14

THE SYNTHETIC APPROACH FOR REGULATORY AND METABOLIC CIRCUITS

Wilson W. Wong and James C. Liao

Chemical and Biomolecular Engineering Department, University of California, Box 951592, Los Angeles, California 90095

14.1 INTRODUCTION

14.1.1 Motivation for Synthetic Approach to Biology

The study of biology and gene regulation has traditionally been conducted using a reductionist approach, where a complex system of biomolecules is reduced to smaller units and each component is individually investigated. These smaller units, however, are always connected *in vivo* to form a network of interacting molecules, thus, the overall properties of the network are rarely the sum of its individual parts. Network connectivity and topology, as well as biochemical properties of individual components, are therefore required to completely describe the behaviors of an organism.

While the reductionist approach aims to determine the biochemical properties of each individual component, the systems approach focuses on elucidating network connectivity. Although these two complementary approaches hold significant promise for characterizing the behavior of biological systems, they often do not readily yield the design principles behind the complex networks. Due to millions years of evolution, existing intracellular networks are complicated by many auxiliary circuits that may

Systems Biology and Synthetic Biology Edited by Pengcheng Fu and Sven Panke

mask the basic design principle of the system. Therefore, to deduce fundamental principles by illuminating each component in the modern-day cell is as difficult as rediscovering fundamental laws of physics by disassembling an automobile. In an alternative approach, dubbed the synthetic approach, hypothetical operating principles are generated and then tested using artificially synthesized networks. The design approach may avoid second-order functions that are not important for the first principle. Furthermore, synthetic networks are not limited by natural biological systems, providing a wider range of test conditions.

The synthetic approach is initiated by educated creativity, much like the design of any engineering system. At this stage, the principle is inspired by physical and mathematical insights, but constrained by biological and chemical realities. A prototype mathematical model that serves as a conceptual blueprint is useful and often necessary. When such a prototype model is constructed, each component needs to be implemented by biological elements. The proper biological components such as promoters, regulators, enzymes, and metabolites are then identified to fulfill design specifications. Finally, the network is "reconstituted" inside the cell to test the properties of the system.

The creation of artificial systems also allows exploration of potential applications that are not displayed by natural design. An example of such application is the dynamic metabolic feedback loop [1] that addresses the fundamental challenge in metabolic engineering of maintaining a balance of the cell's resources, specifically between cell growth and metabolite production. In this circuit, a synthetic feedback controller was constructed in *Escherichia coli* that allows for gene expression of the key enzyme in lycopene production pathway to be under the control of a metabolite, acetyl-phosphate. When grown in glucose, *E. coli* produces acetate, a metabolic waste, when the tricarboxylic acid cycle (TCA) is no longer able to accommodate the incoming glycolytic flux. Acetate production also serves as an indication that the cells have sufficient energy and material resources and therefore represents a prime opportunity to shift cellular resources from cell growth toward the production of metabolites. When the level of acetate increases, its precursor, acetyl-phosphate, would also increase and activate the production of lycopene (Fig. 14-1).

The network synthesis approach is analogous to *in vitro* protein reconstitution commonly performed in the fields of biochemistry and molecular biology. Instead, the network is reconstituted *in vivo* by the selection of the proper genetic and metabolic components. A design flow diagram is illustrated in Figure 14-2. This approach has also been generalized to cell-free systems [2]. The design principle tested using the synthetic approach may or may not be used in real life. However, these principles provide focal points to search for similar designs in the cell and to examine the gap between theoretical prediction and biological reality.

14.1.2 Challenges in Synthetic Biological Circuit Design and Construction

The design of synthetic biological circuits shares many similarities with engineering constructions, but faces a major complication in the form of biological uncertainties. These uncertainties are manifested at two levels. First, the lack of detailed kinetic parameters for biological elements prevents the precise prediction of network

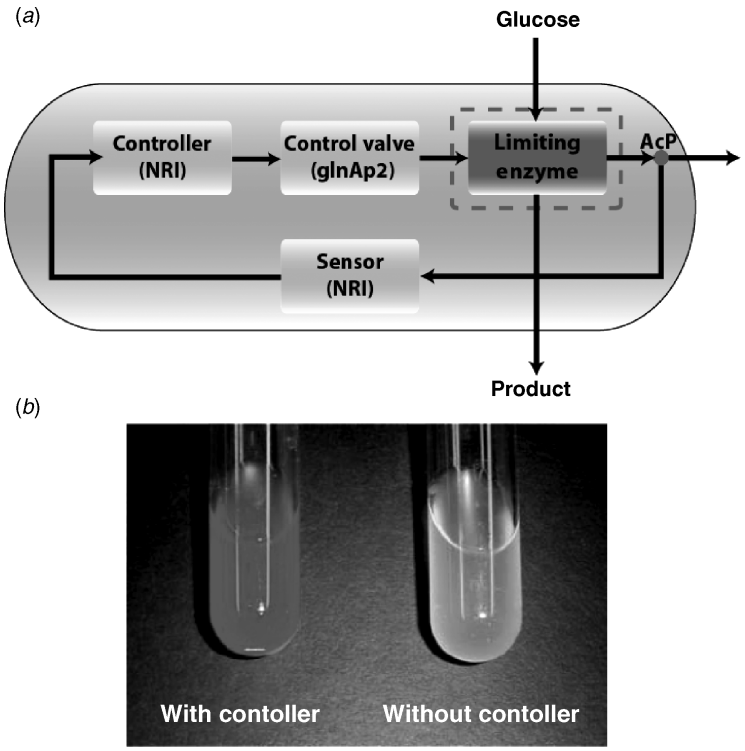

Figure 14-1 Dynamic metabolic feedback controller. (a) As cells grow on glucose, metabolic waste product, acetate, and it precursor acetyl-phosphate, accumulates. To divert cellular resources toward metabolite production when acetyl-phosphate accumulates, the limiting enzymes of the metabolic pathway are placed under the control of *glnA*p2 promoter. The *glnA*p2 promoter is activated by the phosphorylated form of NRI and acetyl-phosphate phosphorylates NRI in the absent of NRII [1]. (b) Production of lycopene, a reddish compound, with and without the controller.

behavior. Second, the interaction of these molecules with other cellular components is even less characterized, which causes additional difficulties. Constructions of synthetic circuits are therefore challenging and often iterative. Efforts have been made to expedite the construction process through combinatorial synthesis [3], directed evolution [4], and the creation of standardized biological parts [5]. Nevertheless, several synthetic circuits have already been demonstrated, which provide valuable insights into the design principles of biological networks [3,6–10]. Most of the recent synthetic circuits are reviewed in other chapters. The focus of this review is on oscillation, intercellular communication, and their interaction with metabolism.

