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Synthetic Biology Toolkits for Metabolic Engineering of Cyanobacteria

Peng-Fei Xia, Hua Ling, Jee Loon Foo, and Matthew Wook Chang**

Cyanobacteria are of great importance to Earth's ecology. Due to their capability in photosynthesis and C1 metabolism, they are ideal microbial chassis that can be engineered for direct conversion of carbon dioxide and solar energy into biofuels and biochemicals. Facilitated by the elucidation of the basic biology of the photoautotrophic microbes and rapid advances in synthetic biology, genetic toolkits have been developed to enable implementation of nonnatural functionalities in engineered cyanobacteria. Hence, cyanobacteria are fast becoming an emerging platform in synthetic biology and metabolic engineering. Herein, the progress made in the synthetic biology toolkits for cyanobacteria and their utilization for transforming cyanobacteria into microbial cell factories for sustainable production of biofuels and biochemicals is outlined. Current techniques in heterologous gene expression, strategies in genome editing, and development of programmable regulatory parts and modules for engineering cyanobacteria towards biochemical production are discussed and prospected. As cyanobacteria synthetic biology is still in its infancy, apart from the achievements made, the difficulties and challenges in applying and developing genetic toolkits in cyanobacteria for biochemical production are also evaluated.

1. Introduction

Cyanobacteria are among the most ancient organisms on Earth and they have been the main players in major biogeochemical cycles through history, including those of carbon, nitrogen, and oxygen. Particularly, cyanobacteria orchestrate the global and local carbon cycle via either direct assimilation of carbon dioxide (CO_2) or photosynthesis-mediated biocalcification.[1] Due to these features, they have attracted much interest in understanding the molecular mechanisms and employing the newfound knowledge in various applications. By studying cyanobacteria, C1 metabolism-related innovations were inspired by the CO_2-fixing Calvin cycle,[2,3] the physiology in circadian rhythm is revealed through the diurnal oscillation,[4,5] and, notably, cyanobacteria themselves have been engineered as a microbial chassis for simultaneous chemical production and atmospheric CO_2 assimilation.[6] In doing so, engineered cyanobacteria could directly utilize atmospheric CO_2 and sunlight as carbon and energy sources, respectively, to produce a wide variety of biofuels and biochemicals without competing with food supply and for arable land to obtain sugar-based carbon sources, thus contributing to carbon conservation and reduction of greenhouse gases.

Driven by rapid advances in synthetic biology, toolkits have been developed for cyanobacteria and played important roles in the progress of metabolic engineering of cyanobacteria for production of valuable chemicals. Standardized components, such as promoters and ribosome-binding sites (RBSs), have been defined in cyanobacteria[7,8] and pathways can be assembled in vitro or in *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*).[9] Basic methodologies have been adapted from other microbes to cyanobacteria (**Figure 1**A), and advanced genetic tools are emerging.[6] As most of the model cyanobacteria strains are naturally transformable, designed genetic materials can be transferred relatively easily into target hosts directly or through conjugation for genetic engineering of cyanobacteria.[10,11] Consequently, in the past decade, production of biofuels and value-added chemicals by engineered cyanobacteria have accelerated, particularly in the model strains *Synechococcus elongatus* PCC7942 (*Synechococcus* 7942), *Synechococcus elongatus* PCC7002 (*Synechococcus* 7002), and *Synechocystis* sp. 6803 (*Synechocystis* 6803).[6,12] For instance, cyanobacteria have been engineered to produce fuels and fuel precursors, such as ethanol,[13,14] isobutyraldehyde,[15] butanol,[16,17] alkenes,[18] fatty acids and lipids,[19,20] and the inventory of products has been expanded to value-added chemicals for manufacturing, food, pharmaceuticals, and cosmetics, e.g., isoprene,[21] squalene,[22] L-lysine,[23] limonene,[24] and lactic acid.[25] Besides the increasingly diverse products, mixotrophic

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growth of these photoautotrophic strains has been achieved by the installation of sugar-utilizing pathways,[26,27] thus establishing synthetic hybrid cyanobacterial platforms capable of continuous growth in diurnal conditions with significantly improved metabolic robustness.[27] These endeavors demonstrate the immense potential of cyanobacteria as a biochemical production host and signal the rise of cyanobacteria as an ideal autotrophic platform for sustainable chemical production.[28]

Herein, we review the synthetic biology toolkits for enabling metabolic engineering in model cyanobacterial strains, including conventional methodologies and recent innovations in gene expression, genome editing, and programmable regulations. Particularly, we focus on providing insights into how synthetic biology toolkits have facilitated strain engineering of cyanobacteria for biochemical production. As cyanobacteria synthetic biology is still an emerging field, the challenges in developing toolkits for taking metabolic engineering of the microbe to the next level are also discussed.

