Synthetic and Systems Biotechnology 5 (2020) 81–91

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/synbio

Toward fine-tuned metabolic networks in industrial microorganisms

Ning Li[a,b,c], Weizhu Zeng[a,b,c], Sha Xu[a,b,c], Jingwen Zhou[a,b,c,*]

[a] Key Laboratory of Industrial Biotechnology, Ministry of Education and School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu, 214122, China
[b] National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu, 214122, China
[c] Jiangsu Provisional Research Center for Bioactive Product Processing Technology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu, 214122, China

ARTICLE INFO

Keywords:
Fine-tuned regulation
Protein engineering
Upregulation
Downregulation
Dynamic regulation

ABSTRACT

There are numerous microorganisms in nature capable of synthesizing diverse useful compounds; however, these natural microorganisms are generally inefficient in the production of target products on an industrial scale, relative to either chemical synthesis or extraction methods. To achieve industrial production of useful compounds, these natural microorganisms must undergo a certain degree of mutation or effective fine-tuning strategies. This review describes how to achieve an ideal metabolic fine-tuned process, including static control strategies and dynamic control strategies. The static control strategies mainly focus on various matabolic engineering strategies, including protein engineering, upregulation/downregulation, and combinatrorial control of these metabolic engineering strategies, to enhance the flexibility of their application in fine-tuned metabolic networks. Then, we focus on the dynamic control strategies for fine-tuned metabolic networks. The design principles derived would guide us to construct microbial cell factories for various useful compounds.

1. Introduction

Microorganisms are widely used for the industrial production of useful chemicals. In the early stage of industrial biotechnology, high-producing strains are screened from the natural environment; however, natural microorganisms usually produce low titers and yields of target products. Additionally, accumulation of byproducts not only decreases the yield, but also makes the downstream process difficult. Mutagenesis and efficient screening techniques can significantly enhance microorganism performance [1]; however, the types of the products capable of being produced remain limited. To address these issues, engineering the microorganisms to achieve efficient production of target compounds has progressed, and the advent of genetic engineering has greatly promoted the development of the fermentation industry. Because microbial fermentation involves the actions of multiple enzymes in the microbial metabolic network, Stephanopoulos [2] and Bailey [3] proposed the concept of metabolic engineering. A major challenge in this field is the construction of appropriate metabolic pathways and regulation of the expression levels of specific genes in these pathways.

In earlier studies, directing metabolic flux to target products involved the use of simple metabolic engineering methods, including knockout and overexpression [4], to modify specific pathways. These methods achieved effective accumulation of target products, while reducing byproduct biosynthesis, resulting in high-performance strains. With continued metabolic modification of strains, researchers found that additional modifications could not further improve target-product accumulation, and that such modifications could lead to serious inhibition of cell growth, thereby adversely affecting target-product biosynthesis. Therefore, the balance of cofactors and energy became a topic of interest [5].

To achieve fine-tuned gene regulation in the metabolic network, various fine-tuned methods have become research hotspots in metabolic engineering and synthetic biology. However, fine-tuned metabolic networks relies on powerful and effective methods and has become a significant challenge. The rapid development of metabolic engineering and synthetic biology allowed the emergence of a variety of gene-regulation methods, some of which are used for gene fine-tuning (e.g., RNAi [6], multivariate modular engineering [7], and CRISPRi [8]). In this review, we first describe what is an ideal fine-tuned metabolic process and the current challenges to building the fine-tuning of metabolic networks, then summarized the relevant static strategies, including protein engineering strategies, upregulation/downregulation strategies and combinatrorial control of these metabolic engineering strategies. Finally, we focus on the dynamic regulationg of these strategies to realize the construction of a fine-tuned metablic networks. The design principles derived would guide us to construct microbial cell

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author. National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu, 214122, China.
E-mail address: zhoujw1982@jiangnan.edu.cn (J. Zhou).

https://doi.org/10.1016/j.synbio.2020.05.002
Received 26 December 2019; Received in revised form 30 March 2020; Accepted 6 May 2020

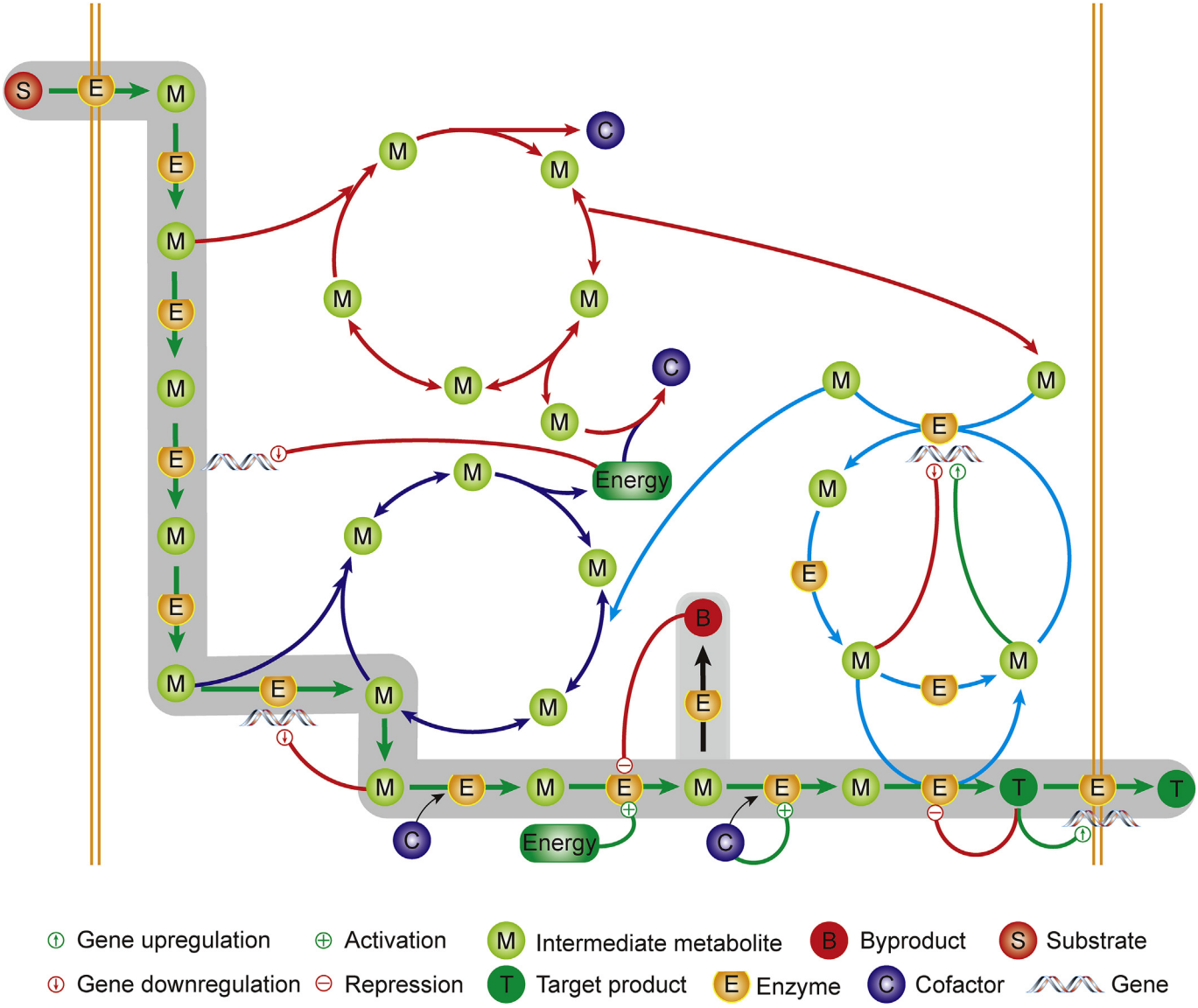

Fig. 1. Ideal fine-tuning metabolic engineering processing. A complete metabolic network contains multiple elements, including the production of target-product, enzyme, cofactor, and an energy source. The concentration of these elements affects the expression level of related genes or the activity of related enzymes. Fine-tuning of which plays an important role in improving target-product accumulation and reducing the byproduct synthesis.

factories for various useful compounds.

2. Current challenges to the fine-tuning of synthetic metabolic networks

An ideal metablic fine-tuned process needs the cooperation of many elements, including senor, transducer, actuator, resulting in a sensor-transducer-actuator system [9]. The sensor can sense environmental change, then the transducer convert signal to the actuator, the actuator actuat transcriptional events to compensate for metabolic activity. Many of these synthetic systems make up an ideal fine-tuning synthetic metabolic network (Fig. 1). The rapid development of metabolic engineering and synthetic biology has allowed the biosynthesis of multiple compounds; however, challenges still remain. The biosynthetic pathways of some compounds remain unknown or are unattainable in some microorganisms. Therefore, an important task involves constructing biosynthetic pathways. Additionally, rate-limiting steps and regulatory factors need to be discovered in order to allow fine-tuning of metabolic pathways (Fig. 2).