14.2 BIOLOGICAL OSCILLATORS

Oscillation is a fascinating and an important phenomenon displayed by biological systems. Biological oscillators are ubiquitous circuits with a wide range of frequencies. They are found in numerous organisms, such as bacteria, plants, insects,

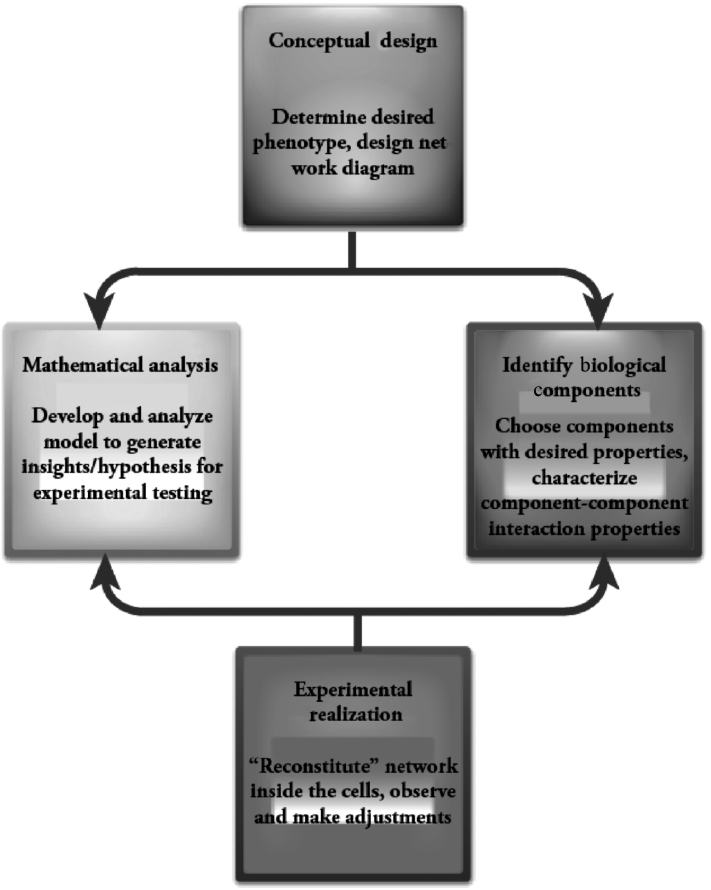

Figure 14-2 Process flow diagram for the design and construction of synthetic biological circuits. As with many engineering projects, the first step is to generate a conceptual design. Biological components are then identified and mathematical models are constructed. After analyzing the feasibility of the concept, the design is implemented inside the cells. Due to uncertainties associated with biological systems, implementation of synthetic circuits tends to be iterative. Efforts are being made to create standardized parts and infrastructures to reduce uncertainties [5].

and mammals. Oscillation also governs numerous vital processes such as global gene expression and cell cycle. Disruptions to circadian rhythm circuits, an oscillator with a 1-day period, have been demonstrated to cause sleep disorders and have also been linked to alcohol [11] and drug abuse [12,13] in mammals. Therefore, understandings of biological oscillators can have far-reaching medical and social implications.

Although many molecular details of biological oscillators have been determined in recent years, the network properties of these oscillators remain elusive. To gain further insight into the design principles of biological oscillators, three synthetic oscillators have been demonstrated so far, each of which is based on a conceptual idea that serves

as a design principle. The first two are isolated modules that do not interact with metabolism, but demonstrate the idea of oscillation at the transcriptional level. The third oscillator integrates gene expression with metabolism to drive the oscillatory circuit. Theoretical discussions of biological oscillation have been sufficiently reviewed by others [14,15].

14.2.1 Synthetic Oscillators

14.2.1.1 Three Mutually Repressible Transcription Factors and Promoters Form an Oscillator The first synthetic biological oscillator to be constructed is the ring oscillator, termed the repressilator, by Elowitz and Leibler [16]. This oscillator involves three mutually repressible promoters that regulate the expression of the three repressors. Repressor 1 inhibits the expression of repressor 2, and similarly repressor 2 inhibits the expression of repressor 3. Finally, repressor 3 inhibits the expression of repressor 1 to complete the circuit (Fig. 14-3a). The final construct used LacI as repressor 1, TetR as repressor 2, and λ cI as repressor 3. As with every oscillator design, not all parameters of the individual components will lead to oscillation. This is where mathematical analysis and intuition can be helpful. Through modeling, Elowitz and Leibler determined that components property such as tightly regulated promoters and shorter protein half-lives can improve the likelihood of oscillation. Hence, promoters were chosen to minimize "leakiness" when fully

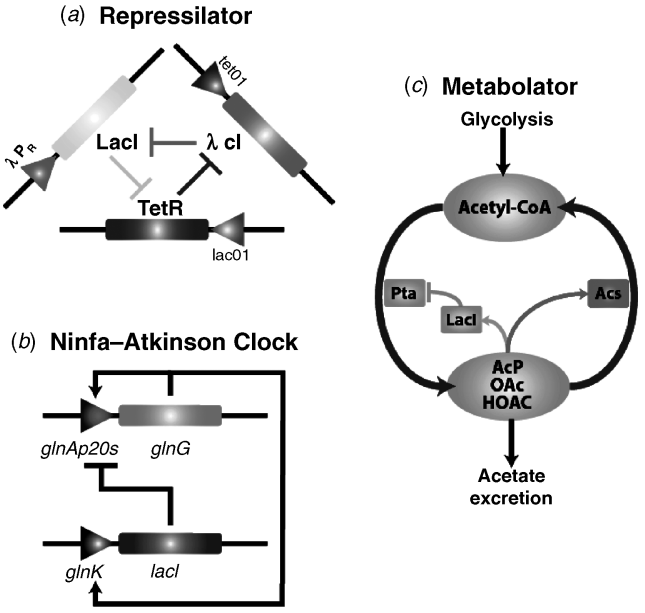

Figure 14-3 Schematic network diagram for (a) repressilator, (b) Ninfa–Atkinson clock, and (c) metabolator. See text for more detailed descriptions.

repressed and degradation peptide sequences were added to each repressor to reduce the half-life of the proteins. To observe the response of the repressilator, green fluorescence protein (*gfp*) was placed under the control of one of the promoters. This system was not designed to be synchronized and therefore the oscillation dynamics can only be observed at the single-cell level. The period of the repressilator is approximately 150 min with 40 percent of the cells exhibiting oscillation.

14.2.1.2 A Predator (lacI)–Prey (glnG) Pair of Regulators Exhibit Oscillation Atkinson et al. [17] later designed a synthetic oscillator (Fig. 14-3b) with a dramatically longer period of 10–20 h and the oscillation dynamics that can be observed in a continuous culture. The conceptual design is reminiscent of the one proposed by Barkai and Leibler [18], which is inspired from observations of naturally occurring oscillators. In this oscillator design, an activator enhances its own gene expression and the expression of another regulatory protein that inactivates the activator. Through simulation, this design consisting of a positive element coupled with the negative element was determined to be relatively noise resistant. The actual design created by Atkinson et al. is slightly different from the one proposed by Barkai and Leibler. This oscillator involves a positive regulator (NtrC) that activates its own expression and the expression of a repressor (LacI). Instead of antagonizing the activity of NtrC, LacI represses the gene expression of NtrC. The activator, NtrC, can be regarded as a prey that "feeds" into the predator LacI, which decreases the population of the prey. Again, the authors relied on both conceptual reasoning and mathematical modeling in their selection of the appropriate biological components. The repressor, LacI, was placed under the control of *glnK* promoter. The activator, NtrC, which is a part of a two-component system involved in the nitrogen starvation response, was placed under the control of a modified *glnA*p2 promoter that contains a LacI-binding site (Fig. 14-3b). Both *glnA*p2 and *glnK* promoters are positively activated by NtrC, but the *glnK* promoter requires higher levels of activated NtrC to be fully induced when compared to the *glnA*p2 promoter. The design by Atkinson et al. did not involve any degradation sequence and the experiments were performed in a continuous bioreactor under constant cell density condition (turbidostat). This oscillator amazingly displayed oscillation dynamics at the population level, even though the oscillation was dampened. The synchronization is probably due to the exposure of IPTG before the experiment, which sets all the cells to the same states. However, it is unclear how this circuit maintained synchronization throughout the experiment.

14.2.1.3 Two Interconverting Pools of Metabolites with Nonequilibrium Fluxes Display Oscillation Naturally occurring oscillators found in many organisms rarely operate independently from the rest of the cell's physiology. In fact, the oscillators are usually linked to the global regulation of gene expression and metabolism. As mentioned earlier, circadian rhythms can regulate alcohol and drug intake. Conversely, alcohol intake can also affect the function of the circadian rhythm [19–22]. This intimate relationship between intracellular oscillators and the environment is a critical property of natural oscillators, which allows the circuit to

sense and respond to environmental changes. The work by Fung et al. [23] mimicked this property by integrating genetic oscillators into the central metabolism of *E. coli*.