2. Expression of Heterologous Genes

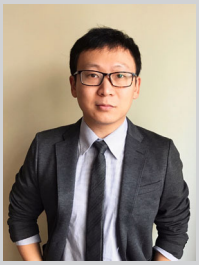
Successful implementation of synthetic metabolic pathways in microbial hosts for bioproduction requires expression of heterologous genes, typically by cloning them into plasmids or integrating them into chromosomes. In *E. coli* and *S. cerevisiae*, self-replicating plasmids are the preferred workhorses for gene expression, especially when high expression levels and sophisticated regulations are necessary, while chromosomal expressions are used to ensure genetic stability. Unlike these model microbes, heterologous expression in cyanobacteria largely relies on gene integration, either into the chromosomes or endogenous plasmids; self-replicating vectors are still relatively limited for cyanobacteria. In this section, we outline how advances in the synthetic biology toolkits for gene expression in cyanobacteria confer desired functionalities and the challenges present.

2.1. Chromosomal Expression

The strategies for heterologous gene expression in cyanobacteria are summarized in **Table 1**. The most commonly used expression method is based on the integration of heterologous genes into neutral sites (NSs) on chromosomes, which are typically intergenic sites or nontranscribing sequences with negligible effect on the cell physiology upon modification. Typically, heterologous genes are carried by locus-targeting vectors and integrated into NSs or loci of interest by homologous recombination (HR). After segregation, a homogenous population with the integrated genes will be obtained (Figure 1B). Several NSs have been identified and employed in cyanobacteria strains for heterologous gene expression without disruption of cellular physiology, including five frequently used NSs in *Synechococcus* and three in *Synechocystis* (Table 1). For a single NS, Tsujimoto et al.[33] demonstrated the insertion of a large 20.8 kb gene cluster into the slr2030-31 site in *Synechocystis* 6803, illustrating the ability to integrate large DNA cassettes into NS for chromosomal expression. To facilitate chromosomal expression, Kim et al.[46] developed an improved system, SyneBrick vectors, for the integration of genes into NS1–3 and to enable inducible expression using the TetR, LacI, and T7 systems in *Synechococcus* 7942. Recent developments have vastly diversified the range of available integrative vectors. Standardized genetic parts, including homologous arms, repressors, activators, promoters, and terminators, can be easily assembled with the vectors using commercialized methods, such as Golden Gate assembly and Gibson assembly,[47,48] and vectors carrying large gene clusters can be constructed using DNA assembler in *S. cerevisiae*.[9,49]

By employing the tools developed for chromosomal expression of heterologous genes, the inventory of biofuels and biochemicals produced in cyanobacteria has been greatly expanded.[6,15,22,23,27,29] For instance, Atsumi et al.[15] expressed four heterologous genes in NS1 and NS2 along with an extra copy of the endogenous *rbcLS* in NS4 (Table 1), leading to a high yield of isobutyraldehyde directly from CO_2. Squalene production was achieved by employing three NSs to express four genes[22] and the synthesis of 3-hydroxybutyrate was realized by expressing five genes in two NSs.[30]

Recently, the model cyanobacterium *Synechococcus* 7942 has been engineered to utilize organic carbon sources, including glucose, xylose, and glycerol,[26,35,50] to grow continuously



