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Review

Genetic Circuit-Assisted Smart Microbial Engineering

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Rapid advances in DNA synthesis, genetic manipulation, and biosensors have greatly improved the ability to engineer microorganisms with complex functions. By accurately integrating quality biosensors and complex genetic circuits, recently emerged smart microorganisms have enabled exciting opportunities for dissecting complex signaling networks and making responses without artificial intervention. However, because of the lack of design principles, developing such smart microorganisms remains challenging. In this review, we propose the concept of smart microbial engineering (SME) and describe the general features of basic SME, including the circuit architecture, components, and design process. We also summarize the latest SME achievements, remaining challenges, and potential solutions in this growing field.

Highlights

SME is a technology trend endowing strains with self-adaptive and decision-making ability.

SME can facilitate the advance of intelligent bioproduction, bioremediation, smart materials, and precision medicine.

Recent advances in genetic circuit engineering and biosensor engineering greatly expand the toolbox for SME; however, many challenges still need to be considered and addressed in the design and implementation of SME.

Emergence of Smart Microorganisms

Synthetic biology (see Glossary) aims to solve industrial and clinical problems through the rational addition of functionality in living systems [1]. Despite the large collection of well-defined synthetic biology tools [2], reprogramming cells with predictable biological function remains challenging because of the existence of evolutionarily conserved and hard-wired cellular regulatory networks [3]. As a result, low intelligence in microbial engineering remains problematic. For example, conventional gene expression technologies depend largely on the external supplementation of artificial inducers (e.g., isopropyl-β-D-thiogalactoside (IPTG) and anhydrotetracycline). This has forced bioengineers to add multiple types of costly inducer during the logarithmic phase of fermentation and has increased the difficulty of implementing **layered genetic circuits** [4,5]. Moreover, when engineered biosynthetic pathways involve toxic metabolite accumulation or feedback inhibition, it is difficult for this static strategy to sense stochastic perturbations and adjust the unfavorable metabolic states to the designed states to improve cellular fitness and productivity [6].

A possible solution to these challenges is to provide microorganisms with autonomous decision-making ability by exploiting ingeniously designed genetic circuits (Figure 1). These genetic circuit-assisted microorganisms can autonomously sense and make adjustments in response to the changing external environment or cellular state without artificial intervention, such as inducer addition [7], sampling monitoring, and high-throughput screening [8]. Compared with conventional microbial engineering, the states of these systems are tightly controlled by the concentration of signal molecules, and system behaviors may change over time or be determined by a specific signal threshold [9]. These intelligent designs can ameliorate cellular fitness and productivity by dynamically decoupling cell growth and production, strain self-directed evolution, compensating for the cellular burden, and diminishing phenotypic heterogeneity. Moreover, they can provide innovative applications, including the record of transiently formed disease-related biomarkers [10] and precise release of therapeutic compounds at desired locations [11] that cannot be accomplished by traditional methods (Table 1).

Here, the strategic design and implementation of smart microorganisms is termed smart microbial engineering (SME) and is a potential future direction of microbial engineering that is discussed in this review. SME is a highly interdisciplinary field that interfaces with component characterization, circuit construction, and systems implementation. In this review, we first map out some general features of these sensor-regulator systems. Next, recent advances in SME tools and strategies, together with exciting application examples, are summarized. Finally, we discuss the current challenges in SME construction and their potential solutions.

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General Features of a Basic SME Design

For conceptual simplicity, the desired behavior of a genetic circuit-assisted SME can be regarded as a composite set of logic operations in which the input and output modules are connected by appropriate logical relationships [12]. Therefore, it is critical to understand some general features of a basic SME design.

Circuit Architecture

To clearly illustrate the architecture of a basic SME design we can perform a bottom-up dissection of an SME into a hierarchy of parts, modules, subsystems, and systems. In this abstract framework, the parts are elementary functional building blocks such as promoters or transcription factors. They can form different modularized modules such as DNA-binding protein-based inducible systems [6], RNAi devices, and two-component systems [13]. Multiple modules can form a subsystem with an appropriate logic gate to perform defined functions such as a sensing subsystem for communication [14], processing subsystem with signal processing, and output subsystem with **actuator** element [15]. By coupling the above subsystems, the final SME system can be constructed for a specific function [16].

Circuit Components

The 'subsystem' plays a vital role in the design of an SME. There are three common subsystems used in the basic SME architecture.

(i) Sensing subsystem. A wide range of nucleic acid- or protein-based **biosensor**s have been identified and employed to construct sensing subsystems, including riboswitches [8], promoters [17], transcription factors [18], and two-component systems [19]. Biosensors can respond to signals from different sources such as environmental signals, including oxygen [20], pH [8], temperature [21], and light [2,22]; extracellular signals such as biomarkers [23], quorum sensing (QS) molecules [6], and environmental pollutants [24,25]; intracellular signals such as intermediate metabolites [9], substrates, or products [26], and the cellular physiological state [7] (Table 1).

(ii) Processing subsystem. To create sophisticated cellular behaviors, the converted information from upstream is further processed by a processing subsystem in a logical manner before an output decision is made. To date, numerous Boolean logic gates have been built by allosteric conformational changes or split protein associations [27,28]. Other complex logic gates [15,27], including 'AND', 'NAND', 'OR', and 'NOR', have been employed for multilayered logic processing, such as amplifiers [18,29], **toggle switches** [30], oscillators [31], and feedback/feedforward loops [32]. Frequently used tools for constructing these multilayered processing subsystems include recombinase, which is capable of engineering DNA inversion or excision [33], **CRISPRi**/a, which is capable of controlling gene expression levels without editing the DNA [34], toehold switches [28], or small transcription-activating RNAs [35], which are capable of controlling gene expression levels at the RNA level, and proteases that are capable of splitting or degrading target proteins [36].

(iii) Output subsystem. According to a decision from the upstream processing subsystem, the actuator accordingly dictates the output of system parameters, such as modulation of gene expression and enzyme proteolysis [37], which ultimately lead to the desired biological functions and phenotypes such as cell growth regulation, cell fate, morphology, or motility change.

Notably, only the sensing subsystem and output subsystem are essential components of an SME design. However, to develop more complex genetic circuits with sophisticated functionalities, a processing subsystem is necessary for processing multiple signals [26].

