Molecular parts and genetic circuits for metabolic engineering of microorganisms

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One sentence summary: This review describes current progress in the design and application of genetic circuits for the metabolic engineering of microorganisms.

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ABSTRACT

Microbial conversion of biomass into value-added biochemicals is a highly sustainable process compared to petroleum-based production. In this regard, microorganisms have been engineered via simple overexpression or deletion of metabolic genes to facilitate the production. However, the producer microorganisms require complex regulatory circuits to maximize productivity and performance. To address this issue, diverse genetic circuits have been developed that allow cells to minimize their metabolic burden, overcome metabolic imbalances and respond to a dynamically changing environment. In this review, we briefly explain the basic strategy for constructing genetic circuits by assembling molecular parts such as input, operation and output modules. Next, we describe recent applications of the circuits in the metabolic engineering of microorganisms to improve biochemical production. Beyond those achievements, genetic circuits will facilitate more innovative approaches to future strain development through mining and engineering new genetic elements and improving the complexity of genetic circuit design.

Keywords: genetic circuit; metabolic engineering; synthetic biology; strain development

INTRODUCTION TO GENETIC CIRCUITRY

The manufacturing of chemicals using microbial cells is considered a promising alternative to petroleum-based production (Branduardi and Porro 2016), since microbial cells can utilize inexpensive and renewable substrates for chemical production (Erickson, Nelson and Winters 2012; Kumar and Kumar 2017). Countless biochemical reactions can be harnessed and assembled to build metabolic pathways to produce diverse chemical products. Furthermore, these biochemical reactions can occur simultaneously within a single or small number of microbial strains (Bernstein and Carlson 2012; Zhou et al. 2015a). Finally, cultivation conditions for microbial cells are much milder than those required for chemical processes, substantially reducing operational costs (Keasling 2010).

Microorganisms must be re-engineered to produce the desired chemicals economically because their primary goal in nature is survival rather than the directed synthesis of specific metabolite products (Holtz and Keasling 2010; Min et al. 2017). Engineering of microorganisms often requires sophisticated gene expression regulation. In addition to the simple
overexpression or knockout, the optimal balance and correct timing of expression of genes in a pathway are crucial for achieving maximum production. For example, excessive expression of pathway genes can lead to deficiencies in cellular resources needed for growth and maintenance (Noh et al. 2017). An imbalance in the expression of pathway genes can lead to the accumulation of toxic intermediates (Dahl et al. 2013; Lim et al. 2016). Unnecessary early expression of pathway genes before reaching sufficient buildup of precursors can diminish product titer and yield. Therefore, pathway gene expression needs to be tightly regulated to ensure the optimal concentrations and timing of pathway enzymes.

Complex gene expression regulation can be realized using genetic circuits. Genetic circuits are biological equivalents of electric circuits in which various input signals are received and computed to control the expression of outputs, the pathway genes. The genetic circuits are composed of modular parts (Brophy and Voigt 2014; Fig. 1). First, an input module recognizes diverse signals such as the substrate, product or cell density and transmits the signal by changing its conformation. An operation module computes the received signals from input module and determines regulation. Finally, an output module expresses the protein of interest (e.g. fluorescent protein, selection marker, metabolic enzyme and another operation module).

In this review, we describe current progress in the design and application of genetic circuits for metabolic engineering of microorganisms. Modular parts and strategies for their assembly are summarized, and the applications of genetic circuits in metabolic engineering are explained.

**Design of Genetic Circuit**

Cells can detect diverse environmental stimuli (metabolite concentration, temperature, light etc.) by using protein- and RNA-based molecules and regulating the expression levels of genes. Gene expression can be switched on (1) or off (0) depending on the existence of an input signal. Simple Boolean operations (YES and NOT gates) can be accomplished by employing the molecular parts. Furthermore, a set of molecular parts can be combined to yield more complex circuits (AND, OR, NAND and NOR gates) for processing multiple inputs or outputs (Moon et al. 2012; Daniel et al. 2013). In this section, the molecular parts (input, operation and output modules) of genetic circuits are explained, and the principles used in constructing biological logic gates are discussed.