2.1. Metabolic pathway construction

Natural existed microorganisms can biosynthesize a wide variety of compounds. However, many of them can only produce little desired compounds and be hard to metabolic engineering, even there are many compounds that fail to find the appropriate biosynthetic pathways in microorganisms. It is premature to discuss how to fine-tune the metabolic network of these compounds which fail to find the appropriate biosynthetic pathways. Therefore, to obtain strains capable of high yields of target compounds, construction of biosynthesis pathways is the first step. Currently, enzymes involved in biosynthesis of target compounds are mostly screened from different sources [10]. The heterologous expression of enzymes may result in products suffering from improper folding, lack of activity, and poor specificity [11], all of which remain challenges to metabolic pathway construction. In addition to enzyme screening and selection, protein engineering provides avenues to address these challenges [12].

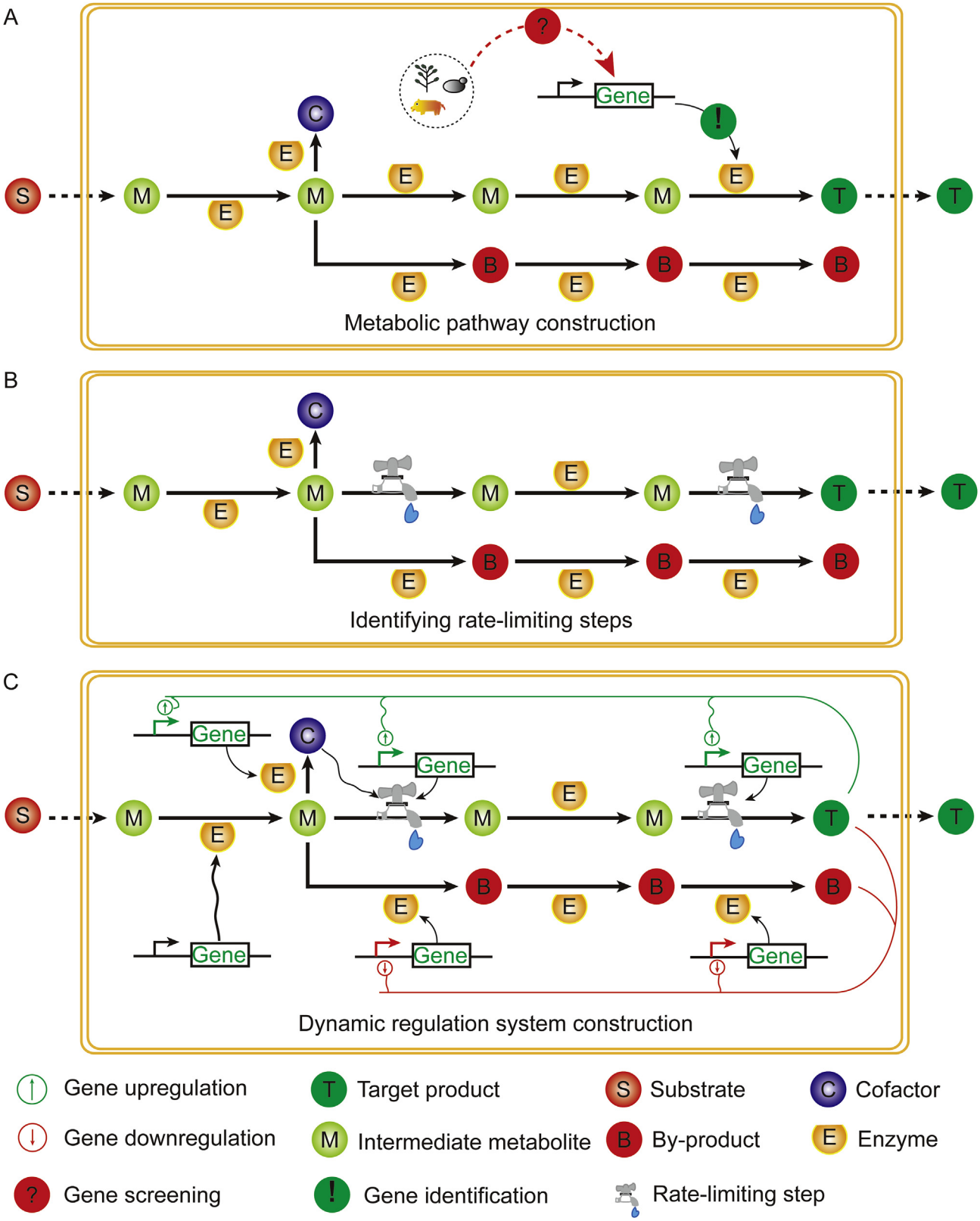

Fig. 2. Current challenges for the fine-tuning of metabolic networks. (A) Construction of metabolic pathway, includes screening and identification of enzymes involved in the pathway. (B) Identifying rate-limiting steps. (C) Construction of dynamic regulation system, includes response to the changes of target products and by-products, then dynamically up-regulate or down-regulate the related gene expression.

2.2. Identifying rate-limiting steps

Target-compound yield is dependent on enzyme-specific rate-limiting steps. To fine-tune metabolic networks, it is necessary to identify these steps and engineer alternative pathways to address their shortcomings in order to maximize target-product yield. Using CRISPR technology, it is possible to identify whether specific genes play an important role [13], and determination of intermediate metabolites also represents an intuitive method of identifying rate-limiting steps.

The development of -omics techniques enables detection and analysis of various factors and cellular metabolic components. Such methods include transcriptome-based identification of rate-limiting steps for feedback inhibition of yeast in order to increase lipid production [14], proteomics-based identification of two rate-limiting activities associated with inefficient isopentenol production [15], and comparative metabolomics to identify key metabolites involved in FK506 production [16]. Monitoring metabolic dynamics is an important step in understanding how cells respond to environmental changes on a real-time scale, which was accomplished by combining high-resolution mass spectrometry to identify evidence of switch-like feedback inhibition in amino acid biosynthesis [17]. Static and dynamic -omics methods can diagnose rate-limiting steps but can only analyze specific metabolic pathways. Complex cellular environments limit their ability; therefore, development of more powerful tools is necessary.

2.3. Dynamic regulation system construction

Currently, many regulatory strategies for fine-tuning metabolic networks focus on the static level, which leads to imbalanced metabolism under various environmental conditions. Dynamic regulation can maintain balanced metabolic flux. QS is a commonly used dynamic regulatory technique [18], but it might not be sufficient for the diversity of metabolic networks. Besides, other methods such as riboswitchs and biosensors also are used to construct the dynamic regulation system [19,20]. Development of methods for dynamic regulation is a slow process, and understanding native dynamic regulation networks in microorganisms is the basis for this development and very complicated. We believe that development of a dynamic metabolic regulatory system will maximize the synthesis of target products in microorganisms.

3. Static control strategies

3.1. Protein-engineering strategies

Target products can be synthesized efficiently in microorganisms according to specific metabolic pathways; however, some hosts lack enzymes related to target-product biosynthesis or harbor enzymes exhibiting low activity and high degrees of promiscuity. Here, we discuss acquisition of optimal enzymes using protein-engineering methods, including protein bioprospecting and directed evolution and rational engineering of proteins (Fig. 3). These protein-engineering strategies will help researchers to construct a series of biosynthetic pathways of compounds and even improve their biosynthesis efficiency [21].

3.1.1. Screening of specific proteins from the natural environment

Many useful compounds exist only in higher-level organisms, and wide availability of such compounds is limited by currently inefficient and high-cost extraction methods. Therefore, to produce these products efficiently by microorganisms, *de novo* designing optimal pathways for the products by screening of specific proteins from natural environment are essential [22]. However, the enzymes from different sources have different properties in the host, screening of the sources is also essential. To develop a pinoresinol bioconversion cascade in *Escherichia coli*, suitable enzymes needed to be identified for scavenging H_2O_2. Lv et al. [23] evaluated all available *E. coli* peroxidases from PeroxiBase and used these as queries against the *E. coli* BL21(DE3) genome sequence through BLAST, ultimately identified two peroxidases displaying efficient activity from eight that were screened from 20 homologous sequences. After genome sequencing, alignment, verification, and annotation of the *Ketogulonicigenium vulgare* WSH-001 genome, different combinations of five L-sorbose dehydrogenases (SDHs) and two L-sorbosone dehydrogenases (SNDHs) from *K. vulgare* WSH-001 were introduced into *Gluconobacter oxydans* WSH-003 for conversion of D-sorbitol to 2-keto-L-gulonic acid to replace the conventional two-step fermentation process with a one-step process, resulting in a good combination of SDH and SNDH [24]. The cited study successfully constructed the metabolic pathway for target products by using currently existing enzymes. However, these successful examples were limited by the library of natural enzymes. Furthermore, insolubility of heterologous proteins can be a major obstacle to efficient biosynthesis of target products. For these limitations, additional efficient enzyme-engineering strategies need to be developed.