Design Process

To accomplish SME, the design process can be summarized in three steps:

Glossary

Actuator: a biological part or device that generates an output (e.g., reporters, motility, death, or antibiotic production).
Bandpass circuit: a gene regulatory filter that responds to a specific range of signal input (enzymes and inducer molecules).
Biosensor: a protein and/or nucleic acid device able to recognize a signal and induce a cellular response.
CRISPRi: an engineered CRISPR system, recruiting a deactivated Cas9 protein and a customizable guide-RNA to a specific coding sequence for blocking transcription.
Layered genetic circuit: a complex genetic circuit that integrates multiple regulation units to create output in a logical manner.
Memory circuit: an artificial genetic circuit that alters the system behavior on transient exposure to a signal. It will continue to exhibit the altered behavior even when the signal input is over.
Metabolic burden: the molecular load placed on an organism, typically caused by the expression of exogenous genes.
Metabolic engineering: a rewiring of the metabolic network of an organism, using genetic modifications, to optimally produce target metabolites and/or decrease undesirable products.
Model strains: typical strains that have been extensively used for research or industrial applications, such as *E. coli* and *Saccharomyces cerevisiae*.
Ribosome binding site (RBS): a sequence of nucleotides upstream of the start codon of an mRNA transcript that is responsible for the recruitment of a ribosome during the initiation of protein translation.
Synthetic biology: a rational and systematic construction of new biological machineries or redesign of the existing biological systems via genetic modifications for specific needs or products.
Toggle switch: a bistable gene regulatory network.

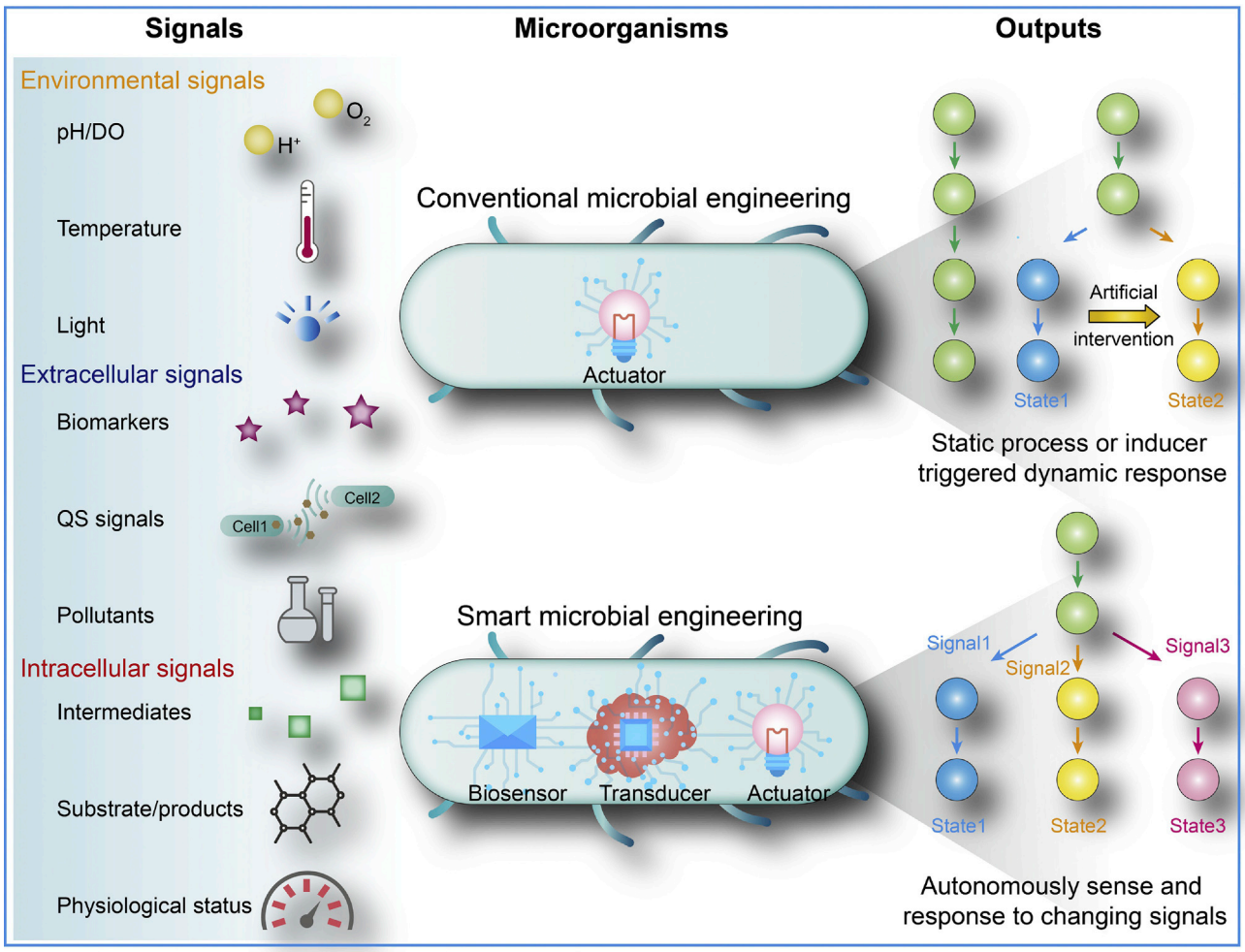


Figure 1. Schematic of Smart Microbial Engineering (SME) and Conventional Microbial Engineering.
Conventional microbial engineering relies on the multivariate optimization of strains to obtain the desired phenotype, which is a static or partially dynamic process driven by exogenously introducing artificial inducers. However, a 'smart' cell must be capable of sensing different signals (including environmental, extracellular, and intracellular signals) and adjusting to different states according to their biosensor and processing modules. Abbreviation: QS, quorum sensing; DO, dissolved oxygen.

Step 1: Top-down decomposition. A complex circuit may be subjected to top-down decomposition into simpler functional subsystems. Some of these subsystems may include a recombinase subsystem that can store memory or cause an irreversible biological function [38], **bandpass circuit** subsystem that can selectively respond to specific signals with relatively narrow input ranges [39], and bistable switch subsystem that confers the ability to alter and retain states [40].