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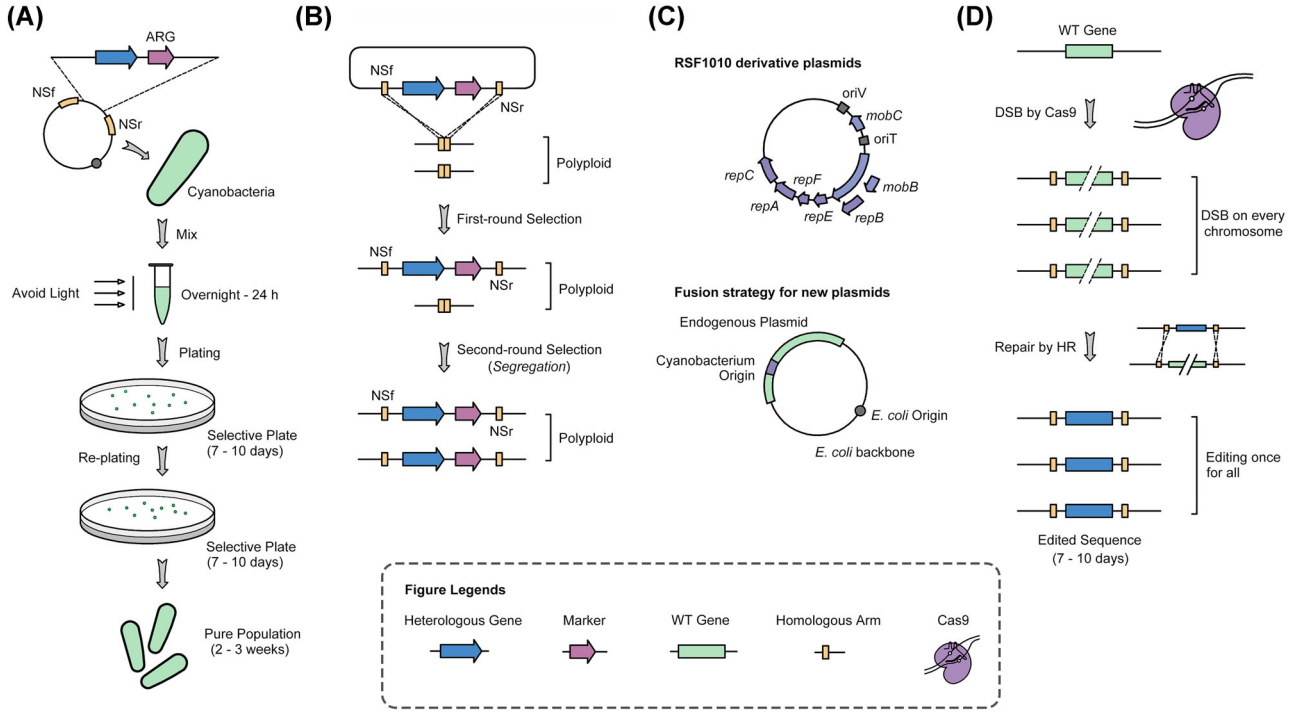

Figure 1. Schemes for cyanobacteria molecular biology. A) Basic laboratory methods for the engineering of cyanobacteria. The scheme depicts the protocol for *Synechococcus*; for *Synechocystis*, avoiding light is not required for transformation. B) Scheme for chromosomal expressions via the integration of target genes into NSs. C) The frequently used RSF1010 plasmid and the strategy to develop novel self-replicating plasmids. D) Class II Type II CRISPR-based genome editing. Briefly, Cas9 cuts the targeting sequences on each chromosome under the guidance of sgRNAs. Then, the donor DNAs, either designed for insertion, deletion, or site mutation, repair the DSBs, accomplishing one round selection genome editing in cyanobacteria. NSf, forward homologous arm of NSs; NSr, reverse homologous arm of NSs; ARG, antibiotic resistance genes; DSBs, double-strand breaks; WT, wild type.

under diurnal conditions. These engineered mixotrophic strains exhibited better performance in chemical production.[27] Besides enhanced production of target chemicals, it also opens the possibility of cultivating cyanobacteria in a natural environment with day–night cycles. In a different approach to improve the metabolism in cyanobacteria, an extra C1 utilization pathway, the 3-hydroxypropionate bicycle, was introduced into *Synechococcus* 7942 to supplement the Calvin cycle, leading to enhanced cellular robustness for biochemical production.[36] However, these endeavors are still in their infancy and have areas that need further improvement. The implementation of sugar utilization pathways increases the cost of feedstock and operational complexity. Additionally, it may cause an imbalance of innate metabolisms and accumulation of toxic intermediate metabolites, such as ribulose-1,5-bisphosphate and methylglyoxal.[35] Although much effort is needed to resolve these challenges, the advances and achievements made in engineered mixotrophic strains signal the potential of transforming the autotrophic cyanobacteria into a hybrid phototrophic chassis for biofuel and biochemical production with higher productivity and yield.

2.2. Episomal Expression

Although chromosomal expression confers genetic stability, which is advantageous and essential for long-term cultivation and bioproduction applications, it is insufficient to accomplish all desired tasks. Compared with chromosomal expression, episomal expression usually exhibits higher expression, which is vital for high bioproduction performance. To this end, endogenous plasmids, which exist in most cyanobacteria, can be exploited for high-level gene expression. Heterologous genes have been integrated into sites on these plasmids to elevate expression levels. Nozzi et al.[31] compared these gene expression approaches by implementing the 2,3-butanediol production pathway at an integrative site on the chromosome and on the endogenous plasmid pAQ1 in *Synechococcus* 7002. The highest titer of the compound was achieved using the high-copy-number pAQ1, exemplifying the potential of using endogenous plasmids for enhancing gene expression and biochemical production.