The integrated gene and metabolic oscillator by Fung et al., termed the metabolator, consists of a flux-carrying network with two interconverting metabolite pools (M1 and M2) catalyzed by two enzymes (E1 and E2), whose expressions are negatively and positively regulated by M2, respectively. In the first stage where the M2 level is low, E1 is expressed, while E2 is not. A high-input metabolic flux converts M1 to M2 rapidly. The accumulation of M2 represses E1 and upregulates E2. When the backward reaction rate exceeds the sum of the forward reaction rate and the output rate, M2 level decreases and M1 level increases. E1 is then expressed again and E2 is degraded, returning to the first stage. On the other hand, if the input flux is low, M2 will not accumulate quickly enough to cause a large swing in gene expression, and thus a stable steady state will be reached. This design allows metabolism to control gene expression cycles, a characteristic commonly seen in circadian regulation.

The experimental design is realized with the promoter *glnA*p2 controlling two genes, *lacI* and *acs* (encoding acetyl coenzyme A synthetase) (Fig. 14-3c). Phosphotransacetylase (Pta), a reversible enzyme that catalyzes the conversion of acetyl coenzyme A (AcCoA) to acetyl phosphate (AcP), is placed under the control of the *lacO1* promoter. The *lacO1* promoter is a synthetic promoter designed to reduce the leakiness of gene expression when repressed, while maintaining a large dynamic range of protein expression. To obtain readout of the circuit, a green fluorescence protein is placed under the control of another LacI repressible promoter. All proteins were fused to an *ssrA* degradation peptide to reduce their half-lives. The promoter *glnA*p2, in the absence of NRII (a bifunctional protein kinase/phosphatase regulation involved in the nitrogen starvation response), can be activated by AcP [1]. Here AcCoA corresponds to M1, AcP corresponds to M2, Pta corresponds to E1, and Acs corresponds to E2. When the AcP level is low, LacI and Acs expression levels are also low, and thus derepressing the *lacO1* promoter, which in turn increases the production of Pta. As Pta is being produced, it converts acetyl coenzyme A into AcP, which activates the *glnAp2* promoter and synthesizes Acs and LacI. Increasing the concentration of LacI represses the transcription rate of *pta*, hence lowering the level of AcP. Meanwhile, as the level of Acs increases, it converts more acetate into AcCoA. This removes the downstream product from the AcP degradation pathway and helps lower the level of AcP. One important aspect of this design is the interconversion of two metabolite pools, AcCoA and AcP, through two enzymes, Pta and Acs, which are controlled by the circuit. These two enzymes in turn, indirectly and directly, respond to AcP.

To gain more insight into the properties of the system, nonlinear differential equations and bifurcation analysis were employed to probe the dynamic properties of the system. The analysis predicts that oscillation will be favored when the metabolic influx is high. The metabolic flux-driven dynamics represents a very important feature of this design. An imbalance of metabolic fluxes destabilizes the steady states and leads to oscillation. This allows the metabolator to respond to the glycolytic influx. To test the prediction, the metabolator was cultured in different carbon sources that support different glycolytic rates. When grown in glucose, fructose, and mannose,

which support high-glycolytic flux, the metabolator exhibits oscillation. When grown in glycerol however, which yields a low-glycolytic flux, the metabolator did not exhibit oscillation. The experimental results therefore confirmed the mathematical predictions. The construction of the metabolator demonstrates that the two-pool network architecture produces oscillation with metabolic fluxes as the driving force for oscillation.

To successfully design and construct the metabolator, we began with an understanding of the interaction between gene regulation and metabolism. Then a conceptual idea of the network was conceived. With the conceptual framework, biological components were identified and the network was constructed using techniques from microbiology and molecular biology. Mathematical models were constructed with reasonable parameters based on the network connectivity and biological components of choice to identify parameter space that leads to oscillation. Modeling can be very helpful in identifying and exploring parameters that have significant impact on the performance of the system. Many pitfalls, however, can also be associated with modeling, such as choosing inaccurate range of parameters. Artifacts from the simulation can lead to surprising results that defy common sense. Therefore, one cannot blindly trust the results from modeling and the results generated from the model should make intuitive sense.

14.2.2 Circadian Circuits from Cyanobacteria Also Form Two Interconverting Pools

Interestingly, the circadian rhythm network structure found in cynaobacteria *Synechococcus elongatus* also possesses two interconverting pools similar to those described in the metabolator (Fig. 14-4). This circuit from *S. elongatus* is remarkably robust. A study at the single-cell level demonstrated that the oscillation is stable at the

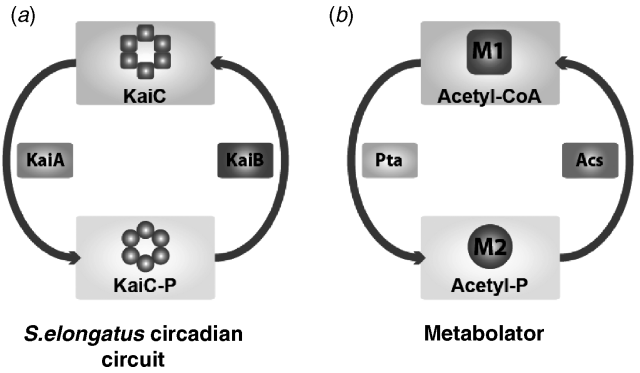

Figure 14-4 Similarities between the circadian circuit from *S. elongatus* and the metabolator. The overall structure of the metabolator is similar to the Kai system from *S. elongatus*. This structure might represent a fundamental motif for oscillation in bacteria. The major difference between the two systems is that the phosphorylated form of KaiC, KaiC-P, probably does not decrease the activity of KaiA. Moreover, KaiC-P can control gene expression at genomic scale in *S. elongatus*.

individual cell level without the need to synchronize with surrounding cells [24]. The central clock components in *S. elongatus* are coded by the genes *kaiA*, *kaiB*, and *kaiC* ("Kai" is a Japanese word that means cycle). KaiC represses its own expression, as well as KaiB, both of which are in the *kaiBC* operon. KaiA activates KaiB and KaiC expression [25]. The traditional model for generating a circadian rhythm is the transcription–translation oscillator (TTO) model. KaiC represses its own gene expression, and this negative feedback loop is the core oscillator component. KaiA sustains the oscillation by enhancing the expression of KaiC. Recent findings, however, report data that contradicts the TTO model. KaiC phosphorylation, rather than the transcription and translation of KaiC, seems to be the dominating factor in controlling oscillation dynamics [26]. Tomita et al. [27] directly tested the TTO model by growing *S. elongates* under constant darkness. In this condition, transcription terminates in *S. elongates*, but the phosphorylation state of KaiC continues to display circadian rhythmicity. More interestingly, in a subsequent report, KaiC phosphorylation was demonstrated to exhibit oscillation in a mixture that contained only purified KaiA, KaiB, KaiC, and ATP *in vitro* [28]. The current model of the Kai circuit is as follows. When KaiC is not phosphorylated, KaiA repeatedly and rapidly associates with KaiC to enhance the phosphorylation of KaiC. When KaiC phosphorylation reaches a sufficiently high level, its binding with KaiB is promoted, which in turn inactivates KaiA leading to its own dephosphorylation. When KaiC is dephosphorylated, KaiB dissociates from KaiC, which then activates KaiA to repeat the cycle [29].