Step 2: Bottom-up assembly. After system design and decomposition, the next critical step involves choosing components and parts assembly. To equip microorganisms with intelligent regulation networks, specific signal–sensor pairs for customized issues are urgently needed. These components can be obtained by either genomic mining with high-throughput screening or rational design/random mutation on current signal–sensor pairs [41]. Moreover, when developing higher level intelligent control, the difficulty of artificial

Table 1. Examples and Applications of SME

Design purpose	Strategies			Refs
	Sensing	Processing	Output and effect	
Population-based dynamic decoupling of bacterial growth and product synthesis	AHL	P_{esaS} promoter will be activated by EsaRI70V regulator and repressed by AHL	5.5-fold titer improvement of myo-inositol; unmeasurable to >0.8 g/l glucaric acid; unmeasurable to >0.1 g/l shikimate	[6]
Layered dynamic regulation for improving metabolic pathway productivity	AHL and *myo*-inositol	*Myo*-inositol-responsive time delay switch and QS circuit	Highest glucaric acid titer (2 g/l) in *E. coli* K12 strain	[9]
Rapid evolution of strains to tolerate multiple organic acids	pH	pH-riboswitch controlled recombinase expression to close mutagenesis	Turn off mutagenesis and enrich acid-tolerant phenotype	[8]
Feedback regulated evolution of phenotype system for strains' productivity improvement	Tyrosine	A tyrosine-responsive TF TyrR was used to regulate expression of the mutator mutD5	5-fold titer improvement of tyrosine	[32]
Automatically compensating cellular burden to improved the host cell fitness	Burden	A burden-responsive P_{htpG1} promoter controlled CRISPRi-based feedback system to regulate construct expression	Tracking cellular burden and reducing burden to improve total protein production	[63]
Population quality control to eliminate heterogeneity	Free fatty acid	Product signal-responsive promoter P_{AR} was used to control the expression of growth-dependent gene	Eliminate low-performing cells and enriching high-performing cells to improve the ensemble production	[67]
Self-assembling mercury-sequestration system for bioremediation	Mercury	A mercury-responsive TF MerR was used to regulate expression of the curli to sequestrate mercury	4.5-fold improvement of mercury bio-sorption capacity	[24]
Self-assembled materials via cell–cell communication	AHL	A QS circuit was used to control the secreting of $CsgA_{His}$ monomer	The composition and structure of materials varied dynamically and autonomously over culture time	[70]
Long-term live diagnostics of inflammation	Tetrathionate	A tetrathionate-responsive P_{ttrBCA} promoter controlled CI/Cro state switch	Engineered diagnostic strain can retain memory of tetrathionate exposure and be a living diagnostic in the gut of mice over 6 months	[10]
Reprogramming microbes to be pathogen-seeking killers	AHL	A *P. aeruginosa* AHL-responsive P_{LasI} promoter controlled gene expression for strain motility and killing	The killing activity against planktonic and mature biofilm *P. aeruginosa* was significantly improved	[77]

circuit design exponentially increases with the complexity of synthetic genetic circuits. A promising strategy to shorten the period of SME from design to optimization is *in silico* modeling of circuit functions [27]. After completing the design, the circuit components must be packaged into vector scaffolds. Recently, new DNA-assembly tools, including the MoClo system [42], GoldenBraid [43], MODAL [44], and PaperClip [45], have been developed [46,47]. These assembly methods can streamline the assembly workflow, create reusable modular DNA parts, and free up space for tuning and debugging gene circuits [48].

Step 3: Iterative optimization. After finishing the conceptual design of genetic construction, the target behavior can be quantitatively tested to evaluate user-defined input and time-dependent output behavior. However, most of these initial design systems may perform imperfectly and may require iterative adjustment. The range of behaviors available to the

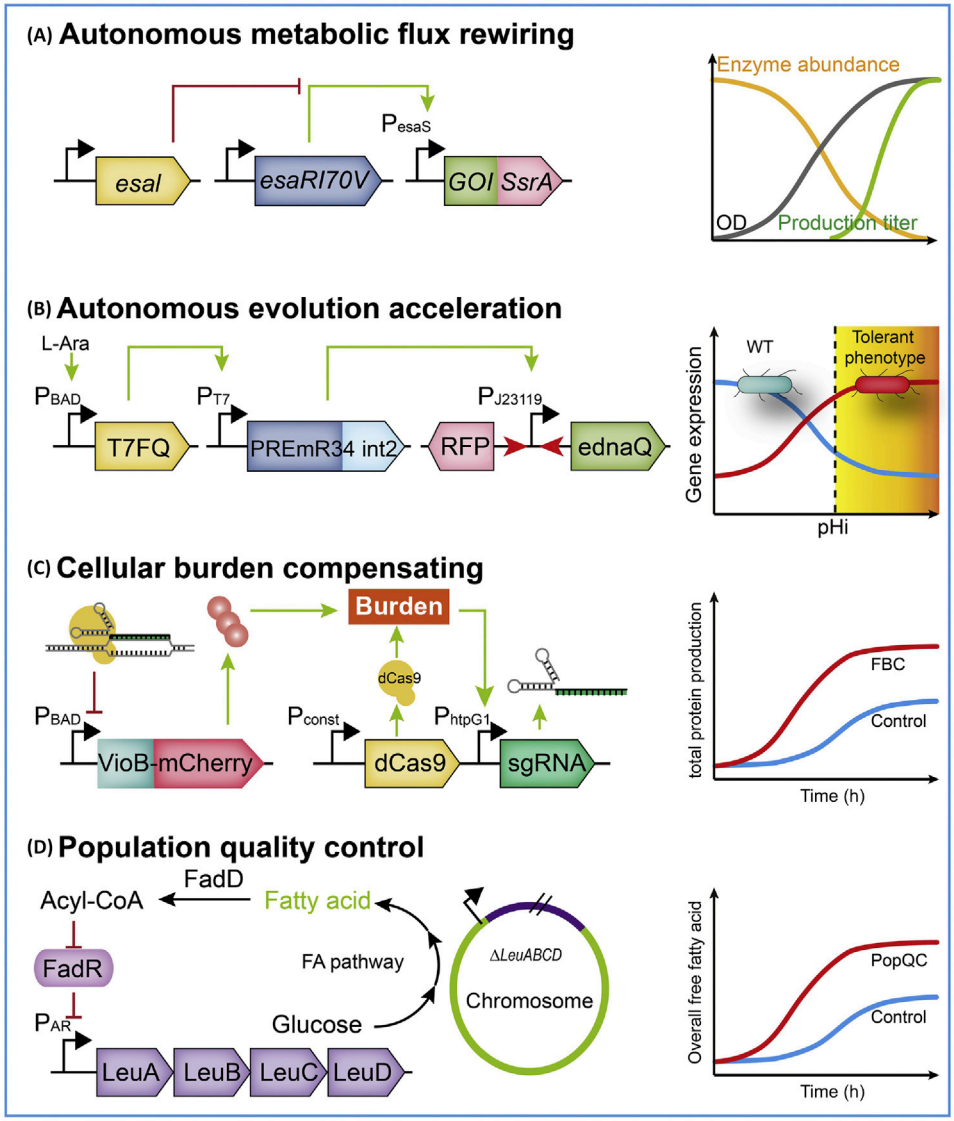


Figure 2. Smart Microbial Engineering (SME) Applications in Industrial Biotechnology.