**Input module**

An input module should recognize a specific signal, typically represented as a concentration of small molecules (Fig. 1A). Cells utilize many sensor-regulator, such as allosteric transcription factors (TFs) consisting of ligand and, usually, DNA-binding domains to recognize the intracellular levels of metabolites to regulate gene expression. Because the ligand-binding domain of TFs specifically interacts with their cognate ligands, the domain can be an input module to accept the signal. In addition, some TFs that regulate the gene expression by cooperative manner were identified, which could lead to a digital-like function (Ptashne et al. 1980; Krell et al. 2007). This cooperativity could lower detection limit, and facilitates a rapid switch from on-to-off and vice versa.

The most well-known and commonly utilized TFs for genetic circuit construction are LacI (binds to isopropyl-β-D-1-thiogalactopyranoside, IPTG), AraC (binds to arabinose), TetR (binds to anhydrotetracycline, aTc) and LuxR (binds to acyl-homoserine lactones, AHL; Shimizu 2013; Min et al. 2017). Although these TFs can detect the input signals, their sensitivity can be further increased by changing the binding affinity to the target ligand. For example, Satya Lakshmi et al. constructed a library of LacI mutants through error-prone PCR and shuffling and obtained four mutants that bind IPTG even at extremely low concentrations (Satya Lakshmi and Rao 2009). The mutants showed a 4–7-fold higher inducibility than the native LacI with an addition of 1 μM IPTG. Furthermore, the affinity of a ligand-binding domain can be modified to bind other molecules; Taylor et al. engineered LacI to recognize lactitol, gentiobiose, fucoside and sucralose (Taylor et al. 2016) rather than lactose or IPTG. Similarly, Trp repressors have been engineered to recognize tryptophan analogs by changing key residues using an evolutionary approach (Ellefson, Ledbetter and Ellington 2018). In
addition to engineering well-known TFs, input signals can be diversified by identifying unknown TFs in microorganisms. Dahl et al. identified a farnesyl pyrophosphate (FPP)-responsive TF and its cognate promoter via whole-genome transcript array (Dahl et al. 2013). Alternatively, Zhou et al. obtained TFs that bind 3-hydroxypropionic acid by analyzing the regulatory region of catabolic genes in Pseudomonas denitrificans (Zhou et al. 2015b). These efforts to expand available input signal sensors are important because concentrations of metabolites of interest are frequent inputs in metabolic engineering applications. Another potential input module is the two-component system, which allows organisms to sense extracellular signals and respond to the changes (Stock, Robinson and Goudreau 2000; Mitrophanov and Grosimov 2008). The system generally consists of a histidine protein kinase that is regulated by environmental stimuli and a corresponding response regulator that bring changes in the gene expressions. The system can be utilized as an input module, for example, Escherichia coli was engineered to sense methanol by fusing a sensing domain FlhS of Paracoccus denitrificans and a catalytic domain EnvZ of E. coli (Selvamani et al. 2017).

In addition to protein-based molecules, RNA can be also utilized as an input module (Jang et al. 2018). As RNA is highly flexible, it is an important cellular element for interacting with small molecules. These regulatory RNA molecules, known as aptamers, are often found in the 5′ upstream sequence of riboswitches. Diverse cellular molecules such as metabolites (Mandal et al. 2004; Serganov, Huang and Patel 2008), metal ions (Ren, Rajashankar and Patel 2012; Dambach et al. 2015; Furukawa et al. 2015) or even proteins (Schilling et al. 2004) can be detected using RNA aptamers. Similar to protein-based input modules, aptamer affinity can be adjusted by introducing mutations or using an alternative aptamer with a different dissociation constant (Kd). A synthetic aptamer can be also artificially developed via a technology known as systematic evolution of ligands by exponential enrichment (Ellington and Szostak 1990; Tuerk and Gold 1990; Darmostuk et al. 2014; Zhuo et al. 2017).