3.1.2. Directed evolution of proteins

Introducing exogenous biosynthetic pathways into microorganisms can enable target-product production, such as the cases of caffeyl alcohol [25] and dihydrochalcones [26]. However, it is possible that some enzymes are incapable of such synthesis due to low substrate-binding capacity, temperature sensitivity, low catalytic efficiency, and promiscuity, resulting in low or no production of target compounds. Protein engineering describes the process of altering the structure of an existing protein to improve its properties [27], and includes three steps: selection of mutation sites, application of mutation methods, and evaluation of modification results. In cases of minimal knowledge regarding the protein structure, there will be a lack of a theoretical basis for the selection of mutation sites; therefore, directed evolution represents a suitable strategy.

Directed evolution [28] is a method that involves simulating and accelerating the natural evolution of enzymes in the laboratory. First, a mutation library of protein-coding genes is constructed using random-mutation methods, such as DNA shuffling [29], a staggered extension process [30], random-priming *in vitro* recombination [31], error-prone polymerase chain reaction (epPCR) [32], and rapidly efficient combinatorial oligonucleotides for directed evolution [33]. An efficient screening method was established for screening dominant mutants from a mutant library according to the intended experimental purposes. The tyrosine phenol lyase (TPL) can be used in the biocatalytic synthesis of L-DOPA, and TLP with high catalytic activity have been reported from several microbes, but substrates and product strongly inhibited TPL activity, resulting in the production of L-DOPA lower than expected. Using TPL derived from *Erwinia herbicola*, Zeng et al. [34] constructed a mutant library by epPCR. Then a high throughput screening method was established to screen high activity TPL, resulting in a L-DOPA high-producing strain. Additionally, to rapidly screen target mutants, a suitable reporter strain was established to perform screening in combination with fluorescence-activated cell sorting [35]. Directed evolution strategy can obtain enzymes with better performance, but it is also time-consuming and laborious. To reduce the workload, the most important strategy is either constructing an efficient screening method or reducing the size of the library.

3.1.3. Rational engineering of proteins

To obtain a protein that meets specific requirements, screening and directed evolution of existing proteins are common methods. Moreover, directed evolution does not require a thorough understanding of the structure and catalytic mechanism of the target enzymes, making it useful for engineering of many proteins. After a blind random mutation, a large library is constructed, followed by screening, with the screening workload determined the efficiency of the method. Therefore, a rational method that can reduce the size of the library needs to be established in order to spend the least amount of time achieving the desired goal. For

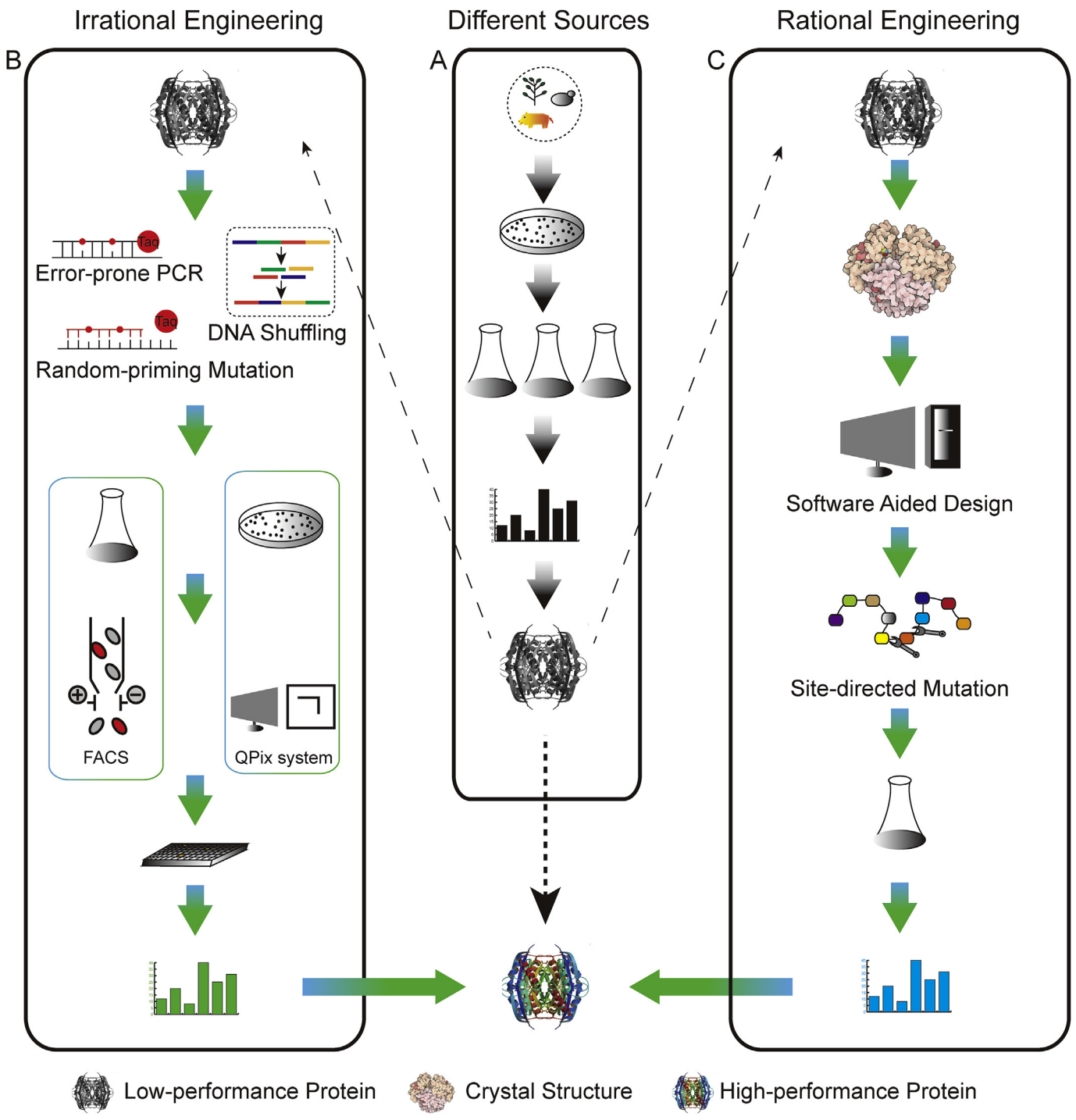

Fig. 3. Protein-engineering strategies. Required proteins can be obtained through (A) screening different sources from the natural environment, strain construction, fermentation culture, and product detection. (B) After directed evolution, strain isolation, fermentation culture, and product detection, a low-performance enzyme can be engineered to become a high-performance enzyme. (C) The crystal structure of a low-performance enzyme is prepared, and after software-aided design, site-directed mutagenesis, fermentation culture, and product detection are performed to obtain a high-performance enzyme.

this process, a thorough knowledge of the target protein is necessary for selection of important regions for engineering. Given the increased availability of protein structures, rational design and rational engineering methods have been widely applied to augment *de novo* protein design [36–38]. One-carbon assimilation pathway is a very important part of ecosystem, but it is difficult to introduce heterologous one-carbon assimilation pathway into the organisms that lack it, so that one carbon compound is the sole carbon source. Siegel et al. [39] created a new enzyme named formolase by *de novo* computational design which can catalyzes the carboligation of three formaldehyde into the central metabolite dihydroxyacetone phosphate (DHAP), resulting in a new pathway. Although protein engineering can change enzyme performance, the degree of the change is sometimes limited, suggesting that further improvements to these methods are required.