(A) Autonomous metabolic flux rewiring system [6]. Left: expression of gene of interest (GOI) under P_{esaS} promoter will be activated by EsaRI70V regulator and repressed by acyl homoserine lactone (AHL) produced by enzyme EsaI. Right: enzyme abundance can be dynamically downregulated to decouple cell growth and product synthesis. SsrA, protein degradation tag. (B) RiDE system [8]. Left: expression of integrase *int2* which catalyzes unidirectional reorientation of a constitutive pJ23119 promoter flanked by its recognition sequences (red arrow) was controlled by a pH-dependent riboswitch PREmR34. The expression switches from mutator *ednaQ* to RFP could occur at pHi = 7.5. Right: acid-tolerant phenotypes can be enriched autonomously as pHi increases. L-Ara, L-arabinose; T7FQ, T7 RNA polymerase variants. (C) Cellular burden compensating system [63]. Left: constitutive P_{const} promoter is used to drive the expression of dCas9, which is directed by single guide (sg) RNA (triggered by a burden-inducible promoter P_{htpG1}) to bind to a specific region of promoter P_{BAD}. Right: cells equipped with feedback controller (FBC) outperformed control cells in terms of total protein yield. (D) Population quality-control system (PopQC) [67]. Higher fatty acid productivity will confer a greater cell growth rate by providing more leucine because the chromosome leucine biosynthetic gene cluster LeuABCD was

(Figure legend continued at the bottom of the next page.)

circuit may be systematically adjusted by changing the strength of the circuit connectivity such as the promoter [49], **ribosome binding site (RBS)** [48], or degradation tag [6], or by rewiring with new control modules [50]. At this stage, omics technology and mathematical modeling such as the toggle switch model [51], reaction-diffusion model [14], bandpass circuit model [36], and oscillator model [31] may help to predict circuit behavior and reduce designers' workload to achieve the desired function.

SME Used in Industrial Biotechnology

Engineering a cell factory to produce valuable chemicals from renewable substrates is one aspect of the societal transition toward sustainability [2,52]. However, using traditional approaches to accomplish this goal remains challenging. One important obstacle is the paradox of carbon flux regulation. Improving the titer, yield, and productivity in engineered cells conflicts with the cell's purpose to proliferate and generate biomass [3]. Other obstacles include high-performance strain development, **metabolic burden** during **metabolic engineering**, and population heterogeneity in scale-up fermentation. To overcome these conflicts, novel SME strategies for sensing the cellular status and controlling bioproduction in response must be developed in the biomanufacturing industry [53].

Autonomous Metabolic Flux Rewiring

A long-standing challenge in metabolic engineering is how to improve the host production capacity through the autonomous distribution of resources between biomass and production [54]. One promising strategy is to develop circuits that can autonomously redistribute metabolic fluxes after a prescribed time. The QS system involves self-produced, diffusible, extracellular chemical signals such as acyl homoserine lactone (AHL) or peptides. These signals can function as proxies for cell density [55]. As a well-characterized genetic timer, the QS system allows microorganisms to produce costly extracellular products only when there is sufficient biomass, which delays the cellular metabolic burden from direct expression of pathway enzymes. For example, by varying the AHL accumulation rate, the transcriptional activity of cell growth-dependent genes can be downregulated at various cell densities over the fermentation course for dynamic redistribution of the metabolic flux in a multipathway [6,9] (Figure 2A). Analogously, because of time differences in gene transcription initiation by different physiological-dependent promoters (such as the stationary-phase promoter [56] and exponential growth phase promoter [57]), target gene expression can be controlled in a cell physiological state-dependent manner. Furthermore, to achieve precise autonomous control, these strategies can be integrated into an AND gate. In this way, metabolic regulation can respond to both the microbial communities and cell physiological state while synergistically improving production performance [7].

Another strategy for autonomously rewiring metabolic flux is to build circuits that sense environmental or intermediate metabolite changes during fermentation. To achieve this goal, specific transcription factors should be engineered to link a metabolic pathway with product synthesis. The first report of this type of design was a feedback genetic circuit in which lycopene was produced only in the presence of excess glucose flux in the cell [58]. Recently, a time-delay switch was designed and implemented for glucaric acid production [9]. Specifically, a hybrid promoter containing a binding site of the transcription factor IpsA, which is responsive for intermediate *myo*-inositol, was constructed for controlling the expression of an unstable *myo*-inositol oxygenase. As a result, a just-in-time production pattern was developed in which the *myo*-inositol oxygenase reaction rate was upregulated only when the *myo*-inositol substrate accumulated.

Figure 2. *Continued*

removed. In all panels, a red line represents repressed activity, and a green line represents induced activity. Abbreviations: FA pathway, fatty acid biosynthesis pathway; FadD, fatty acyl-CoA synthetase; FadR, fatty acid-responsive transcription factor; P_{AR}, promoter repressed by FadR;. VioB, violacein biosynthesis protein; WT, wild type.