14.2.3 Metabolism and Circadian Rhythm

Interactions between the circadian rhythm and metabolism in mammals are well documented in literature [30]. In mammals, the main "clock" that controls the rest of the peripheral systems is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. NPAS2 (or its homologue, Clock) and BMAL1 are the major regulators in circadian rhythm. They form a heterodimer (NPAS2:BMAL1 or Clock:BMAL1) in the nucleus. When this complex is activated, it binds to the DNA and expresses the clock genes *Cry* and *Per* and clock output genes such as lactate dehydrogenase *Ldh*. Rutter et al. [31] have shown that reduced nicotinamide adenine dinucleotide (NAD(P)H) can directly activate the NPAS2:BMAL1 complex with near switch-like response *in vitro*.

Aside from NADH, recent work [32,33] suggests that heme negatively regulates the activity of the NPAS2:BMAL1 complex in the presence of carbon monoxide (CO). The complex, in turn, activates the expression of a heme biosynthesis rate-limiting enzyme aminolevulinate synthase 1 (Alas1). As the level of Alas1 increases, the concentration of heme also increases. A sufficiently high level of heme will eventually induce its own degradation enzyme, called heme oxygenase, which generates CO as a final product. CO inhibits the activity of the NPAS2:BMAL1 complex and therefore, the expression of Alas1 as well. Without Alas1, the heme level eventually reaches a sufficiently low level to allow NPAS2:BMAL1 to become active again, thus continues the cycle.

Glucose has also been shown to interact with circadian gene expression in rat fibroblasts [34]. In transgenic mice, the disruption of BMAL1 and *Clock* upsets glucose homeostasis [35]. These mutant mice display altered diurnal variation of

plasma glucose and triglycerides, as well as glucose intolerance and insulin resistance with a high-fat diet. In another report, mice with a mutation in the *Clock* gene are obese and develop metabolic syndromes such as hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia [36,37]. These works once again demonstrate the intimate link between biological oscillation and metabolism. They also highlight the importance in elucidating the design principle of oscillation for both fundamental understanding and potential medical applications.

14.2.4 Oscillation Frequency and Responses

As demonstrated in electronic circuits, information can be encoded into the frequency of oscillation. Therefore, an oscillator with tunable frequency can be useful in encoding information, thus allowing more information content to be stored and transmitted with fewer signaling molecules. Examples of the frequency-dependent response in natural system have already been identified in calcium oscillation and NFκB oscillation [38–43]. A successful engineering application of encoding information into the frequency, however, entails another challenge of constructing a biological frequency decoder. Currently, it is not known how such decoding is achieved in biological systems.

14.3 CELL–CELL COMMUNICATION IN BACTERIA

Intercellular communication is of paramount importance to the development of higher organisms. Recently, numerous reports have also demonstrated the importance of cell–cell communication in bacterial physiology. Further insight into biological networks requires a better understanding of molecular details and system level analysis of intercellular communication. Using well-characterized model organisms such as *E. coli* and *S. cerevisiae,* one can construct synthetic intercellular circuits to decipher underlying principles, similar to the approach used in developing synthetic intracellular circuits.

14.3.1 Natural Cell–Cell Communication Systems

Bacteria have long been considered to be unicellular organisms that do not interact with other bacteria. This notion is mainly due to the way in which studies were performed using bacteria. Under most laboratory conditions, bacteria are grown in pure culture. In natural environment, however, bacteria rarely live alone in pure culture planktonic condition. Environmental biologists have long recognized the importance of bacterial communities to biogeochemical cycling that maintain the biosphere [44]. Biofilm is one example of a bacterial community. Biofilm is composed of single specie or multiple species. Such communities can live on biotic and abiotic surfaces and perform diverse metabolic functions.

To coordinate population behavior, intercellular communication is essential. In the 1970s, researchers had identified a communication system in *Vibrio fisheri* with

homoserine lactone as the signaling molecule termed autoinducer-1 (AI-1) [45]. *V. fischeri* is a bacterial symbiont that lives in the light-producing organ of the squid *Euprymna scolopes*. At low cell density conditions, *V. fisheri* produces small amounts of AI-1 proportional to growth. Once a threshold concentration of AI-1 is reached, it activates the *lux* operon, which encodes gene products that emits light, while producing more AI-1. Because this gene expression system was found to respond to density, or quorum, this system was termed "quorum" sensing. Since then, numerous examples of quorum sensing have been found in both Gram-positive and Gram-negative bacteria with different types of diffusible molecules and regulation mechanisms. Cellular functions that are under the control of quorum sensing include sporulation, biofilm formation, and virulence factor expression.

In higher organisms, cellular development relies heavily on the signaling cues generated by other cells. Complex spatial patterning has been shown to be the result of multiple intercellular signals coupled with feedbacks. One example is the development of left–right asymmetry in mouse embryos. The heart and other inner organs develop an asymmetrical arrangement during morphogenesis [46]. The expression and relay of TGF-β family signaling molecule, Nodal, has been found to be crucial in the symmetry breaking and differentiation of left–right organs. Nodal couples with extracellular cofactor EGF-CFC to form a positive feedback loop and substantiate its own existence. Moreover, Nodal also induces another intercellular signal called Lefty-2/antivin to form a negative feedback loop. In chicks, Nodal was found to induce a Cre-like molecule, Caronte, to relay the signal to more distal cells [47].

14.3.2 Synthetic Cell–Cell Communication Circuits

Equipping circuits with communication systems will greatly enhance capabilities of circuits, as demonstrated by the marriage of computers and the Internet. Analogously, capabilities of gene circuits can be greatly improved with an intercellular communication network. Engineering a communication system between cells will allow cellular programming at a population level rather than at the single cell level. Basu and Weiss utilized the quorum-sensing system from *V. fisheri* to create a spatiotemporal pulse generator [48] (Fig. 14-5a). This circuit contains sender cells that can produce AI and receiver cells that generate a pulse response. Using a similar concept, but with a different network configuration, Basu et al. created spatial patterns such as a "bulls eye" pattern on solid media (Fig. 14-5b) [49]. These two reports demonstrate that spatial and temporal patterns can be created in single cell organisms, mimicking a powerful capability commonly found in higher organisms.

You et al. [50] developed a circuit in *E. coli* that can sense and control its own cell density. This circuit produces AI continuously and accumulates AI in a cell-density-dependent manner (Fig. 14-6). As the AI level reaches a threshold, it activates a toxic gene, which leads to cell death. This circuit is a negative feedback loop that operates concertedly at the population level. The stability of AI is pH dependent, and therefore the steady-state cell density can be modulated by pH. As with any negative feedback loop, this system can potentially oscillate when operated in the proper parameter space. In this system, the degradation of AI is probably too slow for oscillation to occur.

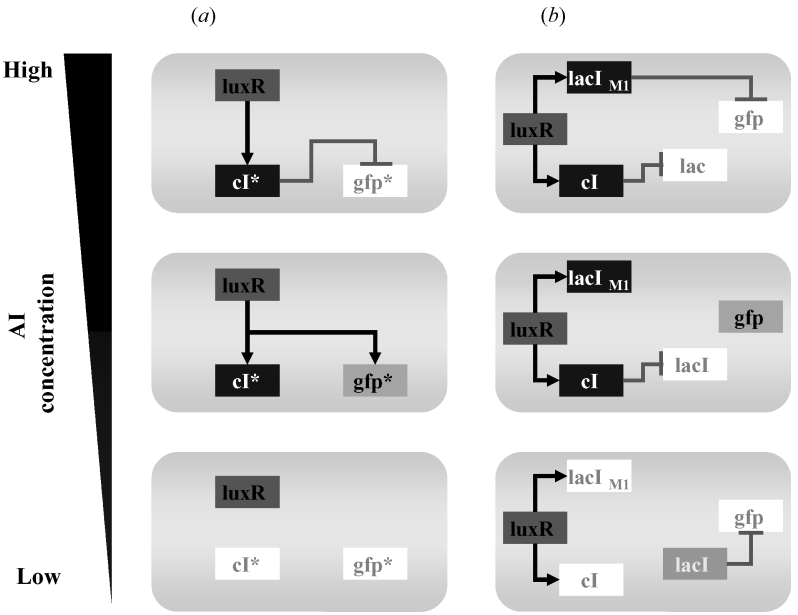

Figure 14-5 Intercellular communication network in *E. coli* based on the lux/AI system from *V. fisheri*. Network configuration for (a) the spatiotemporal pulse generator [48] and (b) the pattern formation [49]. The asterisk denotes that the protein half-life is reduced by the addition of a degradation sequence at the end of the protein. $lacI_{M1}$ is a codon modified mutant of lacI.