Output module

Operation module

Operation modules determine whether an output gene will be expressed (Fig. 1B). For allosteric TFs, their physical binding to a cognate promoter with operator or activator-binding sites governs gene expression. The event of ligand binding to TFs is also closely related to this regulation, which drives changes in the conformation of DNA-binding domain, thereby increasing or lowering affinity to the transcriptional regulatory sites. Common TFs (e.g. LacI, TetR) in the ligand-bound state lose their affinity and detach, allowing transcription to occur (Ellefson, Ledbetter and Ellington 2018). However, some TFs such as TrpR attach to DNA when the ligand binds to itself. As a protein-based operation module, other TFs that do not have their own cognate molecules can be used. The most well-characterized TF is the CI repressor from bacteriophage lambda (Bell et al. 2000; Casjens and Hendrix 2015). Furthermore, heterologous sigma factors can be used, as they specifically recognize unique promoter sequences (Rhodius et al. 2013; Bervoets et al. 2018). These molecular parts, whose expression is controlled by the input modules of the first circuit, function as operation modules for the second circuit.

One important feature of protein-based operation modules is orthogonality, as they are trans-regulatory elements. If the interaction between TFs and cognate promoters are not orthogonal, the output signal can greatly fluctuate and is prone to containing noise. In addition, the orthogonality can be a constraint on the construction of more complex circuits by exploiting more than one operation module. In this regard, Stanton et al. found that orthogonal TFs and cognate operators can be used for genetic circuits while minimizing unintended interactions (Stanton et al. 2014). Using this approach, Stanton et al. tested repressor orthogonality, and 16 repressors were found to exhibit minimal cross-reactions. These sets can obviously be used as potential operation modules for constructing complex genetic circuits with a large set of compatible gates (Fernandez-Rodriguez et al. 2017; Segall-Shapiro, Sontag and Voigt 2018).

In case of RNA regulatory factors, output signals are mostly dependent on the formation of a secondary structure that allows for gene expression. Because most riboswitches are naturally involved in controlling the biosynthesis of metabolites (e.g. lysine), a secondary structure covering the ribosome-binding site is generally formed to downregulate the output gene when the concentration of the target metabolite is high (Vitreschak 2003; Blount et al. 2007; repression type). Some riboswitches also show opposite regulation; genes for degradation are upregulated by corresponding metabolites via riboswitch activation (Mandal et al. 2004). In addition to the riboswitch, other riboregulators can mediate input and output signals. For example, Green et al. reported toehold switches regulating gene expression by interacting with trigger RNAs (Green et al. 2014). The toehold domain can hybridize with trigger RNAs that contain complementary sequences, exposing the ribosome-binding site. Therefore, these sequences can act as triggers to determine whether the output gene is expressed (Pardee et al. 2016; Alam et al. 2017). In addition, Na et al. reported that synthetic small regulatory RNAs can regulate gene expression (Na et al. 2013). The synthetic small regulatory RNAs bind to ribosome-binding sites and interfere with the binding of the ribosome to mRNA, repressing the expression of the downstream gene. Finally, Chappell et al. created synthetic regulatory RNAs known as small transcription-activating RNAs (Chappell, Takahashi and Lucks 2015). Antisense small regulatory RNAs induce terminator formation and inhibit transcription via an attenuator mechanism, and the terminator structure can be disrupted by using small transcription-activating regulatory antisense RNA. These riboregulators can be used to transduce an input signal to an output signal depending on their mapping.