Autonomous Evolution Acceleration

Cell evolution is an effective strategy for obtaining the desired phenotype in metabolic engineering and synthetic biology [59,60]. Traditionally, the mutagenesis process is decoupled from the cellular phenotype such as the production capacity and acid-tolerance capacity. Thus, appropriate screening methods must be developed to select desired mutants from mutant libraries. To construct an automatic evolution system, the mutagenesis rates can be coupled with the desired phenotype using a feedback-regulated loop [8,32]. In one SME design named as TyrR-DnaQ, the cellular mutagenesis rates can be dynamically modulated by coupling a sensor module (tyrosine concentration-responsive transcription factor TyrR) with an actuator module (proofreading exonuclease of DNA polymerase III DnaQ which controls the mutagenesis rates). When evolved strains exhibited a high yield of desired compounds, the mutagenesis cycle was terminated by repressing mutator DnaQ expression [32]. As a result, the evolved strains showed 5× and 3× increases in the tyrosine titer and isopentenyl diphosphate production, respectively [61]. Another SME design employed a narrow-dynamic-range biosensor to accelerate cell evolution. In this RiDE system (Figure 2B), a pH-responsive riboswitch was designed as a sensor module with a site-specific recombinase subsystem designed as the actuator module. By interlinking error-prone DNA replication machinery and fluorescent cell labeling, this design digitalized pH-dependent gene expression for high-resolution memory-based detection of external pH. When mutants exhibit the capacity to buffer against changes in pHi, inversion of the J23119 promoter is triggered by integrase expression. As a result, this design can turn off mutagenesis and turn on RFP expression, allowing programmed *Escherichia coli* to evolve phenotypes automatically and enrich acid-tolerance phenotypes [8].

Cellular Burden Compensating

When maximizing the fermentation index (titer, productivity, and yield), heterologous protein overexpression or sophisticated genetic regulation in the host can cause metabolic burden and ultimately decrease the cell growth fitness and fermentation index. To address this issue, a circuit that can automatically tune the expression level of heterologous protein by sensing the cellular burden signal was designed (Figure 2C). Specifically, a cellular burden-responsive promoter, P_{htpG1}, was mined [62] and arranged to control the transcription of single guide RNA (sgRNA) in the CRISPRi system. When the cellular burden signal (caused by VioB–mCherry protein expression) reached the threshold for triggering the expression of sgRNA, dCas9 protein accompanied by sgRNA bound the target promoter to downregulate heterologous protein expression to dynamically decrease the cellular burden. As a result, cells equipped with this cellular burden compensating circuit maintained robust growth and outperformed control cells without the circuit in terms of foreign protein yield in batch production [63].

Population Quality Control

Phenotypic heterogeneity is an innate bacterial survival strategy for microorganisms to overcome suddenly changing environmental conditions in a flexible manner [64]. This heterogeneity can be caused by various factors, including nutrient depletion [64], bioprocess scale-up from the laboratory level to the industrial scale [65], stochastic gene expression, and random division of metabolites between daughter cells [66]. Although phenotypic heterogeneity is helpful for strain evolution, it is counterproductive in bioprocess development. Low-performing subpopulation cells gradually accumulate and eventually replace high-performing subpopulation cells because of their proliferative advantage during bioprocesses. Thus, diminishing phenotypic heterogeneity may be important for maintaining system robustness and strain productivity. For example, the concept of population quality control was recently proposed (Figure 2D) [67]. In this genetic circuit design, by coupling the production capability with cell growth, selective pressure was introduced to reward the cells with desired phenotype and punish the cheater cells. Specifically, expression of a growth-dependent gene (*leuABCD*) was controlled by the product (free fatty acid) signal-responsive promoter P_{AR}. As a result, a positive feedback circuit was constructed in which a leucine-auxotrophic strain with a high titer produced more leucine to facilitate cell growth compared with the cheater cells [67]. As a result, heterogeneity was alleviated.

SME Used in Environmental Engineering

Environmental pollution is the major roadblock to achieving sustainability. Current methods for detecting pollutants often require costly equipment. Bioremediation using microorganisms is an interesting alternative because of its low cost and sustainability. However, traditional bioremediation methods rely on microorganisms themselves to sequester contaminants, and thus the microbes become pollutant sinks. Consequently, bacterial biomass must be continuously regenerated and the cells are damaged by pollutant enrichment. Moreover, these methods use chemical inducers rather than allowing the cells to dynamically respond to, and capture, environmental pollutants [68]. A recent SME-based bioremediation system was proposed in which engineered *E. coli* was constructed to sense and sequester mercury ions (Figure 3A). Specifically, the mercury-responsive transcriptional regulator MerR was integrated into a mercury-sensor circuit that can control the induction of curli nanofiber synthesis to sequester mercury in an extracellular matrix. Ideally, in the presence of mercury, the MerR repressor undergoes a conformational change and binds and sequesters mercury [24]. The matrix is then generated when the mercury content surpasses a critical level. Once the mercury has been sequestered, and its concentration falls below a certain threshold, matrix biosynthesis ceases and the cell focuses on propagation [69].