Figure 14-6 Schematic diagram of the population controller. AI is the diffusible autoinducer [50]. "Kill" is a gene that when expressed, can inhibit growth of *E. coli*. The "Kill" gene used in this network is *ccdB*, control cell death B, which interferes with DNA gyrase and causes cell death.

When grown in a microchemostat condition, however, the constant washing of the growth chamber facilitates AI removal, which allows the circuit to display cell-density oscillation [51].

14.3.2.1 Artificial Cell–Cell Communication System With only one channel of communication, the amount of information that can be transmitted is limited.

This limitation constrains the capability of all the networks within the system. To increase the information content, one solution is to generate oscillating signals and to encode the information into frequencies. Another solution is to create additional communication channels. The synthetic oscillators discussed in the earlier sections had the potential to generate oscillating intercellular signals. A separate frequency decoder circuit, however, will be needed to complete the scheme. Bulter et al. [52] addressed the later possibility by demonstrating the design and construction of an artificial communication system in *E. coli* using gene and metabolic network with acetate as the signaling molecule. Chen and Weiss later engineered an artificial communication system in *Saccharomyces cerevisiae* by incorporating *Arabidopsis thaliana* signal synthesis and receptor components into the host [53]. Using the *V. fisheri* AI-1 as the basis, Collins et al. created mutants of the transcription factor LuxR that are sensitive to different autoinducers. The design strategy identified here can also serve as a blueprint for further development of more independent communication channels.

An intercellular communication system can be divided into two modules—signal generation and signal detection. In designing a signal generation system, the signaling molecules must be chosen so that it is diffusible. The production of the signal must also be controlled. In designing the signal-detection module, the response to the signal must be specific and cannot be a general toxic or stress response. The detection system should also be sufficiently sensitive to detect the broad range of signal production and tunable.

14.3.2.2 Synthetic Communication System in E. coli In the artificial communication system in *E. coli*, acetate was chosen as the signaling molecule (Fig. 14-7a). Acetate is mainly produced from the *pta/ackA* pathway in *E. coli* [52]. Acetate is typically considered as a waste product during fermentation. When the TCA cycle cannot oxidize the carbon flux from glycolysis, Aceyl-CoA buildup is resulted. Since Acetyl-CoA is the entry point into the TCA cycle, the production of acetate through the *pta/ackA* pathway is intimately linked to the activity of the TCA cycle and the availability of oxygen. When the *pta/ackA* pathway is disrupted, however, a small amount of acetate, about 10 percent of the original level, is still produced through the biosynthesis of arginine and cysteine. This residual production of acetate, however, is no longer sensitive to oxygen levels.

The transport of acetate across the membrane is passive with the permeability of acetic acid across the membrane being three orders of magnitude higher than acetate, the negatively charged conjugate base of acetic acid. The dissociation equilibrium of acetic acid is pH dependent. Since the intracellular pH of *E. coli* is homeostatically regulated near pH 7.6, thus the intracellular acetate concentration depends on the ΔpH, intracellular pH minus extracellular pH, across the membrane. At high extracellular pH, the acid–base equilibrium shifts toward acetate. At low extracellular pH, the reverse is true. Therefore, less acetate is needed to activate the *glnAp2* promoter at low pH and to enhance the sensitivity of the *glnAp2* promoter to acetate. This unique property of weak acids/promoter interaction allows dynamic sensitivity tuning of the system. This tuning property is the opposite of AI. AI stability is lowered when pH decreases, therefore decreasing its sensitivity.

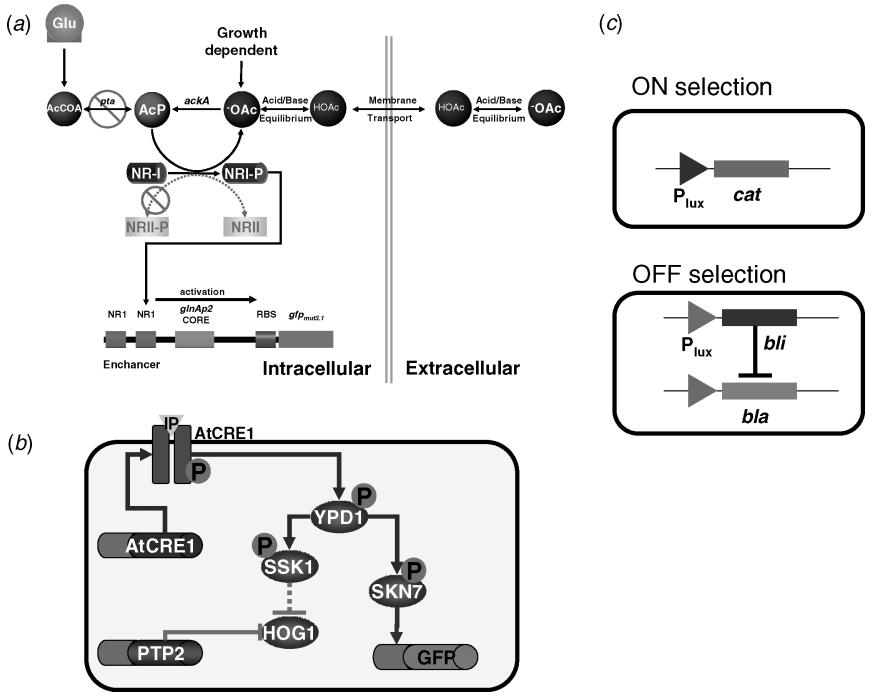

Figure 14-7 *Engineering intercellular-signaling systems.* (a) Artificial cell–cell communication in *E. coli* and (b) *S. cerevisiae*. In (a), the signaling molecule is acetate, a metabolite from the central metabolism. The detection system employs a nitrogen starvation response two-component system, Ntr, to sense acetate. NR-I is responsive to acetate in the absence of it cognate sensor, NR-II [52]. AcCoA, acetyl-CoA; AcP, acetyl-phosphate; OAc, acetate; HOAc, acetic acid. In (b), the signaling molecule and detection module is borrowed from the plant *Arabidopsis thaliana* [53]. For (c), the regulator luxR, which normally is sensitive to 3OC6HSL, is subjected to a dual selection scheme that selects for mutant sensitive to another autoinducer without significant cross talk from the original 3OC6HSL [54]. This selection involves an ON selection that select for luxR with expanded sensitivity to different autoinducer. The ON selection, however, does not eliminate the sensitivity to the original 3OC6HSL. Thus, the OFF selection is employed to select against the sensitivity of 3OC6HSL. *Cat* is a chloramphenicol resistant gene, *bla* is an amplicilin-resistant gene, and *bli* is a gene that inhibits the activity of *bla*.

For the detection module, the *glnAp2* promoter described in the construction of gene-metabolic oscillator was employed. The activity of the promoter was reported by using *gfp*. This circuit can exhibit cell density-dependent gene expression. The sensitivity of this behavior can be modulated by pH and also through promoter engineering. The NRI-P binding sequence was altered to manipulate the enhancer's strength. A stronger and a weaker binding sequence of NRI-P compared to the wild type had been identified and incorporated into the quorum sensing circuit. When performing the quorum sensing experiment with these new enhancer sequences, the strong enhancer requires less cell density to achieve the same level of gene expression as the wild type. Similarly, the wild type required less cell density to achieve the same level of gene expression as the weak enhancer.