Output module

An output module is a protein whose synthesis is determined by the functioning of an operation module in response to a specific input signal. The output module can be a protein for another operation module (e.g. TF) to construct a more layered genetic circuit (Lo et al. 2016). Once the final output is reached, various functional proteins can be used depending on the purpose of the genetic circuit (Fig. 1C). Fluorescent proteins are the most commonly used proteins to facilitate the visualization of output (Gulati et al. 2009; Tracy, Gaida and Papoutsakis 2010), and most complex genetic circuits are initially validated using this form of output (Tamsir, Tabor and Voigt 2011; Moon et al. 2012). Moreover, this approach has broad applicability for strain improvement and enables real-time monitoring of metabolite concentration (Xu et al. 2014; Rogers and Church 2016). Such reporters are often combined with high-throughput screening systems such as fluorescence-activated cell sorting or microfluidic systems to screen for efficient metabolite producers (Tracy, Gaida and Papoutsakis 2010; Jang et al. 2016). Similar to fluorescent proteins, selection markers are used in strain development. Compared to fluorescence screening, there is no limit to the library size that can be covered by growth selection;
both inducers activates transcription. The complex of repressor and small molecule represses the transcription of an output protein, and the signal is converted. (C) AND gate design based on a promoter repressed by two repressors. Both inducers are required to expose the promoter by inactivating the two repressors. (D) OR gate design based on two activators. Only one of the two inducers can activate transcription. (E and F) Complex logic gates using a combination of basic gates. NAND gate can be constructed by linking an AND gate to a NOT gate, and the presence of both inducers inhibits transcription. A NOR gate can be constructed by linking an OR gate to a NOT gate, and the absence of both inducers activates transcription.

The throughput of fluorescence screening is limited (microtiter plates: $10^4$–$10^5$, FACS: $10^6$), while the throughput of growth selection is determined by the size of the culture (Dietrich, McKee and Keasling 2010). Numerous metabolic engineering strategies such as effectors of pathway enzymes and flux optimization have been demonstrated (Boersma, Dröge and Quax 2007; Yang et al. 2013; Seok et al. 2018) with selection markers as the output. Key metabolic enzymes are also often targeted as outputs. Metabolic flux control (Cameron and Collins 2014; Izard et al. 2015; Lo et al. 2016) or rebalancing (Dahl et al. 2013; Doong, Gupta and Prather 2018) has been achieved by regulating gene expression. Moreover, proteins with specific functions can be utilized as output modules. The utilization of CRISPR/Cas9 can allow more freedom to target and regulate genes through the simple substitution of single-guided RNA (Bikard et al. 2013; Qi et al. 2013). In addition, a site-specific recombinase can be applied to construct a digitalized circuit that implements two completely separated states by inverting genetic element of circuit such as promoter, and it is also possible to construct a circuit for multi-state computation (Roquet et al. 2016; Rubens, Selvaggio and Lu 2016; Pham et al. 2017). As such, a variety of proteins can be exploited, generating a genetic circuit that can perform more layered and complex functions (De Rubertis and Davies 2003; Wang, Barahona and Buck 2014; Müller et al. 2017).

**Construction of biological logic gates**

Basic logic gates (YES and NOT) can be constructed by using a combination of input, operation and output modules (Fig. 2). The YES gate makes the output identical to the input (Fig. 2A). This gate can be simply constructed by using allosteric TFs that detach from their promoters in the ligand-bound state. Alternatively, an activation-type riboswitch also can be used. To obtain an inverted output, the NOT gate can be used (Fig. 2B). To construct this gate, TFs that bind operators in the ligand-bound state should be used; a repression-type riboswitch also forms the NOT gate (Taton et al. 2017). Both YES and NOT gates can turn on/off expression of the output depending on the addition of ligand, and these gates have been widely utilized for inducible expression/repression of metabolic genes (Soma et al. 2017).

As necessary, basic logic gates can be further combined to yield more complex logic gates (AND, OR, NAND and NOR) with multiple inputs. In the AND gate, all inputs should be 1 (ON) to obtain 1 as an output signal (Fig. 2C). The simple AND gate can be built by combining multiple YES gates, and all inducers (ligand) are required to be included to detach the repressors from the DNA to allow transcription initiation. Additionally, the promoter itself is that is activated by a complex of two gene-products can also form an AND gate (Moon et al. 2012; Teo and Chang 2014). The OR gate results in 0 (OFF) as an output only if all inputs are 0 (Fig. 2D). Two independent sets of TFs and their cognate promoters can construct the OR gate (Tamsir, Tabor and Voigt 2011). Moreover, more complex logic gates are composed of the logic combinations described above, for example, a NAND gate can be constructed by connecting an AND gate to a NOT gate, and an NOR gate can be constructed by linking an OR gate to a NOT gate (Fig. 2E and F; Tamsir, Tabor and Voigt 2011).