3.2. *Upregulation strategies*

To improve the metabolic flux of specific pathways in

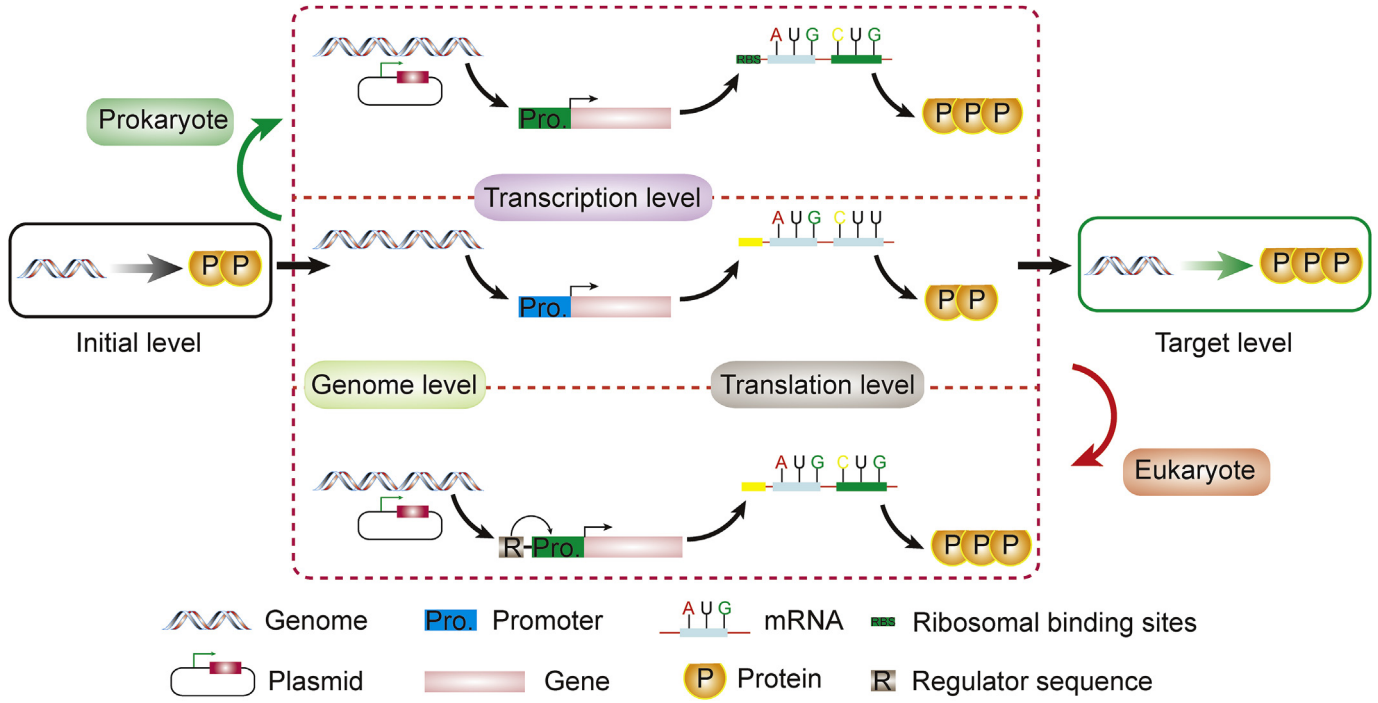

Fig. 4. Strategies for upregulating genes in industrial microorganisms. (A) For prokaryotes, gene upregulation can be achieved through plasmid expression, strong promoter replacement, strong RBS replacement, and codon optimization at the genome, transcription, and translation levels. (B) For eukaryotes, gene upregulation can be achieved through plasmid expression, regulatory sequence modification, strong promoter replacement, and codon optimization at the genome, transcription, and translation levels.

microorganisms, the most common method used involves enhanced expression of the specific genes involved in this pathway. Fig. 4 describes upregulation strategies, including vector-based, promoter-based, ribosome-binding site (RBS)-based, and codon-based strategies, that are involved in altering gene copy number, transcription initiation, translation initiation, and translation extension, respectively.

3.2.1. *Vector-based strategies*

Genes encode proteins following transcription to mRNA. Some organisms harbor multiple gene copies, which can facilitate high levels of expression under specific conditions. Therefore, increasing gene-copy number in the genome might represent a feasible strategy for upregulating expression levels. However, genome-specific increases in copy number are inefficient. Plasmids are a key aspect of genetic engineering, using a series of plasmids harboring different copy numbers to increase the gene-copy number [40], modular optimization strategies have improved the titer of some compounds, such as fatty acids [41] and terpenes [42]. To optimize (2S)-pinocembrin production from D-glucose, Wu et al. [43] used three modules in four vectors (pCDFDuet-1, pETDuet-1, pACYCDuet-1, and pRSFDuet-1) harboring different copy numbers. Guo et al. [44] designed a metabolic strategy using plasmids for the overexpression of two subunits of pyruvate dehydrogenase components in order to reduce the accumulation of pyruvate. Moreover, to increase the supply of 5-aminolevulinate, a precursor for haem synthesis, Zhao et al. [45] increased the expression of synthetic genes using high-copy plasmids to achieve higher accumulation of haem. Although multi-plasmid strategy can regulate gene-expression levels by increasing gene-copy number, this can also put a burden on strains.

3.2.2. *Promoter strategies*

Regulation of gene expression can be fine-tuned at various levels, including at the transcription and translation levels [46]. Transcriptional regulation alters metabolic flux by controlling the transcript abundance of key genes at their promoter regions [47]. Recently, increasing efforts have focused on promoter alteration, which attempts to modulate transcriptional capacity through mutation or direct insertion of a different promoter [48]. Different promoters affect transcription differently, making their selection a critical component of metabolic engineering design [49]. Previous studies screened promoters displaying different strength, followed by selection according to the requirements of gene expression in metabolic pathways [50]. Strong promoters can overexpress specific genes. The T7 promoter [51] is a high-strength phage-derived promoters often used for overexpression of heterologous proteins in prokaryotes, whereas the strong constitutive promoters TEF [52], GPD [53], and HXT7 [54] are typically used for metabolic engineering purposes in *Saccharomyces cerevisiae*. Hu et al. [55] screened promoters by proteomics analysis, resulting in overexpression of D-sorbitol dehydrogenase in *Gluconobacter oxydans* following incorporation of the new promoter and resulting in increased titers and productivity of L-sorbose synthesis from D-sorbitol. Upregulation of gene-expression levels can be achieved using specific promoters, with different strengths of the promoters capable of regulating expression levels. However, different promoters have different properties in different strains, making their use frequently strain-specific. Additionally, promoter screening is time-consuming, thereby making its practice a limiting factor in engineering processes.

3.2.3. *RBS-based strategies*

RNA translation involves four steps: initiation, elongation, termination, and ribosome turnover. Translation initiation is the rate-limiting step and determined by multiple interactions, including hybridization of 16S ribosomal RNA to the ribosome binding site (RBS) and binding of translation RNA (tRNA) to the start codon [56]. Efficient translation initiation is primarily determined by the RBS, which comprises the sequence upstream of AUG/GUG in an mRNA transcript and that can be accurately recognized by the ribosome [57]. Therefore, gene upregulation can be achieved by RBSs exhibiting high ribosomal-binding capacity. To upregulate gene expression via 104 promoters with varying strengths, Zhou et al. [58] integrated strong transcriptional promoters harboring strong RBSs. To improve *recET* expression in

Corynebacterium glutamicum, Wang et al. [59] incorporated RBSs with different strength and ultimately achieved heterologous expression of *recET* at an appropriate level. By incorporating high-strength RBSs, specific gene expression can be upregulated to fine-tune the regulation of metabolic flux; however, results can differ between strains like promoter. Therefore, the universality of this strategy requires further improvement.

3.2.4. Codon-based strategies

Protein translation occurs following generation of gene transcripts. Promoter strength and mRNA stability determine the levels of intracellular mRNA, which affect translation efficiency [60]. Synonymous codon usage in protein-coding sequences are not random, and codon-usage bias is a distinct feature of the genome of each organism [61]. Recent studies report codon usage as a factor involved in mRNA stability [62]. Besides, each codon is matched to a specific tRNA, and limited concentrations of tRNAs targeting rare codons in mRNAs will reduce the translation rate. Therefore, codon distribution in mRNA influences protein translation and ultimately determines the level of gene expression [63]. Codon optimization can increase protein expression, suggesting that codon usage plays an important role in gene expression [64]. In addition to the influence on gene expression, non-optimal codon usage can toxic to strains. Mittal et al. [65] found many synonymous variants that are toxic to *E. coli*. Unlike previous studies of the effect of synonymous mutations, the effect is independent of translation, but it depends on the production of toxic mRNA molecules. Finally, the toxicity was prevented by reducing mRNA level. These phenomena showed that codon optimization can improve gene expression, but sometimes it can also cause the production of toxic mRNA, which is not conducive to gene expression.

3.3. Downregulation strategies

During target-product synthesis, some metabolic pathways compete with others for metabolic flux, making it necessary to limit the metabolic flux of competing pathways. Downregulation genes is a conventional method used to accomplish this. In addition to some strategies such as promoter strategies and codon-based strategies similar to upregulation strategies, there are sRNA-based strategies and CRISPR-based strategies, with Fig. 5 showing strategies used for this activity.

3.3.1. sRNA-based strategies

Regulatory RNAs are key modulators of gene expression through their interactions with DNA, RNA, proteins, and metabolites at every step of gene expression. These regulatory RNAs include synthetic sRNA, RNA interference (RNAi), and antisense RNA (asRNA). sRNA includes a MicC scaffold and a target-specific sequence. The MicC scaffold recruits the Hfq protein, which binds the scaffold to impede translation of the target mRNA bound to sRNA via Watson–Crick base-pairing [66]. RNAi involves activity by the RNA-silencing complex (RISC) to silence translation of specific mRNAs and can be used to fine-tune the regulation of metabolic flux following binding of small-interfering RNA to mRNA targets, thereby restricting RISC-mediated translation. Moreover, asRNA binding of mRNA targets impedes ribosome binding to the mRNA, thereby blocking translation [67].