Acetate is produced in a wide variety of organisms. Moreover, AcP is also a regulator molecule in many bacteria. Two-component systems such the Che, Pho, and Ntr had been shown to be capable of sensing AcP. Those two-component systems are present in many different bacteria as well. Many other bacteria are known to possess similar two-component systems, which can be modified to respond to AcP. These facts suggest that this cell–cell communication system can be universally adapted to other bacteria. Hence, the artificial system presented here also possesses the possibility for interspecies communication. This system can serve as a model system to understand how cell–cell communication that differs between different species can lead to various behaviors and phenotypes observed in nature.

14.3.2.3 Synthetic Communication System in Saccharomyces cerevisiae In the yeast communication system, cytokinin isopentenyladenine (IP) from the plant *Arabidopsis thaliana* was chosen [53] as the signaling molecule (Fig. 14-7b). IP can be generated by adenylate isopentenyl-transferases. To sense this signal, the cytokine receptor AtCRE1 from the same plant was chosen. This receptor can interact with yeast's endogenous phosphorylation-signaling pathway YPD1/SKN7 to activate gene expression. In the wild-type strain, YPD1/SKN7 is part of a phosphorylation pathway with SLN1, a cell–surface osmosensor hisdine kinase, and SSK1, an aspartate response regulator. Under normal condition, SLN1 phosphorylates YPD1, which in turn phosphorylates SSK1 and represses HOG1 activity. HOG1 activity is crucial for survival in high osmolarity conditions. In normal condition, however, HOG1 activity is lethal. The constant phosphorylation of YPD1 by SLN1 is therefore a problem because it will render the system insensitive to IP. Deletion of SLN1, however, results in activation of HOG1. To circumvent this problem, Chen and Weiss removed SLN1, but overexpressed an endogenous HOG1 phosphatase to decrease HOG1 activity. This artificial system exhibits quorum-sensing behavior when both the signal generation module and the receiver module are present in the same cell.

14.3.2.4 Engineering Specificity of Autoinducer Variants in E. coli Numerous quorum-sensing signals that are based on acyl-homoserine lactones have been identified in nature. These signaling molecules share homoserine lactones as the same core structure, but are differentiated by their unique side chains. The most well-studied acyl-homoserine lactone is 3OC6HSL from *Vibrio fisheri*. 3OC6HSL is synthesized by LuxI. When 3OC6HSL is bound to the transcription LuxR, it activates gene expression from the P_{lux} promoter. LuxR and its homologues had been shown to have crosswalk among different acyl-homoserine lactones. To utilize homoserine lactone variants as signaling molecules, Collins et al. employed a dual selection strategy to evolve and select for LuxR mutants from *V. fisheri* that are only specific to another acyl-homoserine lactone [54]. This dual selection strategy selected for LuxR variants that activate gene expression when different AIs are present, but remain inactive when the original AI is present (Fig. 14-7c).

The above examples represent different strategies for creating artificial communication networks. In the case with acetate, an endogenously produced metabolite is

converted to a signaling molecule. In the case for *S. cerevisiae*, however, the communication system was transplanted from another organism.

Moreover, naturally occurring signal sensors can be evolved to enhance specificity to other signaling molecules, thus creating more channels of communication system with less cross talk.

14.3.3 Information and Cell–Cell Communication

In cell–cell communication, gene expression is dependent on the concentration of small molecules, such as acetate, cytokines, or various forms of autoinducers from natural systems. It is important to note the difference between cell–cell communication system and other small molecule inducible promoters, such as the *lac* promoter with IPTG as the inducer. The difference between these systems lies in the information that the signaling molecule carries. Since cells are unable to produce IPTG, the operator of the experiment needs to add this inducer to the culture. The inducer carries only the desire of the operator to activate gene expression and nothing else. The level of the inducer does not reflect any information of the cells. In quorum sensing, the signaling molecules carry cell density information, thus encoding one aspect of the physiological state. Although the artificial cell–cell communication network is incorporated into a quorum sensing system, the use of primary metabolites allows encoding of metabolic states as well. When *pta* is disrupted, the production of acetate is proportional to growth. With intact *pta*, acetate can be used to encode the metabolic state of the central metabolism. The *pta* gene can also be transcriptionally fused to other promoters to transfer information sensed by the promoter to other cells.

14.3.4 Identifying Communication Molecules

Every endogenously produced diffusible small molecule has the potential to be a signaling molecule for cell–cell communication. How to differentiate a *bona fide* communication signal from other metabolites? Winzer et al. [55] contends that the key criterion for signaling molecules is that its ability to respond to the signal should extend beyond the needs to detoxify and metabolize the signals. The autoinducer-1 system seems to satisfy the criteria [55,56]. Whether another autoinducer system, AI-2, satisfies this criteria is less certain. AI-2 is proposed to be a universal signaling molecule because of its primary synthesis gene, *LuxS,* is present in many different organisms. AI-2 is produced from *S*-adenosylmethionine, SAM. SAM is used as a methyl donor to DNA, RNA, and other metabolites, giving *S*-adenosylhomocysteine (SAH). The subsequent step of metabolizing SAH is critical because SAH is a potent inhibitor for SAM-dependent methyltransferases and the cells need to regenerate their building blocks. An AI-2 uptake system, Lsr transporter, has also been discovered. The activation of this transporter is cyclic AMP (cAMP) dependent [57]. Hence, in the presence of glucose, AI-2 can accumulate to a high level whereas, with glycerol, AI-2 will be consumed. The reason why the cells would excrete and later internalize AI-2 remains unclear. Winzer et al. argues that the production of AI-2 is a metabolic side-product used to metabolize SAH, and later reuptake in a controlled catabolite

repression-dependent manner. This excretion and reuptake mechanism is very similar to acetate, our artificial signaling molecule. One major difference between the synthetic systems and the AI-2 system is the response to the signal. The acetate system in *E. coli* is purely artificial. The response to AI-2, although unknown, seems to be metabolic. Our work highlights the difficulties in defining a signaling molecule in natural systems. Before the disruption of NRII, acetate is generally considered as a metabolic waste product. After NRII is disrupted, acetate becomes a cell–cell communication signal. One can imagine a scenario where a metabolic waste product in one condition can become a signaling molecule in another condition. Whether a molecule is a signaling molecule can be condition dependent.

14.4 CONCLUSION

Some of the synthetic circuits constructed, especially the ones involve complex dynamics such as oscillation, perform poorly when compared to their naturally evolved counterparts. Such constructions highlight the major challenge in biological network engineering—dealing with biological uncertainties. Therefore, the construction of synthetic circuits can be tedious. Nonetheless, synthetic circuits have served as proof of concept and generated new insights. The shortcomings of these circuits also raise important issues regarding biological network design, such as those related to how fluctuation of the parameters in individual parts can affect the overall system's robustness. Many exciting works have been done recently to quantify the stochasticity, or noise, in gene expression [58–61] and the source of such stochasticity [62–66]. Based on a bioinformatics study, noise in gene expression seems to be minimized for essential genes, suggesting the importance of noise regulation in fitness enhancement [67]. Noise had also been demonstrated to play an important role in *E. coli pap* operon pilli expression [68,69], bacteriophage lytic decision [70], and HIV viral latency decision [71]. Incorporating these findings into the design of synthetic circuits will improve the performance of the system.