SME Used in Smart Materials

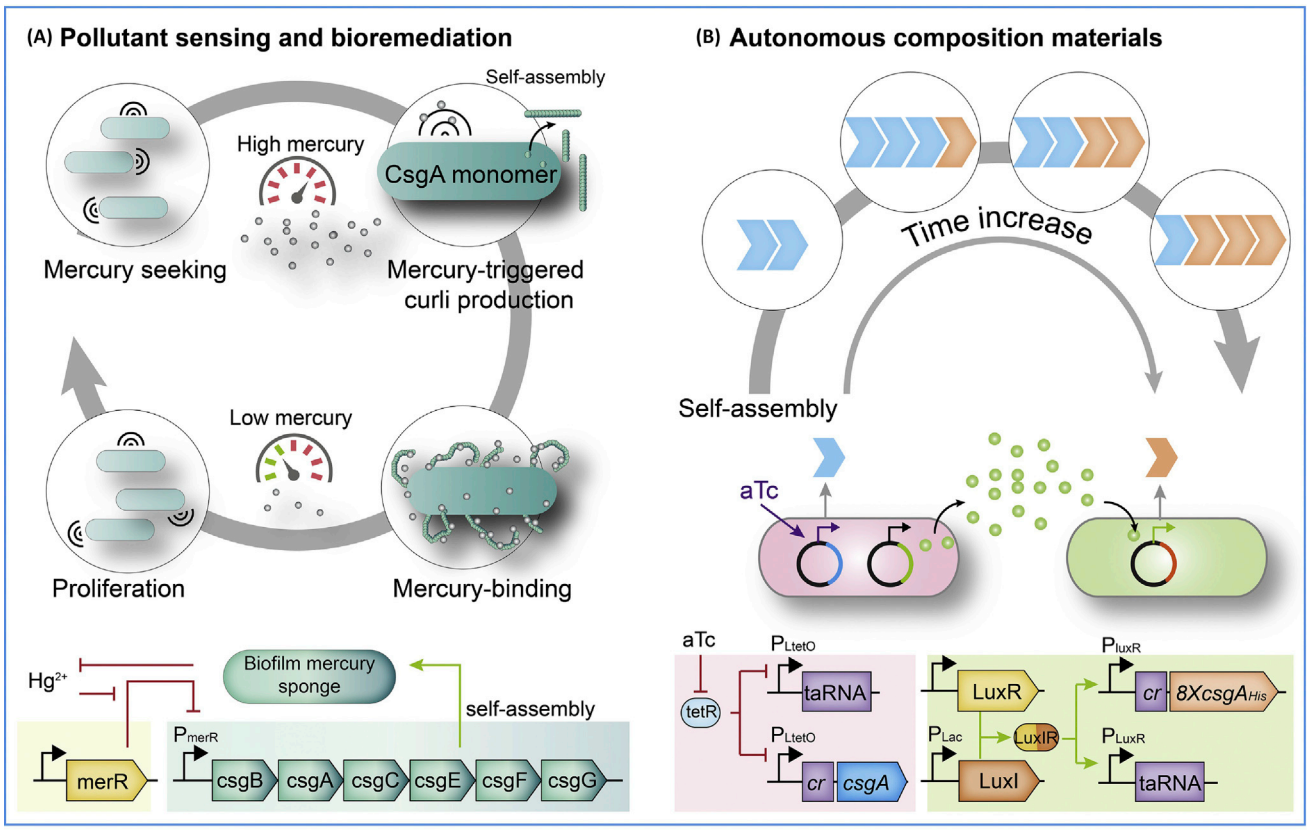
With advances in synthetic biology, current technologies have been used to produce living systems that can be engineered to self-assemble materials [70]. For example, by engineering *E. coli* to sense blue light [71] or chemicals [72], the assembly of biofilm combined with various functional inorganic nanoparticles can be spatially controlled to perform hybrid enzyme–inorganic reaction cascades. To autonomously pattern a biological material, *E. coli* biofilms are engineered with artificial gene circuits to dynamically control the synthesis of extracellular curli amyloid nanofibers. Specifically, in this design, one CsgA-secreting *E. coli* was engineered to constitutively produce the QS molecule AHL. In other recombinant *E. coli*, secretion of the $CsgA_{His}$ monomer was controlled by the AHL concentration in the medium. When these two strains were cocultured, the proportion of $CsgA_{His}$ increased. As a result, the synthetic biofilms generated materials whose nanoscale-to-microscale composition and structure varied dynamically and autonomously over the culture time [70] (Figure 3B). A similar design can be introduced into strains that produce other materials. For instance, by equipping cells with a QS system on the bacterial cellulose-producing strain *Komagataeibacter rhaeticus*, a programmable cell-to-cell communication system, useful for boundary detection or potential self-repairing, was constructed within growing bacterial cellulose pellicles [73]. Going forward, by integrating genetic logic gates that link cell-to-cell communication, microorganisms capable of decision-making may produce variable types of functional materials according to external and internal stimuli.

SME Used in Intelligent Therapeutics

Microorganisms have been used as therapeutics for several centuries. However, advances in synthetic biology have resulted in the production of microorganisms that can sense disease, target lesions, and produce drug molecules, making 'smart' therapeutics a tangible reality [13].

Disease Monitoring

Some transient molecules are difficult to capture and quantify using traditional noninvasive tests because these molecules are easily degraded or absorbed *in vivo*. Thus, by measuring the levels of these specific biomarkers, microorganism-based *in vivo* diagnostics can be used to reveal physiological changes during the asymptomatic phase [49,74]. Moreover, such *in vivo* diagnostics should consist of a memory subsystem to record cellular events either instantaneously or continuously in real time. Recently, an *E. coli* strain was engineered with a diagnostic circuit to sense and record exposure to the transiently formed biomarker tetrathionate (a specific biomarker in inflammatory disease) in mice [10,23] (Figure 4A). Specifically, when this engineered *E. coli* detected the trigger signal of tetrathionate in the mouse gut, the **memory circuit** would switch the output from the OFF (CI^+/Cro and β-gal^-) to the ON (CI^-/Cro and β-gal^+) state. Compared with traditional tomographic methods,



Trends in Microbiology

Figure 3. Smart Microbial Engineering (SME) Applications in Environmental Engineering and Materials.
(A) Schematic of mercury bioremediation circuit [24]. A mercury-responsive transcription factor (TF) MerR was used to regulate expression of the curli monomer *csgBACEFG* controlled by the P_{merR} promoter. When the mercury concentration is high, the above curli monomer can self-assemble to form curli nanofibers in order to sequestrate mercury. However, when the mercury concentration falls below a certain threshold, cells focus on propagation. (B) Synthetic cellular communication for dynamic, autonomous material production [70]. The *trans*-activating RNA (taRNA) prevents the *cis*-repressive sequence (cr) from blocking the ribosome binding site (RBS) controlling translation of the mRNA transcript. Addition of anhydrotetracycline (aTc) induces transcription of *csgA* mRNA and taRNA, thus enabling CsgA monomer production. Quorum sensing (QS) signal molecule is produced by LuxI, whose expression is controlled by a constitutive P_{Lac} promoter. The $CsgA_{His}$ monomer production is dynamically increased by cell density. As a result, the composition and structure of the synthetic biofilm materials can vary dynamically and autonomously over culture time. In all panels, thick gray arrows represent the dynamic change in the system state over time. Red lines represent repressed activity, and green lines represent induced activity.
Abbreviation: P_{LtetO}, hybrid promoter derived from promoter P_L containing binding sites of TetR operator.

the limited system output of memory design can support 6 months of disease progress monitoring [10]. Additionally, continuous recording of cellular events can be achieved by the microorganism-based *in vivo* diagnostics. For instance, a CRISPR biological tape recorder is engineered to monitor continuous real-time cellular events [75]. By transforming signals to DNA abundance, writing DNA to genomic CRISPR array, and resequencing CRISPR arrays, multiplex signals, including the biomarker fucose, can be recorded and reconstructed over 3 days [75].