**APPLICATION OF GENETIC CIRCUITS FOR METABOLIC ENGINEERING**

Genetic circuits with biological logic gates have been extensively applied in metabolic engineering for strain development because of their numerous advantages. First, the use of a genetic circuit can minimize the metabolic burden caused by introduction of heterologous gene or amplification of product synthesis pathway (Fernandez-de-Cossio-Diaz, Leon and Mulet 2017). In addition, genetic circuits can give cells the ability to dynamically adapt to environmental changes and robustly produce chemicals (Xu et al. 2014). Finally, genetic circuits can direct the evolution of proteins and microorganisms to improve biochemical production (Yang et al. 2013; Rogers, Taylor and Church 2016; Seok et al. 2018). In this section, three major examples of genetic circuits in metabolic engineering applications will be explained: toggle switch, feedback loops and evolutionary genetic circuits (Fig. 3 and Table 1).

**Metabolic toggle switch**

To maximize product formation, carbon flux should be directed in favor of product synthesis via upregulation of pathway genes. However, excessive expression of genes in the early stage of fermentation or a severe reduction in essential carbon flux can inhibit cell growth, resulting in low production of biochemicals (Min et al. 2017; Noh et al. 2017). To overcome this problem, temporal separation of the growth and production phases was used in many studies (Brockman and Prather 2015; Gupta et al. 2017). During the initial fermentation phase, genes for cell growth are expressed. When a sufficient biomass is obtained, gene expression is changed to favor product synthesis. For this regulation, a metabolic toggle switch can be constructed by introducing of a series of YES or NOT gates (Farmer and Liao 2000; Taton et al. 2017; Noh et al. 2018); most examples utilized allosteric TFs and a cognate promoter system (Solomon, Sanders and Prather 2012; Torella et al. 2013; Zong et al. 2017). Brockman et al. developed a single-input metabolic toggle switch for myo-inositol production by using the TetR repressor and Ptet promoter (Brockman and Prather 2015). They initially attached an SsrA degradation tag (recognized by the SspB–ClpXP

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**Figure 2.** Genetic logic gates by layering multiple biosensors. (A) YES gate design. The addition of small-molecule detaches the repressor from the promoter, and the output protein is expressed. (B) NOT gate design. The complex of repressor and small molecule represses the transcription of an output protein, and the signal is converted. (C) AND gate design based on a promoter repressed by two repressors. Both inducers are required to expose the promoter by inactivating the two repressors. (D) OR gate design based on two activators. Only one of the two inducers can activate transcription. (E and F) Complex logic gates using a combination of basic gates. NAND gate can be constructed by linking an AND gate to a NOT gate, and the presence of both inducers inhibits transcription. A NOR gate can be constructed by linking an OR gate to a NOT gate, and the absence of both inducers activates transcription.
protease complex) to 6-phosphofructokinase-I (Pfk-I) but allowed active glycolysis in the early phase of fermentation without expressing the SspB protein. Next, the mode was switched for myo-inositol production by adding aTc to express sspB under the Psp promoter. The switch led to a 2-fold improvement in the titers of myo-inositol. Similarly, Soma et al. designed a metabolic toggle switch for isopropanol production (Soma et al. 2017). The expression of gltA-encoding citrate synthase, which is responsible for the entry reaction of citric acid (TCA) cycle, was repressed by adding IPTG by using a NOT gate with the Lac and TetR repressors. In contrast, genes for isopropanol production were expressed by using a YES gate. Using this metabolic toggle switch, acetyl-CoA was efficiently converted to produce isopropanol, the titer was improved by more than 3-fold.