The 6S RNA in *E. coli* represents an sRNA that interacts directly with α^{70}-RNA polymerase and is involved in downregulating the expression of many genes [68]. In *E. coli*, > 80 sRNAs have been identified [69,70], resulting in construction of a database and investigation into their use as regulatory mechanisms in synthetic biology. *Clostridium* is a promising microbial host for the production of valuable industrial chemicals; however, genetic manipulation of *Clostridium* is very difficult. Cho et al. [71] developed an sRNA-based system to fine-tune gene expression in *Clostridium acetobutylicum*, where knockdown of the *adhE1* and *pta* genes decreased acetic acid production and enhanced butanol production. For prokaryotes, such as *E. coli*, use of sRNA allows rapid downregulation of gene expression [72]; however, in higher-order eukaryotes, RNAi is used to fine-tune gene expression by reducing

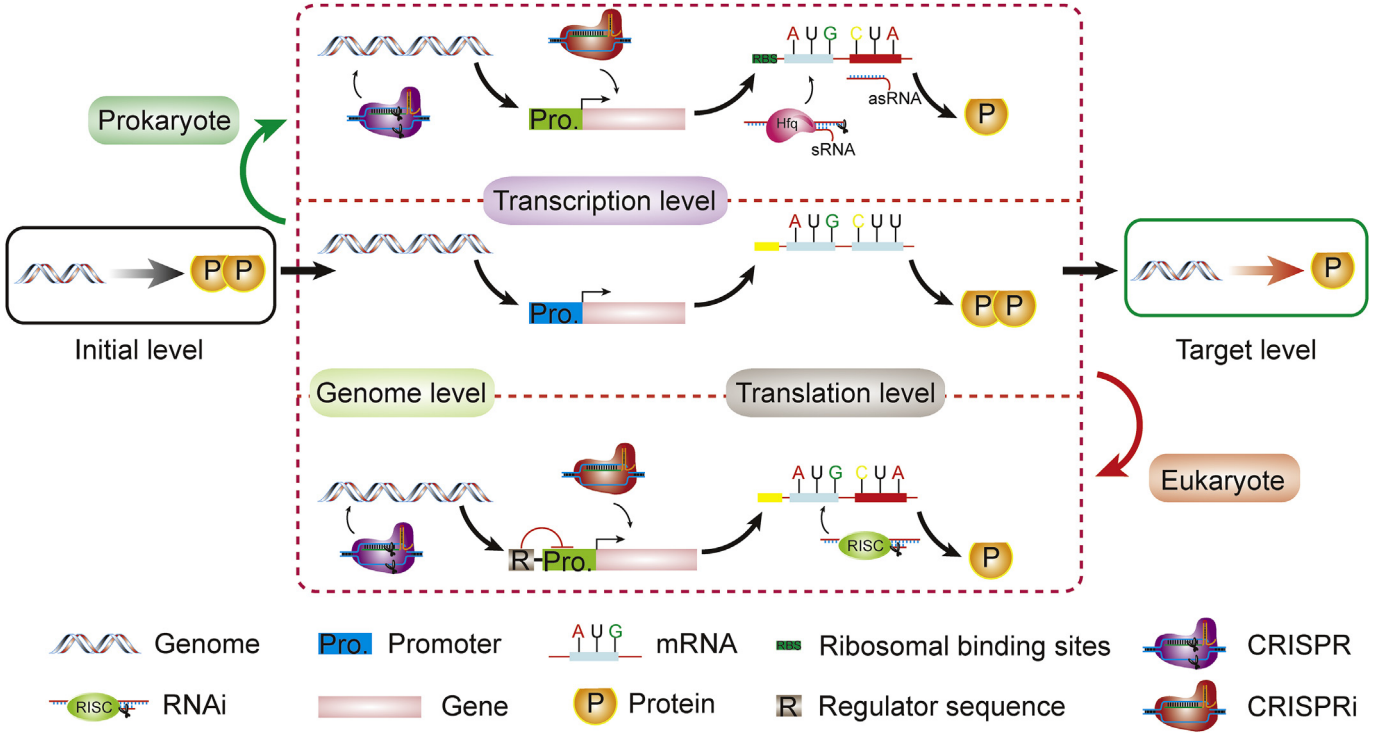

Fig. 5. Strategies for downregulating genes in industrial microorganisms. (A) For prokaryotes, gene downregulation can be achieved using CRISPR, CRISPRi, weak-promoter replacement, weak-RBS replacement, rare-code substitution, sRNA, and antisense RNA at the genome, transcription, and translation levels. (B) For eukaryotes, gene downregulation can be achieved using CRISPR, CRISPRi, regulatory sequence modification, weak-promoter replacement, rare-code substitution, and RNA interference at the genome, transcription, and translation levels.

mRNA levels. Crook et al. [73] optimized a yeast RNAi system to construct a series of hairpin RNA-expression cassettes and then used RNAi to quickly identify metabolic pathways associated with itaconic acid production, which also reduced the cost of the design-build-evaluate cycle in yeast. Wu et al. [74] established an efficient asRNA system that balanced the need for malonyl-CoA between cell growth and target-product biosynthesis. Despite its effectiveness, sRNA specificity remains a limitation; therefore, further development of RNA-based strategies is necessary.

3.3.2. CRISPR-based strategies

CRISPR is used as a defensive system for prokaryotes, including bacteria and archaea [75]. In 2013, Cong et al. [76] engineered two different type II CRISPR systems and created CRISPR/Cas9 for use in humans and mice. Mali et al. [77] engineered the type II bacterial CRISPR system in human cells, whereas Hwang et al. [78] reported the ability of the CRISPR-Cas system to function *in vivo* to induce targeted genetic modifications in zebrafish. This method remains a precise technique for genome editing in various biological systems, including humans [79], mice [80], yeast [81], Bacillus subtilis [82], *E. coli* [83], and *Caenorhabditis elegans* [84]. CRISPR enables rapid genome editing in order to obtain knockout strains, thereby enabling its use to fine-tune gene expression. In the CRISPR/Cas9 system, Cas9 is a nuclease capable of cleavage of target DNA. The CRISPRi system includes a catalytically inactive Cas9 protein (dCas9), with the dCas9–single-guide RNA capable of binding target DNA but be not able to cut it, resulting in hindered RNA polymerase activity [85]. Sander et al. [86] used CRISPRi to silence candidate genes and increase arginine production.

CRISPR has been a great benefit to synthetic biology and other fields; however, there remain issues concerning off-target effects. Recent versions of the Cas9 protein have been designed, including Sniper-Cas9 [87], eSpCas9(1.1) [88], and SpCas9-HF1 [89], that reduce the off-target efficiency of the CRISPR system. To enhance CRISPR-Cas9 accuracy, Chen et al. [90] designed a new hyper-accurate Cas9 variant (HypaCas9) to investigate the mechanism of target discrimination, finding that minor changes in the REC3 domain of Cas9 affected differences in on- and off-target editing. It is expected that continued optimization of the CRISPR system will enhance its role in synthetic biology and metabolic engineering.

3.4. Combinatorial control strategies

For multi-gene pathways, it is very difficult to balance metabolic flux. Flexible use of the above up/down regulation strategies would be an important and innovative method. To increase fatty acid production in *E. coli*, Xu et al. blocked the fatty acid degradation pathway and relieved the allosteric inhibition of acetyl-CoA carboxylase, but only a minor increase in fatty acid production [91]. The coexpression of glycolysis pathway and fatty acid biosynthesis pathway indicated that the imbalance between acetyl-CoA supply and malonyl-CoA consumption resulted in low fatty acid production. Considering the large subsets of engineering targets, exhaustively exploring the entire metablic space is impractical. To circumvent these limitations, Xu et al. report a modular engineering strategy that divided the fatty acid biosynthesis pathway into three modules: acetyl-CoA formation module, acetyl-CoA activation module, fatty acid biosynthase module [91]. Using the ePathBrick gene assembly and pathway construction [92], the three modules were successfully on the five compatible vectors, with varying promoter strength, plasmid copy number, antibiotic resistance marker, and additional RBS strength. These results indicated that the balance of the multiple gene pathways metabolism can be achieved through combinatorial control.

4. Dynamic control strategies

Metabolic engineering and synthetic biology endow many microbes with the ability to produce a wide variety of useful compounds. To reduce metabolic flow to non-target products, previous studies focused on direct overexpression and knockdown/knockout of target genes [93]. However, some genes are essential for cell growth, and their knockdown/knockout adversely affects cell growth/survival [94]. However, excess overexpression of a gene can also burden microorganisms [95]. Therefore, it is important to use appropriate metabolic regulation methods to regulate genes at the right time in order to avoid unintended consequences.

4.1. Identifying naturally existing dynamic regulatory networks

Microorganisms have evolved exquisited regulatory networks and exhibit a variety of metabolic activation, repression, and regulation activities induced by the surrounding environment [96]. Quorum sensing (QS) is a dynamic regulatory system in bacteria used to coordinate complex population-wide phenotypes through space and time [97]. QS constitutes cell-cell communication achieved through a group of signaling molecule called autoinducers, such as 3-oxohexanoylhomoserine lactone (AHL) synthesized by LasI [98], and that allows microorganisms to sense their population and actuat gene transcription. Additionally, microorganisms can react to changes in metabolite/substrate levels in a process called substrate-feedback regulation. Cellular malonyl-CoA levels represent a metabolic signal that regulates the expression of genes involved in mediating fatty acid synthesis, such as *fad* and *fab* in *E. coli* [99] and *fap* in *Bacillus subtilis* [100]. Furthermore, microorganisms harbor a variety of other regulatory systems that could potentially be utilized in engineering applications.