Precision Medicine

Engineering microorganisms that can respond to specific pathogens, as well as distinguish between malignant and healthy cells, is another promising direction for smart living therapeutics [76]. To achieve this goal, *E. coli* was equipped with a 'sense–kill' circuit to enable it to specifically recognize (perceive the AHL secreted by *Pseudomonas aeruginosa*), migrate toward (induce CheZ protein

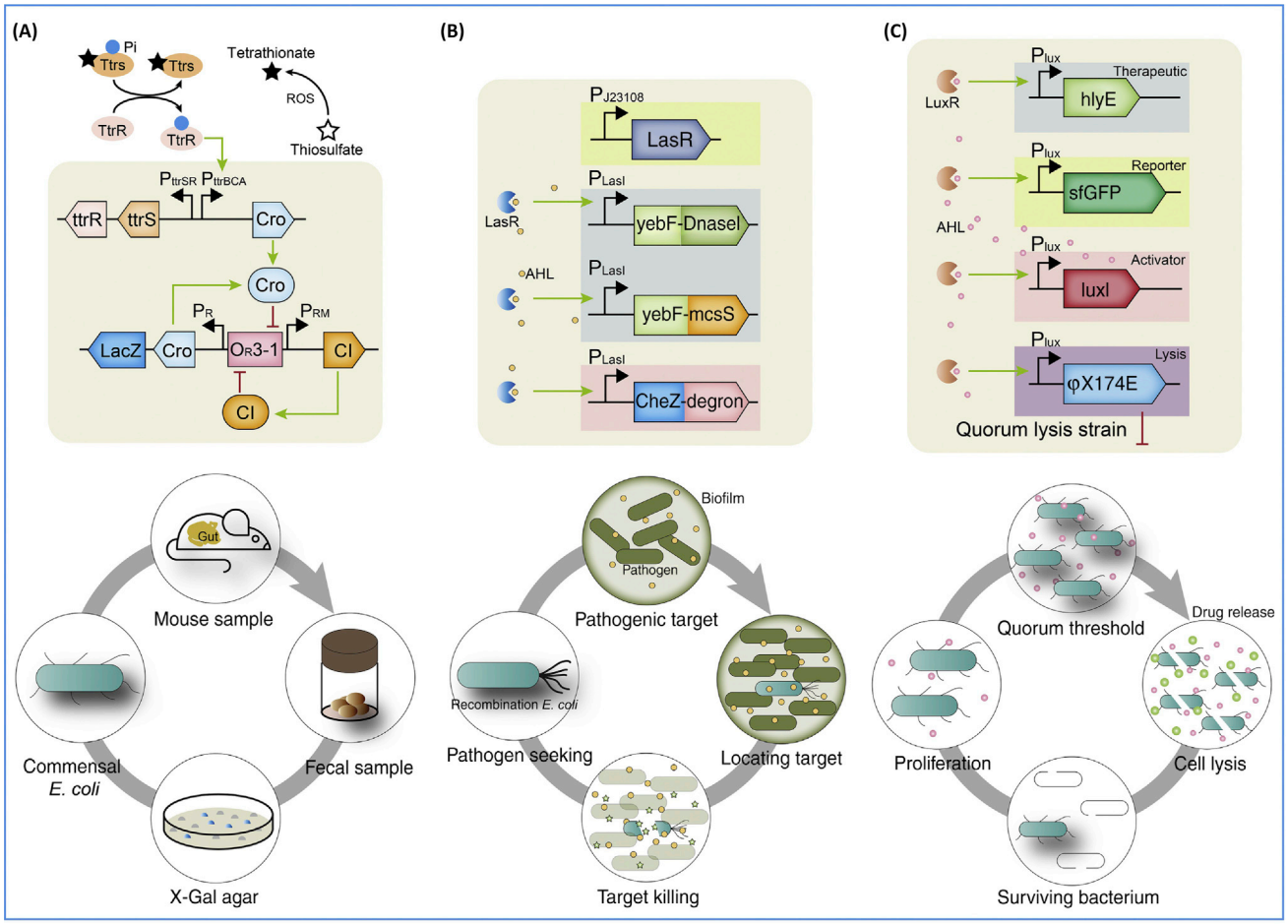


Figure 4. Smart Microbial Engineering (SME) Applications in Intelligent Therapeutics.

(A) Schematic of bacterial memory device PAS638 for registering inflammation events in the gut [10]. The tetrathionate sensor is based on a two-component TtrR/TtrS sensor system expressed from the P_{ttrSR} promoter; its activation through phosphorylation (Pi) leads to the expression of the repressor Cro from the P_{ttrBCA} promoter, which in turn shuts down expression of the repressor CI from P_{RM} promoter and enables long-lasting expression of the reporter β-Gal from the P_R promoter. Fecal samples can be detected by growing the bacteria on agar plates supplemented with the substrate X-Gal. (B) Schematic of chemotactic *Escherichia coli* motility toward *Pseudomonas aeruginosa* PAO1 [77]. The antibiofilm protein DNaseI and antimicrobial peptide microcin S (encoded by *McsS*) combined with the chemotaxis-behavior-controlled protein CheZ were expressed under the control of a *P. aeruginosa* acyl homoserine lactone (AHL)-responsive LasI/LasR quorum sensing (QS) circuit. (C) Schematic of a synchronized lysis circuit. The promoter P_{Lux} drives transcription of a bacteriophage lysis gene (*ϕ*X174 E), *LuxI* (encoding enzyme for synthesizing AHL signals), and *sfGFP* or *hylE* (encoding hemolysin E protein, a pore-forming antitumor toxin) as the reporter module. In all panels, thick gray arrows represent the dynamic change in the system state over time. Red lines represent repressed activity, and green lines represent induced activity.

Abbreviations: YebF, secretion tag; Degron, sequence for promoting protein degradation.

expression), and eradicate (secrete antibiofilm protein DNaseI and antimicrobial peptide microcin S) the pathogenic *P. aeruginosa* [77] (Figure 4B). Recently, using a similar design principle, this strategy was further developed against *in vivo P. aeruginosa* infections in two animal models (*Caenorhabditis elegans* and mice) [78].