Because such genetic circuits require the addition of costly inducers (Lo et al. 2016), inducer-free metabolic toggle switches have been developed (Anesiadis et al. 2013; Williams et al. 2015; Min et al. 2017). These circuits sense AHL, a quorum-sensing molecule, as their input signal. Gupta et al. developed a metabolic toggle switch with the quorum-sensing system from Pantoea stewartii and demonstrated efficient glucaric acid production (Gupta et al. 2017). In the system, low concentrations of AHL allow the TF EsaR170V to bind the PesaS promoter thereby activating transcription. When the cell density is high, the accumulated AHL binds to EsaR170V that is then released from the promoter, resulting in decreased gene expression from the Pes promoter. Placing the gene of interest under the control of the Pes promoter facilitates dynamic expression control without the need to add an inducer. For example, placing pfrA (encoding Pfk-I) under the control of Pes resulted in autonomous switching from the growth to the production phase. The timing of this switching was precisely adjusted by controlled expression of eas1, encoding Acyl-homoserine-lactone synthase (Fig. 3A). Because the addition of an inducer is avoided, this approach has wide applicability for industrial production processes.

Two different TFs can mutually repress the expression of each other to construct a bistable toggle switch (Lac represses the expression of tetR under Plac and TetR represses the expression of lacI under Ptet) (Solomon, Sanders and Prather 2012; Chan et al. 2016; Lee et al. 2016). Compared to the monostable toggle switch, a distinct key feature is the ability to revert from the current state to the original state by adding a cognate inducer. Recently, Bothfeld et al. introduced this bistable toggle switch for polyhydrobutyrate (PHB) production (Bothfeld, Kapov and Tyo 2017). They placed PHB synthetic genes under the Ptet promoter, thereby allowing PHB production upon addition of aTc. Use of the toggle switch improved PHB productivity compared to the titer with constitutive expression of the genes. Furthermore, they successfully modified the circuit to detect glucose starvation because aTc should be avoided in industrial production because of its high cost. For this, lacI was placed under a glucose-responsive promoter. They observed autonomous induction when glucose was depleted; production was stably maintained even after glucose feeding.

**Metabolic feedback loop**

If a biochemical production pathway is not balanced, an intermediate can accumulate to a toxic level and induce global stress responses in cells (Dahl et al. 2013; Xu et al. 2014; Lim et al. 2016). Therefore, maintaining the concentration of potentially toxic intermediates at sub-inhibitory levels can be an effective strategy for improving productivity. In this regard, negative feedback loops that mimic a natural cellular mechanism to maintain a

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**Figure 3. Applications of genetic circuits in strain development.** (A) Quorum sensing elements were utilized to construct a metabolic toggle switch, which switches to the glucaric acid production phase from the growth phase. The transcriptional regulator EsaR170V can bind the Pes promoter and activate transcription. AHL-bound EsaR170V is released by the accumulation of AHL induced by cell density. The expression of pfk-I responsible for glycolysis was regulated by Pes. (B) Malonyl-CoA-dependent feedback loop was designed for fatty acid synthesis. The transcriptional regulator FabR and its cognate promoters dynamically regulate malonyl-CoA synthesis and fatty acid synthesis. (C) Acid-resistant mutants were evolved using evolutionary genetic circuits composed of a pH-responsive riboswitch and a site-specific recombinase. The recombinase allowed mutant strains with high intracellular pH to stop mutagenesis and express RFP.
Table 1. Application of genetic circuits in strain development.

<table>
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<tr>
<th>Product</th>
<th>Genetic elements</th>
<th>Input/output module</th>
<th>Input</th>
<th>Output</th>
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<th>References</th>
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<td>Expression of SspB, degradation of PfkA</td>
<td>Increasing G6P pool</td>
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<td>LacI/P&lt;sub&gt;Plac&lt;/sub&gt;, TetR/P&lt;sub&gt;PTet&lt;/sub&gt;</td>
<td>LBD of LacI/DBD of LacI, P&lt;sub&gt;plac&lt;/sub&gt;, LBD of TetR/DBD of TetR, P&lt;sub&gt;PTet&lt;/sub&gt;</td>
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<td>Knock-down of GHA, expression of product synthetic pathway genes</td>
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<td>Gluconic acid</td>
<td>Quorum sensing</td>
<td>LBD of EsaR170V/DBD of EsaR170V, P&lt;sub&gt;esas&lt;/sub&gt;</td>
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<td>Glucose-responsive promoter</td>
<td>LBD of CRP/DBD of CRP, P&lt;sub&gt;crp&lt;/sub&gt;</td>
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*The LBD and DBD are abbreviations of ligand-binding domain and DNA-binding domain, respectively.*
stable internal environment have been constructed (Dahl et al. 2013; Xu et al. 2014). Typically, a metabolic feedback loop can be constructed using TEs and their cognate promoters that respond directly to the target intermediate. If the promoter is activated by the intermediate, genes for its consumption can be expressed under the promoter. Alternatively, genes for the supply of the intermediate can be placed under the control of a promoter that is repressed by the intermediate.