The first generation of circuits is designed to operate mainly at the transcription level in simple organisms such as *E. coli* and *S. cerevisiae*. However, life is rarely that one dimensional. Rather, it is organized and regulated at multiple levels. To engineer more complex behaviors, more layers of controls are needed to incorporate into synthetic circuits. Our group is focused on integrating metabolic and transcriptional regulation. Others have engineered control at the translation level by manipulating the three dimensional structure of mRNA and the interaction between ribosomes and mRNA [72–74]. Protein-signaling cascades can also be altered and manipulated by rewiring the input and output domains [75–77]. Borrowing from enzymes' powerful chemical synthetic capabilities, biosynthetic pathways have been engineered and rewired to improve the yield of high-valued compounds and to create completely new compounds [78–87]. Works have also been done to engineer circuits, such as toggle switches, in mammalian cells [88,89]. Coupled with the intercellular communication circuits described earlier, sophisticated networks that operate on a global level and involve multiple species that mimic

natural multicellular organisms can be created as a platform for understanding the emergence of complex behavior. More importantly, these systems can also be exploited for biotechnological and medical applications.

REFERENCES

1. Farmer WR, Liao JC. Improving lycopene production in *Escherichia coli* by engineering metabolic control. *Nat Biotechnol* 2000;18:533–537.
2. Noireaux V, Bar-Ziv R, Libchaber A. Principles of cell-free genetic circuit assembly. *Proc Natl Acad Sci USA* 2003;100:12672–12677.
3. Guet C, Elowitz MB, Hsing W, Leibler S. Combinatorial synthesis of genetic networks. *Science* 2002;296:1466–1470.
4. Yokobayashi Y, Weiss R, Arnold FH. Directed evolution of a genetic circuit. *Proc Natl Acad Sci USA* 2002;99:16587–16591.
5. Endy D. Foundations for engineering biology. *Nature* 2005;438:449–453.
6. Alon U. Biological networks: the tinkerer as an engineer. *Science* 2003;301:1866–1867.
7. Becskei A, Serrano L. Engineering stability in gene networks by autoregulation. *Nature* 2000;405:590–593.
8. Becskei A, Seraphin B, Serrano L. Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. *EMBO J* 2001;20:2528–2535.
9. Gardner TS, Cantor CR, Collins JJ. Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 2000;403:339–342.
10. Kobayashi H, Kaern M, Araki M, Chung K, Gardner TS, Cantor CR, Collins JJ. Programmable cells: interfacing natural and engineered gene networks. *Proc Natl Acad Sci USA* 2004;101:8414–8419.
11. Spanagel R, Pendyala G, Abarca C, Zghoul T, Sanchis-Segura C, Magnone MC, Lascorz J, Depner M, Holzberg D, Soyka M, et al. The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nat Med* 2005;11:35–42.
12. McClung CA, Sidiropoulou K, Vitaterna M, Takahashi JS, White FJ, Cooper DC, Nestler EJ. Regulation of dopaminergic transmission and cocaine reward by the Clock gene. *Proc Natl Acad Sci USA* 2005;102:9377–9381.
13. Abarca C, Albrecht U, Spanagel R. Cocaine sensitization and reward are under the influence of circadian genes and rhythm. *Proc Natl Acad Sci USA* 2002;99:9026–9030.
14. Goldbeter A. Biochemical Oscillations and Cellular Rhythms: The Molecular Bases of Periodic and Chaotic Behaviour. New York: Cambridge University Press, 1996.
15. Goldbeter A. Computational approaches to cellular rhythms. *Nature* 2002;420:238–245.
16. Elowitz MB, Leibler S. A synthetic oscillatory network of transcriptional regulators. *Nature* 2000;403:335–338.
17. Atkinson MR, Savageau MA, Myers JT, Ninfa AJ. Development of genetic circuitry exhibiting toggle switch or oscillatory behavior in *Escherichia coli*. *Cell* 2003;113:597–607.
18. Barkai N, Leibler S. Circadian clocks limited by noise. *Nature* 2000;403:267–268.

19. Spanagel R, Rosenwasser AM, Schumann G, Sarkar DK. Alcohol consumption and the body's biological clock. *Alcohol Clin Exp Res* 2005;29:1550–1557.
20. Rosenwasser AM. Alcohol, antidepressants, and circadian rhythms. Human and animal models. *Alcohol Res Health* 2001;25:126–135.
21. Wasielewski JA, Holloway FA. Alcohol's interactions with circadian rhythms. A focus on body temperature. *Alcohol Res Health* 2001;25:94–100.
22. Chen CP, Kuhn P, Advis JP, Sarkar DK. Chronic ethanol consumption impairs the circadian rhythm of pro-opiomelanocortin and period genes mRNA expression in the hypothalamus of the male rat. *J Neurochem* 2004;88:1547–1554.
23. Fung E, Wong WW, Suen JK, Bulter T, Lee SG, Liao JC. A synthetic gene-metabolic oscillator. *Nature* 2005;435:118–122.
24. Mihalcescu I, Hsing W, Leibler S. Resilient circadian oscillator revealed in individual cyanobacteria. *Nature* 2004;430:81–85.
25. Ishiura M, Kutsuna S, Aoki S, Iwasaki H, Andersson CR, Tanabe A, Golden SS, Johnson CH, Kondo T. Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. *Science* 1998;281:1519–1523.
26. Xu Y, Mori T, Johnson CH. Cyanobacterial circadian clockwork: roles of KaiA, KaiB and the kaiBC promoter in regulating KaiC. *EMBO J* 2003;22:2117–2126.
27. Tomita J, Nakajima M, Kondo T, Iwasaki H. No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science* 2005;307:251–254.
28. Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, Oyama T, Kondo T. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation *in vitro*. *Science* 2005;308:414–415.
29. Kageyama H, Nishiwaki T, Nakajima M, Iwasaki H, Oyama T, Kondo T. Cyanobacterial circadian pacemaker: Kai protein complex dynamics in the KaiC phosphorylation cycle *in vitro*. *Mol Cell* 2006;23:161–171.
30. Rutter J, Reick M, McKnight SL. Metabolism and the control of circadian rhythms. *Annu Rev Biochem* 2002;71:307–331.
31. Rutter J, Reick M, Wu LC, McKnight SL. Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* 2001;293:510–514.
32. Kaasik K, Lee CC. Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. *Nature* 2004;430:467–471.
33. Reick M, Garcia JA, Dudley C, McKnight SL. NPAS2: an analog of clock operative in the mammalian forebrain. *Science* 2001;293:506–509.
34. Hirota T, Okano T, Kokame K, Shirotani-Ikejima H, Miyata T, Fukada Y. Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. *J Biol Chem* 2002;277:44244–44251.
35. Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB, Fitzgerald GA. BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol* 2004;2:e377.
36. Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR,et al. AT Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* 2005;308:1043–1045.
37. Staels B. When the Clock stops ticking, metabolic syndrome explodes. (Discussion 55.) *Nat Med* 2006;12:54–55.

38. Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 1998;392:933–936.
39. Allen GJ, Chu SP, Harrington CL, Schumacher K, Hoffmann T, Tang YY, Grill E, Schroeder JI. A defined range of guard cell calcium oscillation parameters encodes stomatal movements. *Nature* 2001;411:1053–1057.
40. Nelson DE, Ihekwaba AE, Elliott M, Johnson JR, Gibney CA, Foreman BE, Nelson G, See V, Horton CA, Spiller DG,et al. Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science* 2004;306:704–708.
41. Hu Q, Deshpande S, Irani K, Ziegelstein RC. [$Ca^{(2+)}$](i) oscillation frequency regulates agonist-stimulated NF-kappaB transcriptional activity. *J Biol Chem* 1999;274: 33995–33998.
42. Lewis RS. Calcium oscillations in T-cells: mechanisms and consequences for gene expression. *Biochem Soc Trans* 2003;31:925–929.
43. Li W, Llopis J, Whitney M, Zlokarnik G, Tsien RY. Cell-permeant caged InsP3 ester shows that Ca^{2+} spike frequency can optimize gene expression. *Nature* 1998;392:936–941.
44. Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* 2000;64:847–867.
45. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* 2005;21:319–346.
46. Gaio U, Schweickert A, Fischer A, Garratt AN, Muller T, Ozcelik C, Lankes W, Strehle M, Britsch S, Blum M,et al. A role of the cryptic gene in the correct establishment of the left–right axis. *Curr Biol* 1999;9:1339–1342.
47. Freeman M. Feedback control of intercellular signalling in development. *Nature* 2000;408:313–319.
48. Basu S, Mehreja R, Thiberge S, Chen MT, Weiss R. Spatiotemporal control of gene expression with pulse-generating networks. *Proc Natl Acad Sci USA* 2004;101:6355–6360.
49. Basu S, Gerchman Y, Collins CH, Arnold FH, Weiss R. A synthetic multicellular system for programmed pattern formation. *Nature* 2005;434:1130–1134.
50. You L, Cox RS, 3rd, Weiss R, Arnold FH. Programmed population control by cell–cell communication and regulated killing. *Nature* 2004;428:868–871.
51. Balagadde FK, You L, Hansen CL, Arnold FH, Quake SR. Long-term monitoring of bacteria undergoing programmed population control in a microchemostat. *Science* 2005;309:137–140.
52. Bulter T, Lee SG, Wong WW, Fung E, Connor MR, Liao JC. Design of artificial cell-cell communication using gene and metabolic networks. *Proc Natl Acad Sci USA* 2004;101:2299–2304.
53. Chen MT, Weiss R. Artificial cell-cell communication in yeast *Saccharomyces cerevisiae* using signaling elements from *Arabidopsis thaliana*. *Nat Biotechnol* 2005;23:1551–1555.
54. Collins CH, Leadbetter JR, Arnold FH. Dual selection enhances the signaling specificity of a variant of the quorum-sensing transcriptional activator LuxR. *Nat Biotechnol* 2006;24:708–712.
55. Winzer K, Hardie KR, Williams P. Bacterial cell-to-cell communication: sorry, can't talk now—gone to lunch! *Curr Opin Microbiol* 2002;5:216–222.
56. Xavier KB, Bassler BL LuxS quorum sensing: more than just a numbers game. *Curr Opin Microbiol* 2003;6:191–197.

57. Xavier KB, Bassler BL. Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. *J Bacteriol* 2005;187:238–248.

58. Elowitz MB, Levine AJ, Siggia ED, Swain PS. Stochastic gene expression in a single cell. *Science* 2002;297:1183–1186.

59. Raser JM, O'Shea EK. Control of stochasticity in eukaryotic gene expression. *Science* 2004;304:1811–1814.

60. Ozbudak EM, Thattai M, Kurtser I, Grossman AD, van Oudenaarden A. Regulation of noise in the expression of a single gene. *Nat Genet* 2002;31:69–73.

61. Pedraza JM, van Oudenaarden A. Noise propagation in gene networks. *Science* 2005;307:1965–1969.

62. Becskei A, Kaufmann BB, van Oudenaarden A. Contributions of low molecule number and chromosomal positioning to stochastic gene expression. *Nat Genet* 2005;37:937–944.

63. Rosenfeld N, Young JW, Alon U, Swain PS, Elowitz MB. Gene regulation at the single-cell level. *Science* 2005;307:1962–1965.

64. Volfson D, Marciniak J, Blake WJ, Ostroff N, Tsimring LS, Hasty J. Origins of extrinsic variability in eukaryotic gene expression. *Nature* 2006;439:861–864.

65. Austin DW, Allen MS, McCollum JM, Dar RD, Wilgus JR, Sayler GS, Samatova NF, Cox CD, Simpson ML. Gene network shaping of inherent noise spectra. *Nature* 2006;439:608–611.

66. Colman-Lerner A, Gordon A, Serra E, Chin T, Resnekov O, Endy D, Pesce CG, Brent R. Regulated cell-to-cell variation in a cell-fate decision system. *Nature* 2005;437:699–706.

67. Fraser HB, Hirsh AE, Giaever G, Kumm J, Eisen MB. Noise minimization in eukaryotic gene expression. *PLoS Biol* 2004;2:e137.

68. Zhou B, Beckwith D, Jarboe LR, Liao JC. Markov Chain modeling of pyelonephritis-associated pili expression in uropathogenic *Escherichia coli*. *Biophys J* 2005;88:2541–2553.

69. Jarboe LR, Beckwith D, Liao JC. Stochastic modeling of the phase-variable pap operon regulation in uropathogenic *Escherichia coli*. *Biotechnol Bioeng* 2004;88:189–203.

70. Arkin A, Ross J, McAdams HH. Stochastic kinetic analysis of developmental pathway bifurcation in phage lambda-infected *Escherichia coli* cells. *Genetics* 1998;149:1633–1648.

71. Weinberger LS, Burnett JC, Toettcher JE, Arkin AP, Schaffer DV. Stochastic gene expression in a lentiviral positive-feedback loop: HIV-1 Tat fluctuations drive phenotypic diversity. *Cell* 2005;122:169–182.

72. Pfleger BF, Pitera DJ, Smolke CD, Keasling JD. Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. *Nat Biotechnol* 2006;24:1027–1032.

73. Bayer TS, Smolke CD. Programmable ligand-controlled riboregulators of eukaryotic gene expression. *Nat Biotechnol* 2005;23:337–343.

74. Isaacs FJ, Dwyer DJ, Ding C, Pervouchine DD, Cantor CR, Collins JJ. Engineered riboregulators enable post-transcriptional control of gene expression. *Nat Biotechnol* 2004;22:841–847.

75. Bhattacharyya RP, Remenyi A, Yeh BJ, Lim WA. Domains, motifs, and scaffolds: the role of modular interactions in the evolution and wiring of cell signaling circuits. *Annu Rev Biochem* 2006;75:655–680.

76. Dueber JE, Yeh BJ, Chak K, Lim WA. Reprogramming control of an allosteric signaling switch through modular recombination. *Science* 2003;301:1904–1908.
77. Park SH, Zarrinpar A, Lim WA. Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. *Science* 2003;299:1061–1064.
78. Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J,et al. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 2006;440:940–943.
79. Yoshikuni Y, Ferrin TE, Keasling JD. Designed divergent evolution of enzyme function. *Nature* 2006;440:1078–1082.
80. Lee TS, Khosla C, Tang Y. Engineered biosynthesis of aklanonic acid analogues. *J Am Chem Soc* 2005;127:12254–12262.
81. Tang Y, Lee TS, Khosla C. Engineered biosynthesis of regioselectively modified aromatic polyketides using bimodular polyketide synthases. *PLoS Biol* 2004;2:E31.
82. Schmidt-Dannert C. Engineering novel carotenoids in microorganisms. *Curr Opin Biotechnol* 2000;11:255–261.
83. Schmidt-Dannert C, Umeno D, Arnold FH. Molecular breeding of carotenoid biosynthetic pathways. *Nat Biotechnol* 2000;18:750–753.
84. Achkar J, Xian M, Zhao H, Frost JW. Biosynthesis of phloroglucinol. *J Am Chem Soc* 2005;127:5332–5333.
85. Guo J, Frost JW. Biosynthesis of 1-deoxy-1-imino-D-erythrose 4-phosphate: a defining metabolite in the aminoshikimate pathway. *J Am Chem Soc* 2002;124:528–529.
86. Wang C, Oh MK, Liao JC. Directed evolution of metabolically engineered *Escherichia coli* for carotenoid production. *Biotechnol Prog* 2000;16:922–926.
87. Wang CW, Oh MK, Liao JC. Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*. *Biotechnol Bioeng* 1999;62:235–241.
88. Kramer BP, Fussenegger M. Hysteresis in a synthetic mammalian gene network. *Proc Natl Acad Sci USA* 2005;102:9517–9522.
89. Kramer BP, Viretta AU, Daoud-El-Baba M, Aubel D, Weber W, Fussenegger M. An engineered epigenetic transgene switch in mammalian cells. *Nat Biotechnol* 2004;22:867–870.