4.2. Reconstruction of dynamic regulation networks

There are many native biological systems in microorganisms, we can mimick those and use a dynamic regulatory nework to eventually construct a microbial cell factory. However, the native biological systems control the metabolic networks of the original strains, and are difficult to fine-tuning regulate the new metabolic network directly [101]. Therefore, it is necessary to modify such systems in a way suitable for other strains. Gupta et al. [102] integrated parts of the QS system from *Pantoea stewartii* into the *E. coli* genome, resulting in dynamic control of endogenous essential genes involved in glycolysis and aromatic amino acid biosynthesis. Victor et al. [103] reengineered the native QS regulon to guide high-level production of rhamnolipid biosurfactant in *Burkholderia thailandensis* E264. To address the severe metabolic burden on host cells after genetic modification, QS can be linked with an RNAi module to enable target-gene silencing after reaching high cell-population density [104]. These examples show the applications of QS to enable dynamic regulation of target genes following introduction of an exogenous QS system.

In addition to QS, substrate feedback plays an important role in regulating metabolic networks. Recently, De et al. [105] created chimeric biosensors for the desired luteolin-specific response by introducing parts of a donor regulatory circuit from *Sinorhizobium meliloti*. Their use of a modified biosensor achieved a specific luteolin transition from 27.5% to 95.3%. QS and substrate feedback use one sensor to regulate gene expression; however, in complex intracellular systems, metabolic pathways are not controlled by a single factor, but are often regulated coordinately by several factors. Therefore, the combination of several different factors capable of regulating a system will constitute an adequate construction of a dynamic control system.

4.3. Logic gate regulation

Regulation of gene expression can be described as a form of logical gate. Genetic logic gates enable several input signals to be integrated [106] and can integrate multiple input signals, followed by their integration and translation into a specific activity involving one or more

genes. This could be used to determine levels of dissolved oxygen and substrates in order to determine gene-expression profiles. Wang et al. [107] constructed a novel real-time control system for gene regulation in *E. coli* that included an enhancement element (duplex DNA aptamers and an upstream promoter) and a repression element (RNA aptamers and an upstream RBS). Ligand (thrombin) binding to the duplex DNA aptamers initiated target-gene expression, whereas ligand (vascular endothelial growth factor) binding to the RNA aptamer repressed mRNA translation. Combining the two approaches in one gene regulatory system allowed control based on different concentrations of multiple ligands. Logic gate regulation can integrate signals and respond to changes in environmental conditions; however, the discovery and modification of regulatory factors and related trans-acting elements restricts its development.

One central goal of synthetic biology is to integrate multiple gene circuits and achieve dynamic control in living biological systems. As a carbon donor, many value-added compounds have been synthesized by regulating the metabolism of malonyl-CoA. Malonyl-CoA is a precursor of fatty acid biosynthesis, the regulation of the malonyl-CoA source pathway and sink pathway is very necessary for fatty acid production. Xu et al. designed a T7-based malonyl-CoA that can sense malonyl-CoA based on the trans-regulatory protein FapR and the cis-regulatory element *fapO* [108]. Next another malony-CoA sensor based on pGAP also was engineered based on FapR and *fapO* [101]. The pGAP-based malonyl-CoA sensor was markedly different from the T7-based malonyl-CoA. When the level of malonyl-CoA was low, FapR activated the transcriptional activity of the pGAP-based promoter and repressed the transcriptional activity of the T7-based promoter, then the transcription levels were opposite when the malonyl level was high. By intergrating the malonyl-CoA-regulated T7 and pGAP promoter, a logic gate regulation of fatty acid production was constructed in *E. coli*, resulting in an oscillatory malonyl-CoA pattern and a balance between cell growth and fatty acid production.

5. Future perspective

Building a variety of useful compound-producing strains to replace chemical synthesis is a goal of metabolic engineers and synthetic biologists. However, the main problems they are facing are the construction of target product biosynthesis pathway and the improvement of biosynthesis efficiency. For the construction of biosynthesis pathway, the selection of enzymes that can catalyze specific reactions is the basis. In addition, the specificity and catalytic activity of the enzymes have an important influence on the biosynthesis efficiency. In order to improve the efficiency of biosynthesis, metabolic engineers and synthetic biologists are also trying to replace those complex and inefficient biosynthesis pathways with simpler and more efficient ones, all of which would be achieved through the above mentioned protein-engineering stategies [109].

Through protein engineering, we can construct the target biosynthesis pathways and slightly improve the biosynthesis efficiency. In addition to the performance of enzymes, the imbalance of metabolism also affects the biosynthesis efficiency. Therefore, the biosynthesis efficiency problem still needs the assistance and cooperation of fine-tuned strategies to get a more perfect solution. Previous studies have shown that static control strategies can solve the problem of metabolic imbalance, while dynamic control strategies can solve it better [110]. Many systems are based on synthetic promoters and inducible transcription factors, which are limited by the number of metabolite-responsive transcriptional factors. No biological process has the opportunity to equip such systems. Although similar dynamic control systems can be used in different strains, specific methods should be constantly constructed and improved. The metabolite-responsive transcriptional factors identified by traditional sequencing, comparison and analysis can not meet the needs of the rapid development of synthetic biology. With the maturity of next-generation sequencing technology, the improvement of high-throughput screening technology and the extensive application of artificial intelligence, it will be faster and more efficient to screen and identify highly specific and efficient enzymes, construct and optimize the dynamic regulatory system if we can reasonably use these technologies [111].

Acknowledgements

This work was supported by the National Key Research and Development Program of China (2017YFC1600403), the National Science Fund for Excellent Young Scholars (21822806), the National Natural Science Foundation of China (31670095, 31770097), the Fundamental Research Funds for the Central Universities (JUSRP51701A), the National First-class Discipline Program of Light Industry Technology and Engineering (LITE2018-08).

References

[1] Li YJ, Wei HB, Wang T, Xu QY, et al. Current status on metabolic engineering for the production of L-aspartate family amino acids and derivatives. Bioresour Technol 2017;245:1588–602.
[2] Stephanopoulos G, Vallino JJ. Network rigidity and metabolic engineering in metabolite overproduction. Science 1991;252:1675–81.
[3] Bailey JE. Toward a science of metabolic engineering. Science 1991;252:1668–75.
[4] Abbott DA, Suir E, Duong GH, de Hulster E, et al. Catalase overexpression reduces lactic acid-induced oxidative stress in *Saccharomyces cerevisiae*. Appl Environ Microbiol 2009;75:2320–5.
[5] Larson MH, Gilbert LA, Wang XW, Lim WA, et al. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat Protoc 2013;8:2180–96.
[6] Karunanithi S, Oruganti V, Marker S, Rodriguez-Viana AM, et al. Exogenous RNAi mechanisms contribute to transcriptome adaptation by phased siRNA clusters in *Paramecium*. Nucleic Acids Res 2019;47:8036–49.
[7] Ajikumar PK, Xiao W-H, Tyo KEJ, Wang Y, et al. Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. Science 2010;330:70–4.
[8] Tian T, Kang JW, Kang A, Lee TS. Redirecting metabolic flux via combinatorial multiplex CRISPRi-mediated repression for isopentenol production in *Escherichia coli*. ACS Synth Biol 2019;8:391–402.
[9] Lv YK, Qian S, Du GC, Chen J, et al. Coupling feedback genetic circuits with growth phenotype for dynamic population control and intelligent bioproduction. Metab Eng 2019;54:109–16.
[10] Scalcinati G, Knuf C, Partow S, Chen Y, et al. Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquitepene alpha-santalene in a fed-batch mode. Metab Eng 2012;14:91–103.
[11] Hummel A, Brüsehaber E, Böttcher D, Trauthwein H, et al. Isoenzymes of pig-liver esterase reveal striking differences in enantioselectivities. Angew Chem, Int Ed Engl 2007;46:8492.
[12] Gajewski J, Buelens F, Serdjukow S, Janssen M, et al. Engineering fatty acid synthases for directed polyketide production. Nat Chem Biol 2017;13:363.
[13] Huang JF, Shen ZY, Mao QL, Zhang XM, et al. Systematic analysis of bottlenecks in a multibranched and multilevel regulated pathway: the molecular fundamentals of l-methionine biosynthesis in *Escherichia coli*. ACS Synth Biol 2018;7:2577–89.
[14] Peng HD, He LZ, Haritos VS. Flow-cytometry-based physiological characterisation and transcriptome analyses reveal a mechanism for reduced cell viability in yeast engineered for increased lipid content. Biotechnol Biofuels 2019;12.
[15] George KW, Chen A, Jain A, Batth TS, et al. Correlation analysis of targeted proteins and metabolites to assess and engineer microbial isopentenol production. Biotechnol Bioeng 2014;111:1648–58.
[16] Xia ML, Huang D, Li SS, Wen JP, et al. Enhanced FK506 production in *Streptomyces tsukubaensis* by rational feeding strategies based on comparative metabolic profiling analysis. Biotechnol Bioeng 2013;110:2717–30.
[17] Link H, Fuhrer T, Gerosa L, Zamboni N, Sauer U. Real-time metabolome profiling of the metabolic switch between starvation and growth. Nat Methods 2015;12:1091–7.
[18] Pietschke C, Treitz C, Foret S, Schultze A, et al. Host modification of a bacterial quorum-sensing signal induces a phenotypic switch in bacterial symbionts. Proc. Natl. Acad. Sci. U.S.A 2017;114:E8488–97.
[19] Dwidar M, Yokobayashi Y. Riboswitch signal amplification by controlling plasmid copy number. ACS Synth Biol 2019;8:245–50.
[20] Li L, Tu R, Song G, Cheng J, et al. Development of a synthetic 3-dehydroshikimate biosensor in *Escherichia coli* for metabolite monitoring and genetic screening. ACS Synth Biol 2019;8:297–306.
[21] Walther T, Topham CM, Irague R, Auriol C, et al. Construction of a synthetic metabolic pathway for biosynthesis of the non-natural methionine precursor 2,4-dihydroxybutyric acid. Nat Commun 2017;8:15828.
[22] Wu JJ, Du GC, Zhou JW, Chen J. Systems metabolic engineering of microorganisms to achieve large-scale production of flavonoid scaffolds. J Biotechnol 2014;188:72–80.
[23] Lv Y, Cheng X, Du G, Zhou J, Chen J. Engineering of an H2O2 auto-scavenging in vivo cascade for pinoresinol production. Biotechnol Bioeng 2017;114:2066–74.
[24] Gao LL, Hu YD, Liu J, Du GC, et al. Stepwise metabolic engineering of