Dahl et al. successfully improved amorphadiene production by introducing feedback regulation of the toxic intermediate FPP (Dahl et al. 2013). Initially, they investigated FPP-responsive promoters in the Escherichia coli genome via transcriptomic analysis. They found that several promoters were either activated (e.g. P_{tetA}) or repressed (e.g. P_{gadE}) by FPP. Thereafter, as described above, they expressed a gene encoding FPP-consuming amorphadiene synthase under P_{tetA}; they also expressed genes (MevT and MBIS) for FPP supply under P_{gadE} to avoid FPP accumulation. This dual feedback regulation showed 2-fold improved production of amorphadiene compared to that of the conventional system. More recently, Xu et al. used a FapR-based feedback loop to control the pool of malonyl-CoA, a key intermediate in fatty acid synthesis (Xu et al. 2014). They found that malonyl-CoA-bound FapR can either activate or repress the expression from P_{Fap} and P_{Tyr}, with FapO sites (Fig. 3B). Regulation of malonyl-CoA supply and consumption pathways under these promoters enabled dynamic optimization of fatty acid synthesis, resulting in a 2-fold increase in titer. Similarly, Zhou et al. constructed a feedback loop by using a repression-type lysine riboswitch to improve lysine production (Zhou and Zeng 2015). They expressed gldA in Corynebacterium glutamicum under control of the riboswitch. When the concentration of lysine was high, the expression of gldA was decreased; this accelerated lysine production by gradually decreasing carbon flux to the TCA cycle.

Evolutionary genetic circuit

Evolutionary genetic circuits have been designed to screen for high-performance producers from a large library via sensing the intracellular concentration of the desired product at a single-cell level (Jang et al. 2015; Lim et al. 2018). These circuits can accelerate the evolution of proteins, metabolic pathways and even microorganisms themselves toward biochemical production. As described above, fluorescence proteins or selection markers are employed as an output module to obtain high throughput (Yang et al. 2013; Rogers, Taylor and Church 2016; Seok et al. 2018).

In this regard, one recent study successfully improved 3-hydroxypropionic acid (3-HP) production by screening for an evolved aldehyde dehydrogenase (Seok et al. 2018). Initially, an evolutionary circuit was constructed with three molecular parts: a 3-HP responsive TF, its cognate promoter and a tetracycline resistance protein. Using this circuit, they screened for an improved enzyme by simple cultivation in tetracycline-containing medium. The resulting mutant aldehyde dehydrogenase (ALDH) exhibited 2.8-fold higher specific efficiency and a 25% increase in productivity. In a similar manner, Tang et al. improved triacetic acid lactone production (Tang et al. 2013). They screened for an evolved 2-pyrene synthase using an AraC-based genetic circuit and demonstrated a 19-fold increase of in the triacetate acid lactone production. Furthermore, a ribozyme-based approach was applied for glucosamine-6-phosphate (GlcN6P) production in Saccharomyces cerevisiae (Lee and Oh 2015). A self-cleaving ribozyme (gln5) in response to GlcN6P was integrated in 3’-untranslated region of FCY1, encoding cytosine deaminase. When the concentration of GlcN6P is not enough, FCY1, which produces toxic fluorouracil from fluorocytosine, can be expressed. Therefore, this circuit can be used to evolve GlcN6P-producing enzymes as only high producers can survive.

