exploring the potential role of computer-aided design software in synthetic biology, applying engineering principles to biological networks, and understanding the factors that contribute to the demise of engineered biological networks over evolutionary time. He was one of the founding developers of the Systems Biology Markup Language, the Systems Biology Workbench, and one of the early proponents and developers of Metabolic Control Analysis. In 2008 he started the synthetic biology standards consortium (<http://www.sbolstandard.org/>), which has now grown to seven institutions and represents a community effort to develop exchange standards for synthetic biology design. In the past he has owned two small companies, a software consultancy business for large financial companies such as the Financial Times and GE Capital, and a company

that specialized in developing interactive educational software for K–12 and college-level students. He also is a proponent of open-source and affordable textbooks and online educational materials. His websites are at www.sys-bio.org, www.sysbiobooks.com, www.sbolstandards.org, synbio.washington.edu, and www.futureskill.com.

Gregory Stephanopoulos, Ph.D., received his degrees in chemical engineering (B.S., NTU Athens; M.S., University of Florida; and Ph.D., University of Minnesota, 1978). He taught at Caltech (1978–1985), after which he was appointed professor of chemical engineering at MIT. He served as Associate Director of the Biotechnology Process Engineering Center (1990–1997) and is also the Taplin Professor of HST (2001–present), Instructor of Bioengineering at Harvard Medical School (1997–present), and the W. H. Dow Professor of Chemical Engineering and Biotechnology.

Professor Stephanopoulos' current research focuses on *metabolic engineering*, the engineering of microbes for the production of fuels and chemicals. He has co-authored or co-edited 5 books, more than 320 papers, and 35 patents and supervised more than 110 graduate and postdoctoral students. He is presently the Editor-in-Chief of *Metabolic Engineering* and *Current Opinion in Biotechnology* and serves on the editorial boards of seven scientific journals and the advisory boards of five chemical engineering departments. He has been recognized with numerous awards (Dreyfus, Excellence in Teaching-Caltech, AIChE Technical Achievement Award, PYI, AIChE-FPBE Division Award, M.J. Johnson Award of the American Chemical Society (ACS), Merck Award in Metabolic Engineering, the R.H. Wilhelm Award in Chemical Reaction Engineering of AIChE, and the Amgen Award in Biochemical Engineering). In 2002 he was elected to the AIChE Board of Directors, in 2003 to the National Academy of Engineering, and in 2005 was awarded an honorary doctorate degree (*doctor technices honoris causa*) by the Technical University of Denmark. In 2007 he won the C. Thom Award from SIM and the Founders Award from AIChE and in 2010 the ACS E. V. Murphree Award in Industrial and Engineering Chemistry and the George Washington Carver Award of BIO.

Professor Stephanopoulos has taught undergraduate and graduate courses of the core of chemical engineering and biotechnology at Caltech and MIT and co-authored the first textbook on metabolic engineering. He is presently directing a research group of approximately 25 researchers.

Christopher Voigt, Ph.D., is a synthetic biologist and an associate professor at UCSF. He holds a joint appointment as a Chemist Scientist at Lawrence Berkeley National Labs. He is an adjunct professor at the Korea Advanced Institute of Science and Technology and an Honorary Fellow at Imperial College. He has been honored as a Sloan Fellow, Pew Scholar, Packard Fellow, MIT TR35, NSF CAREER Award, and Vaughan Lecturer. He received his B.S.E. in chemical

engineering from the University of Michigan (1998) and Ph.D. in biochemistry/biophysics at the California Institute of Technology (2002). Dr. Voigt reprograms cells to do new things by changing their DNA. Part of his work is foundational, involving the construction of new genetic sensors, circuits, and actuators and the tools required to combine these parts to create more complex programs. To push the engineering, his lab has constructed “toy systems,” including strains of bacteria that can take a photograph, perform an edge detection algorithm, swim to different targets in response to an external signal, and perform complex calculations through intercellular communication. The new techniques developed using the toy systems are then applied to problems in biotechnology, including human therapeutics, green materials, and bioenergy.

Hans V. Westerhoff, Ph.D., received his Ph.D. *cum laude* from the University of Amsterdam on the topic mosaic nonequilibrium thermodynamics and (the control of) biological free-energy transduction (1983). After a brief postdoc at the University of Padua he became Visiting Scientist at the NIH (USA) to work on DNA supercoiling, regulation and control, and antimicrobial peptides. After 6 years at the Netherlands Cancer Institute he became full professor of microbial physiology at the VU University Amsterdam, and professor of mathematical biochemistry at the University of Amsterdam. From 2005 he also holds the AstraZeneca Chair for Systems Biology at the University of Manchester, where he is director of the Manchester Centre for Integrative Systems Biology and the Doctoral Training Centre Integrative Systems Biology. Also as Director of the biennial FEBS advanced lecture course Systems Biology he actively promotes systems biology. His research interests include integrated experimental and computational systems biology of microorganisms, systems biology of cancer, carbon and energy metabolism, regulation of cell function, the silicon human, systems biology, and multifactorial disease. His more than 500 publications, cited more than 10,000 times, include a monograph and multiple success stories in synthetic and systems biology.