Gluconobacter oxydans WSH-003 for the direct production of 2-keto-L-gulonic acid from D-sorbitol. Metab Eng 2014;24:30–7.
[25] Chen ZY, Sun XX, Li Y, Yan YJ, Yuan QP. Metabolic engineering of *Escherichia coli* for microbial synthesis of monolignols. Metab Eng 2017;39:102–9.
[26] Eichenberger M, Lehka BJ, Folly C, Fischer D, et al. Metabolic engineering of *Saccharomyces cerevisiae* for de novo production of dihydrochalcones with known antioxidant, antidiabetic, and sweet tasting properties. Metab Eng 2017;39:80–9.
[27] Kazlauskas RJ, Bornscheuer UT. Finding better protein engineering strategies. Nat Chem Biol 2009;5:526–9.
[28] Bassalo MC, Liu RM, Gill RT. Directed evolution and synthetic biology applications to microbial systems. Curr Opin Biotechnol 2016;39:126–33.
[29] Stemmer WP. Rapid evolution of a protein in vitro by DNA shuffling. Nature 1994;370:389.
[30] Zhao H, Giver L, Shao Z, Affholter JA, Arnold FH. Molecular evolution by staggered extension process (StEP) in vitro recombination. Nat Biotechnol 1998;16:258.
[31] Shao Z, Zhao H, Giver L, Arnold FH. Random-priming in vitro recombination: an effective tool for directed evolution. Nucleic Acids Res 1998;26:681.
[32] Wintrode PL, Miyazaki K, Arnold FH. Cold adaptation of a mesophilic subtilisin-like protease by laboratory evolution. J Biol Chem 2000;275:31635–40.
[33] Jin P, Kang Z, Zhang JL, Zhang LP, et al. Combinatorial evolution of enzymes and synthetic pathways using one-step PCR. ACS Synth Biol 2016;5:259–68.
[34] Zeng W, Xu B, Du G, Chen J, Zhou J. Integrating enzyme evolution and high-throughput screening for efficient biosynthesis of L-DOPA. J Ind Microbiol Biotechnol 2019;46:1631–41.
[35] Kortmann M, Mack C, Baumgart M, Bott M. Pyruvate carboxylase variants enabling improved lysine production from glucose identified by biosensor-based high-throughput fluorescence-activated cell sorting screening. ACS Synth Biol 2019;8:274–81.
[36] Hilvert D. Design of protein catalysts. Annu Rev Biochem 2013;82:447–70.
[37] Davey JA, Chica RA. Multistate approaches in computational protein design. Protein Sci 2012;21:1241–52.
[38] Chae TU, Choi SY, Kim JW, Ko Y-S, Lee SY. Recent advances in systems metabolic engineering tools and strategies. Curr Opin Biotechnol 2017;47:67–82.
[39] Siegel JB, Smith AL, Poust S, Wargacki AJ, et al. Computational protein design enables a novel one-carbon assimilation pathway. Proc. Natl. Acad. Sci. U.S.A 2015;112:3704–9.
[40] Tolia NH, Joshua-Tor L. Strategies for protein coexpression in *Escherichia coli*. Nat Methods 2006;3:55–64.
[41] Xu JZ, Zhang JL, Guo YF, Zai YG, Zhang WG. Improvement of cell growth and l-lysine production by genetically modified *Corynebacterium glutamicum* during growth on molasses. J Ind Microbiol Biotechnol 2013;40:1423–32.
[42] Zhou YJ, Gao W, Rong Q, Jin G, et al. Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. J Am Chem Soc 2012;134:3234–41.
[43] Wu JJ, Du GC, Zhou JW, Chen J. Metabolic engineering of *Escherichia coli* for (2S)-pinocembrin production from glucose by a modular metabolic strategy. Metab Eng 2013;16:48–55.
[44] Guo HW, Madzak C, Du GC, Zhou JW, Chen J. Effects of pyruvate dehydrogenase subunits overexpression on the alpha-ketoglutarate production in *Yarrowia lipolytica* WSH-Z06. Appl Microbiol Biotechnol 2014;98:7003–12.
[45] Zhao XR, Choi KR, Lee SY. Metabolic engineering of *Escherichia coli* for secretory production of free haem. Nat. Cata. 2018;1:720–8.
[46] Auslander S, Auslander D, Fussenegger M. Synthetic biology-the synthesis of biology. Angew Chem, Int Ed Engl 2017;56:6396–419.
[47] Ptashne M, Gann A. Transcriptional activation by recruitment. Nature 1997;386:569–77.
[48] Rytter JV, Helmark S, Chen J, Lezyk MJ, et al. Synthetic promoter libraries for *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 2014;98:2617–23.
[49] Blazeck J, Alper HS. Promoter engineering: recent advances in controlling transcription at the most fundamental level. Biotechnol J 2013;8:46.
[50] Zhou SH, Lyu YB, Li HZ, Koffas MAG, Zhou JW. Fine-tuning the (2S)-naringenin synthetic pathway using an iterative high-throughput balancing strategy. Biotechnol Bioeng 2019;116:1392–404.
[51] Jin HJ, Lindblad P, Bhaya D. Building an inducible T7 RNA polymerase/T7 promoter circuit in *Synechocystis sp.* PCC6803. ACS Synth Biol 2019;8:655–60.
[52] Gatignol A, Dassain M, Tiraby G. Cloning of *Saccharomyces cerevisiae* promoters using a probe vector based on phleomycin resistance. Gene 1990;91:35–41.
[53] Tang YL, Ren WK, Li FN, Liu G, et al. Methionine deficiency in culture medium promotes apoptosis in intestinal epithelial cells infected with enterotoxigenic *Escherichia coli*. Amino Acids 2013;45. 603-603.
[54] Reifenberger E, Freidel K, Ciriacy M. Identification of novel HXT genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on qlycolytic flux. Mol Microbiol 1995;16:157.
[55] Hu YD, Wan H, Li JG, Zhou JW. Enhanced production of L-sorbose in an industrial *Gluconobacter oxydans* strain by identification of a strong promoter based on proteomics analysis. J Ind Microbiol Biotechnol 2015;42:1039–47.
[56] Vianna JF, Bezerra KS, Oliveira JIN, Albuquerque EL, Fulco UL. Binding energies of the drugs capreomycin and streptomycin in complex with tuberculosis bacterial ribosome subunits. Phys Chem Chem Phys 2019;21:19192–200.
[57] Eriksen M, Sneppen K, Pedersen S, Mitarai N. Occlusion of the ribosome binding site connects the translational initiation frequency, mRNA stability and premature transcription termination. Front Microbiol 2017;8:7.
[58] Zhou SH, Ding RP, Chen J, Du GC, et al. Obtaining a panel of cascade promoter-5'-UTR complexes in *Escherichia coli*. ACS Synth Biol 2017;6:1065–75.
[59] Wang B, Hu Q, Zhang Y, Shi R, et al. A RecET-assisted CRISPR-Cas9 genome editing in *Corynebacterium glutamicum*. Microb Cell Factories 2018;17:63.
[60] Gingold H, Pilpel Y. Determinants of translation efficiency and accuracy. Mol Syst Biol 2011;7:481.
[61] Ikemura T. Codon usage and tRNA content in unicellular and multicellular organisms. Mol Biol Evol 1985;2:13–34.
[62] Rojano Nisimura AM, Haning K, Janovsky J, Vasquez KA, et al. Codon selection affects recruitment of ribosome-associating factors during translation. ACS Synth Biol 2019;9:329–42.
[63] Yu CH, Dang Y, Zhou Z, Wu C, et al. Codon usage influences the local rate of translation elongation to regulate co-translational protein folding. Mol Cell 2015;59:744.
[64] Bornelov S, Selmi T, Flad S, Dietmann S, Frye M. Codon usage optimization in pluripotent embryonic stem cells. Genome Biol 2019;20.
[65] Mittal P, Brindle J, Stephen J, Plotkin JB, Kudla G. Codon usage influences fitness through RNA toxicity. Proc. Natl. Acad. Sci. U.S.A 2018;115:8639–44.
[66] Cameron TA, Matz LM, Sinha D, De Lay NR. Polynucleotide phosphorylase promotes the stability and function of Hfq-binding sRNAs by degrading target mRNA-derived fragments. Nucleic Acids Res 2019;47:8821–37.
[67] Wang MM, Yu HM, Shen ZY. Antisense RNA-based strategy for enhancing surfactin production in *Bacillus subtilis* TS1726 via overexpression of the unconventional biotin carboxylase II to enhance ACCase activity. ACS Synth Biol 2019;8:251–6.
[68] Hindley J. Fractionation of 32P-labelled ribonucleic acids on polyacrylamide gels and their characterization by fingerprinting. J Mol Biol 1967;30:125.
[69] Parker A, Cureoglu S, De Lay N, Majdalani N, Gottesman S. Alternative pathways for *Escherichia coli* biofilm formation revealed by sRNA overproduction. Mol Microbiol 2017;105:309–25.
[70] Lee HJ, Gottesman S. sRNA roles in regulating transcriptional regulators: lrp and SoxS regulation by sRNAs. Nucleic Acids Res 2016;44:6907–23.
[71] Cho C, Lee SY. Efficient gene knockdown in *Clostridium acetobutylicum* by synthetic small regulatory RNAs. Biotechnol Bioeng 2017;114:374.
[72] Na D, Yoo SM, Chung H, Park H, et al. Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs. Nat Biotechnol 2013;31:170–4.
[73] Crook NC, Schmitz AC, Alper HS. Optimization of a yeast RNA interference system for controlling gene expression and enabling rapid metabolic engineering. ACS Synth Biol 2014;3:307.
[74] Wu JJ, Yu O, Du GC, Zhou JW, Chen J. Fine-tuning of the fatty acid pathway by synthetic antisense RNA for enhanced (2S)-Naringenin production from L-tyrosine in *Escherichia coli*. Appl Environ Microbiol 2014;80:7283–92.
[75] Brouns SJJ, Jore MM, Lundgren M, Westra ER, et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 2008;321:960–4.
[76] Cong L, Ran FA, Cox D, Lin SL, et al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013;339:819–23.
[77] Mali P, Yang LH, Esvelt KM, Aach J, et al. RNA-guided human genome engineering via Cas9. Science 2013;339:823–6.
[78] Hwang WY, Fu YF, Reyon D, Maeder ML, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 2013;31:227–9.
[79] Cho SW, Kim S, Kim JM, Kim JS. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 2013;31:230–2.
[80] Wang HY, Yang H, Shivalila CS, Dawlaty MM, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-Mediated genome engineering. Cell 2013;153:910–8.
[81] Zhang Y, Wang J, Wang Z, Zhang Y, et al. A gRNA-tRNA array for CRISPR-Cas9 based rapid multiplexed genome editing in *Saccharomyces cerevisiae*. Nat Commun 2019;10:1053.
[82] Liu D, Huang C, Guo J, Zhang P, et al. Development and characterization of a CRISPR/Cas9n-based multiplex genome editing system for *Bacillus subtilis*. Biotechnol Biofuels 2019;12:197.
[83] Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 2013;31:233–9.
[84] Friedland AE, Tzur YB, Esvelt KM, Colaiacovo MP, et al. Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. Nat Methods 2013;10:741–3.
[85] Gilbert LA, Larson MH, Morsut L, Liu ZR, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 2013;154:442–51.
[86] Sander T, Wang CY, Glatter T, Link H. CRISPRi-based downregulation of transcriptional feedback improves growth and metabolism of arginine overproducing *E. coli*. ACS Synth Biol 2019;8:1983–90.
[87] Lee JK, Jeong E, Lee J, Jung M, et al. Directed evolution of CRISPR-Cas9 to increase its specificity. Nat Commun 2018;9:3048.
[88] Jensen KT, Floe L, Petersen TS, Huang JR, et al. Chromatin accessibility and guide sequence secondary structure affect CRISPR-Cas9 gene editing efficiency. FEBS Lett 2017;591:1892–901.
[89] Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature 2016;529:490.
[90] Chen JS, Dagdas YS, Kleinstiver BP, Welch MM, et al. Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. Nature 2017;550:407–10.
[91] Xu P, Gu Q, Wang W, Wong L, et al. Modular optimization of multi-gene pathways for fatty acids production in *E. coli*. Nat Commun 2013;4:1409.
[92] Xu P, Vansiri A, Bhan N, Koffas MAG. ePathBrick: a synthetic biology platform for engineering metabolic pathways in *E. coli*. ACS Synth Biol 2012;1:256–66.
[93] Diaz CAC, Bennett RK, Papoutsakis ET, Antoniewicz MR. Deletion of four genes in *Escherichia coli* enables preferential consumption of xylose and secretion of glucose. Metab Eng 2019;52:168–77.
[94] Zhang Y, Shang X, Lai S, Zhang Y, et al. Reprogramming one-carbon metabolic pathways to decouple l-serine catabolism from cell growth in *Corynebacterium glutamicum*. ACS Synth Biol 2018;7:635–46.