To efficiently kill tumors without harming healthy cells, adaptive therapies are being developed with innovative genetic circuits that can precisely control the timing, duration, and localization of therapeutic outputs. For example, to restrict the secretion of anticancer proteins localized to tumors, *E. coli* can be engineered to target and invade cancer cells by regulating heterologous invasin

expression under specific transcriptional operons. Those transcriptional operons can be activated under environmental signals specific to the hypoxic tumor microenvironment [79]. Nonpathogenic *Salmonella* can preferentially proliferate in a hypoxic microenvironment, such as in a tumor. Thus, this species is a good candidate for bacterial therapies. To limit the bacterial population during therapy, a synchronized lysis circuit using coupled positive and negative feedback loops was developed to initiate pulsatile drug delivery in the tightly packed colonies within tumors (Figure 4C) [11,80]. Specifically, in this design, QS-induced lysis was triggered by the accumulation of AHL signals when the population of *Salmonella* reached a critical cell density within the tumors. After lysis, therapeutic toxins inside the cells were released [11], and the surviving bacteria began producing AHL anew, allowing the process to be repeated in a cyclical manner. This administration method can reduce the cellular burden and attenuate tumor resistance in bacterial cancer therapy [31].

Other Emerging Applications

Microbes can be programmed to perform intelligent cellular behaviors in basic research. For example, light-responsive genetic programs such as edge detection [14] and bacterial photography [81] have been successfully developed. In the former, dark sensor, cell–cell communication, and X AND (NOT Y) genetic logic modules are constructed as an independent genetic circuit and assembled into a full algorithm to permit temporal and spatial gene expression control and enable edge detection [14]. Similar approaches can be used in an SME design for sensing multiple light inputs and performing complex decision-making functions. For instance, four modules, including a phytochrome sensor, NOT gates, resource allocator, and pigment-producing actuators, are combined to achieve a colored pattern after programmed illumination [81].

Challenges in SME Implementation

SME confers the autonomous and adaptive characteristics of living microorganisms, which show vast potential as basic tools for overcoming the challenges in many existing and future fields. Although emerging tools for synthetic biology are being updated, significant gaps remain between artificial genetic circuits and real-world applications. To address these challenges, synthetic biologists should take precautionary steps and pay special attention to the following challenges before initiating SME implementation.

Lack of Genetic Tools in Nonmodel Strains

Because of the clear genetic background and availability of various genetic manipulation tools, **model strains** are preferred hosts for building and testing most microbial genetic circuits. However, their uncertain safety in the food and healthcare fields, uncompetitive chemical production capacity, and sensitivity to extreme environmental conditions have limited the application of these model strains in many areas. Many nonmodel microorganisms possess advantageous traits related to their innate physiologies [53]. For example, cyanobacteria can be engineered as a promising photoautotrophic refinery for producing high-value chemicals [82]; *Streptomyces* can produce various secondary metabolites through their complex secondary metabolic pathways [83]; *Pichia pastoris* can be used for biopharmaceutical enzyme production because of its powerful protein synthesis capacity [84]; *Yarrowia lipolytica* can function as a cell factory for biofuel production based on its excellent lipid accumulation capacity [85]; and *Halomonas bluephagenesis* is an attractive candidate for polyhydroxyalkanoate production in a continuous open unsterile fermentation environment [86]. Recently, many genetic tools useful for nonmodel organisms have been developed [82–86]. However, the lack of available genetic tools remains the main limitation of SME in constructing complex circuits in those nonmodel microorganisms. Thus, novel and facile genetic tools in nonmodel and biotechnologically relevant organisms must be developed.

Genetic Stability in SME Implementation

An important factor that may affect synthetic genetic circuit behavior is retroactivity. Signal feedback occurs from the downstream to upstream system in the processing or actuator module in a complex

system [87]. One way to improve genetic circuit stability is to apply insulators in the circuit which can attenuate retroactivity and mitigate hysteresis behavior [88–90].

Moreover, current SME designs can function effectively under constraint conditions at relative small scales (up to benchtop bioreactor), but they may not function nicely on an industrial scale or in the natural environment [91]. One reason for this is phenotypic heterogeneity. In a small biochemical reaction system with relatively few molecules (such as genetic circuits), system noise is prominent and may be generated by stochastic processes such as intracellular gene transcription and translation or molecular or energetic reaction heterogeneity [92]. This disturbance is noticeable and will markedly decrease system robustness when the genetic circuit contains multiple layers of signal transduction. Some potential solutions for repressing the noise and combating metabolic heterogeneity may include: (i) using digital rather than analog circuits to attenuate noise and transmission [93]; (ii) designing a robust system based on network topology analysis [94]; (iii) filtering noise by genetic bandpass circuit design [36,95]; or (iv) adding a population quality-control design [67].

Metabolic Burden Caused by SME

During the engineering of microorganisms, a heterogeneous genetic circuit with a relatively high copy number and overloaded protein expression causes resource competition with endogenous cell metabolism [63,96]. Complex resource allocation in multilayer competition affects both cell proliferation and genetic circuit output [97,98]. The key to solving the competition effect is to decrease the intracellular resource occupancy by using artificial gene circuits. For example, when selecting the regulatory elements of a toggle switch, a recombinase-based design [99] is superior to a protease-driven design [100] or repressor-based design [30] for its limited output. Balancing the benefits of equipping delicate but complex circuits and their cost of cellular resource occupancy remains challenging but necessary work in the design and implementation of SME.

Outstanding Questions

How can we improve the efficiency of signal transmission from different subsystems in a complex circuit?

How can we integrate cellular physiology and genetic circuits in order to create an output of desired cell behaviors with high temporal and spatial resolution?

How can we design and implement SME when handling composite signals for multiple output?

How can we develop efficiency methods or standards for weighing the pros and cons in SME implementation?

How can we incorporate recent advances in artificial intelligence and machine learning to upgrade the current synthetic biology tools and strategies for designing predictable SME?

What can be learned from nature in the design and implementation of SME if we use the idea of bionics?

Concluding Remarks

In summary, our growing capability to predictably manipulate genetic parts, modules, and systems has enabled intelligence-driven rewiring of genetic circuits. On this basis, the SME design can endow autonomous and adaptive characteristics of living microorganisms with enormous potential and utility in numerous fields. Currently, these prospects are in an emerging state (see Outstanding Questions), but through SME design, novel areas that interface with synthetic biology will reveal a new paradigm that revolutionizes how biological systems are designed to solve major challenges facing the 21st century.

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