The evolutionary genetic circuit could be also employed for optimization of metabolic pathway for efficient production of target product. This pathway-level optimization was demonstrated by Yang et al. (2013). In their study, they combined a repression type lysine riboswitch with the tetracycline resistance gene (tetA) to construct a Riboselector. Using this device, a lysine high producer with low expression of tetA was enriched in the presence of nickel ion (tetracycline resistance protein is a dual-selectable marker; the cell that expresses tetA become resistant to tetracycline but is sensitive to nickel ion; Nomura and Yokobayashi 2007). They precisely tuned the activity of the anaerobic pathway to maximize lysine production. Raman et al. also demonstrated evolution-guided optimization of pathways (Raman et al. 2014). They prepared a library using the multiplex automated genome engineering (MAGE) system (Wang et al. 2009) to produce altered transcription and translation efficiency, start codons and a frame-shift mutation in the naringenin and glucaric acid production pathways. Screening of the improved strain under selection pressure yielded 36- and 22-fold increases, respectively.

Recent evolutionary genetic circuits were adapted to utilize randomly generated mutations similar to natural evolution rather than using a pre-determined library (Chou and Keasling 2013; Pham et al. 2017). Chou et al. developed a feedback-regulated system composed of mutator and reporter circuits for evolving phenotype (Chou and Keasling 2013). The mutator circuit was designed to regulate mutant DnaQ (proofreading exonuclease of DNA polymerase III) by tyrosine-responsive TF (TyrR), which allowed for adaptive evolution for tyrosine production. The reporter circuit consisted of the same TF and red fluorescent protein (RFP) as the output module, which was used to screen for the desired mutant. The evolved strain showed a 5-fold increased tyrosine production, and the same principle was successfully applied to isopentenyl diphosphate production, allowing a 3-fold increase in the titer. Similarly, adaptive evolution using a genetic circuit yielded an acid-tolerant phenotype (Pham et al. 2017). A pH-responsive riboswitch and site-specific recombinase were employed as an operation module and output module, respectively. The resulting circuit induced a state conversion from a mutagenesis state expressing the mutant DnaQ to a reporting state expressing RFP in mutants with the desired phenotype (Fig. 3C). These circuit-based systems achieved efficient library construction in a high-throughput manner, suggesting its potential for application in strain development.

CONCLUDING REMARKS

Various genetic circuits have been constructed by assembling well-characterized molecular parts for input, operation and output. Their construction allowed for programmable cellular behaviors in the presence of changing environmental signals. Moreover, their applications in metabolic engineering significantly improved biochemical production. Because an input module consisting of protein or RNA molecules is highly flexible and designable, the choice of the input signal can be expanded by excavation or re-engineering of naturally existing parts, or de novo design (Seo et al. 2014; Hanko, Minton and Maleys 2017; Jang et al. 2017). These efforts, which increase the number of
detectable chemicals, can enable the use of a genetic circuit in metabolic engineering.

The function of genetic circuits could be also expanded by employing novel proteins as output modules (Williams et al. 2015; Roquet et al. 2016; Rubens, Selvaggio and Lu 2016). The use of site-specific recombinase as an output module could allow the analogue-to-digital signal converter in a living cell (Rubens, Selvaggio and Lu 2016). In addition, genetic circuits that perform diverse functions such as threshold setting, a band-pass filter, multiple-input logic gate and 2-bit-computation have been demonstrated by use of recombinase (Rubens, Selvaggio and Lu 2016; Müller et al. 2017; Weinberg et al. 2017). Similarly, the utilization of genetic elements capable of performing complicated computation could be proposed or demonstrated in construction of genetic circuits; CRISPR/Cas9 system (Larson et al. 2013; Brophy and Voigt 2014; Weinberg et al. 2017), RNA polymerase (De Rubertis and Davies 2003) and chaperone (Wang, Barahona and Buck 2014).

Furthermore, more complex operations with multiple inputs can be performed by implementing a multi-layered logic gate (Lo et al. 2016; Hoynes-O’Connor et al. 2017). Notably, in addition to logic gates, other operation modules such as oscillators and transistors, which are essential in electronic circuit design, were recently developed in living cells (Müller et al. 2017). Such improvements will provide the ability to sketch more sophisticated biological networks, and maximize the potential of biorefinery for sustainable biochemical production.

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