[95] Takeno S, Hori K, Ohtani S, Mimura A, et al. L-Lysine production independent of the oxidative pentose phosphate pathway by *Corynebacterium glutamicum* with the *Streptococcus mutans gapN* gene. Metab Eng 2016;37:1–10.
[96] Xu P. Production of chemicals using dynamic control of metabolic fluxes. Curr Opin Biotechnol 2018;53:12–9.
[97] Torres-Cerna CE, Morales JA, Hernandez-Vargas EA. Modeling quorum sensing dynamics and interference on *Escherichia coli*. Front Microbiol 2019;10.
[98] Chuang SK, Vrla GD, Frohlich KS, Gitai Z. Surface association sensitizes *Pseudomonas aeruginosa* to quorum sensing. Nat Commun 2019;10.
[99] Fujita Y, Matsuoka H, Hirooka K. Regulation of fatty acid metabolism in bacteria. Mol Microbiol 2007;66:829–39.
[100] Schujman GE, Paoletti L, Grossman AD, de Mendoza D. FapR, a bacterial transcription factor involved in global regulation of membrane lipid biosynthesis. Dev Cell 2003;4:663–72.
[101] Xu P, Li LY, Zhang FM, Stephanopoulos G, Koffas M. Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. Proc. Natl. Acad. Sci. U.S.A 2014;111:11299–304.
[102] Gupta A, Reizman IMB, Reisch CR, Prather KLJ. Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit. Nat Biotechnol 2017;35:273.
[103] Victor IU, Kwiencien M, Tripathi L, Cobice D, et al. Quorum sensing as a potential target for increased production of rhamnolipid biosurfactant in *Burkholderia thailandensis* E264. Appl Microbiol Biotechnol 2019;103:6505–17.
[104] Williams TC, Averesch NJH, Winter G, Plan MR, et al. Quorum-sensing linked RNA interference for dynamic metabolic pathway control in *Saccharomyces cerevisiae*. Metab Eng 2015;29:124–34.
[105] De Paepe B, Maertens J, Vanholme B, De Mey M. Chimeric LysR-Type transcriptional biosensors for customizing ligand specificity profiles toward flavonoids. ACS Synth Biol 2019;8:318–31.
[106] Smanski MJ, Zhou H, Claesen J, Shen B, et al. Synthetic biology to access and expand nature's chemical diversity. Nat Rev Microbiol 2016;14:135–49.
[107] Wang J, Cui X, Yang L, Zhang Z, et al. A real-time control system of gene expression using ligand-bound nucleic acid aptamer for metabolic engineering. Metab Eng 2017;42:85–97.
[108] Xu P, Wang W, Li L, Bhan N, et al. Design and kinetic analysis of a hybrid promoter-regulator system for malonyl-CoA sensing in *Escherichia coli*. ACS Chem Biol 2014;9:451–8.
[109] Li C, Zhang R, Wang J, Wilson LM, Yan Y. Protein engineering for improving and diversifying natural product biosynthesis. Trends Biotechnol 2020. https://doi.org/10.1016/j.tibtech.2019.12.008.
[110] Miano A, Liao MJ, Hasty J. Inducible cell-to-cell signaling for tunable dynamics in microbial communities. Nat Commun 2020;11:1193.
[111] Zeng W, Guo L, Xu S, Chen J, Zhou J. High-throughput screening technology in industrial biotechnology. Trends Biotechnol 2020. https://doi.org/10.1016/j.tibtech.2020.01.001.