Self-replicating plasmids, which are more commonly used in model hosts such as *E. coli* and *S. cerevisiae*, are promising alternatives for high-level expression of heterologous genes. For instance, Lin et al. combined the utilization of both an endogenous plasmid and an exogenous plasmid to produce limonene,[24] and Miao et al.[16] achieved higher yield and titer of isobutanol when a synthetic pathway was expressed on an RSF1010-derived pEEK2 plasmid. In addition to high expression levels, self-replicating plasmids are easy to manipulate, and hence can be used to rapidly generate different pathway designs for functional studies and optimization. This was exemplified by a

Table 1. Advances in the metabolic engineering of cyanobacterial chassis.

Strain	Strategy[a]	Loci/vector[b]	Genes	Marker[c]	Target/objective	Titers	References
Bioconversion							
Synechococcus 7942	Epi	pCB4	*pdc*, *adh*	$Spec^R$, Kan^R, Cm^R	Ethanol	70 mg L^{-1}	[13]
	Chr	NS1, NS2, NS4	*kivD*, *ilvCD*, *alaS*, *rbcLS*	$Spec^R$, Kan^R, Cm^R	Isobutyraldehyde	1.1 g L^{-1}	[15]
	Chr	NS1, NS5	*alaSD*, *adh*, *xylEAB*	$Gent^R$, $Spec^R$	2,3-Butanediol	5.7 g L^{-1}	[36]
	Chr	NS2	*idi*, *ispS*, *dxs*	$Spec^R$	Isoprene	1.3 g L^{-1}	[21]
	Chr	NS1, NS2, NS3	*dxs*, *idi*, *ispA*, *SQS*	$Spec^R$, Kan^R, Cm^R	Squalene	0.05 g L^{-1}	[22]
	Chr	NS1, NS2, NS3	*sps*, *glgC*, *cscB*	$Spec^R$, $Gent^R$, Cm^R	Sucrose	2.6 g L^{-1}	[29]
	Chr	NS1, NS2	*nphT7*, *tesB*, *phaB*, *phaA*, *pptesB*	$Spec^R$, Kan^R	3-Hydroxybutyrate	1.2 g L^{-1}	[30]
Synechococcus 7002	Chr + Epi	NS5, *AquI*, *Mrr*/ pAQ1	*alaSD*, *adh*	$Gent^R$, Kan^R	2,3-Butanediol	1.6 g L^{-1}	[31]
	Chr	*fadD*	Truncated *tesA*, *rbcLS*	$Spec^R$	Fatty acid	0.1 g L^{-1}	[20]
	Chr	*A2543*, *A1838*, *A2842*[d]	*ybjE*, *lysC*, *dapa*, *ppc*	$Gent^R$, Kan^R	L-Lysine	0.4 g L^{-1}	[23]
Synechocystis 6803	Chr	*phaAB* (*slr1992*, *slr1993*)	*Pdc*, *slr1192*	Kan^R	Ethanol	5.5 g L^{-1}	[14]
	Chr/Epi	*slr1556*/RSF1010	*kivd*, *adh*	Kan^R	Isobutanol	0.6 g L^{-1}	[16]
	Chr	*slr0168*	*ldh*	$Spec^R$	Lactic acid	0.8 g L^{-1}	[25]
	Chr	*slr0168*, *slr1556*	*sll0208*, *sll0209*, *sll1969*	Kan^R, $Spec^R$	Alkene	26 mg L^{-1}	[18]
	Chr + Epi	pCC5.2/RSF1010	*Lim*, *rpi*, *rpe*, *gpps*	Kan^R, $Spec^R$	Limonene	7 mg L^{-1}	[24]
	Chr	*slr2132*	*tesA137*	KanR[e]	Fatty acid	197 mg L^{-1}	[19]
	Chr + Epi	*psbA1*, *slr2030-31*/ RSF1010	*dCas9*, *sgRNA*, *maqu*, *DPW*	$Spec^R$, Kan^R, Cm^R	Fatty alcohol	10.3 mg g^{-1}	[32]
	Chr	*slr2030-31*, *psbA1*	*nif* gene cluster,[f] *cnfR*	$Spec^R$, Kan^R	Nitrogen fixation	—	[33]
Synechococcus 2973	Chr	*M744_RS12430*	*cscB*	Cm^R	Sucrose	3.3 g L^{-1}	[34]
Hybrid chassis							
Synechococcus 7942	Chr	NS1	*galP*, *xylEAB*	Spec	Sugar utilization	—	[26]
	Chr	NS1, NS2	*glpFKD*, *gldA*, *tpiA*, *dhaKLM*	Kan^R, $Spec^R$	Glycerol utilization	—	[35]
	Chr	NS1, NS3	*mcr*, *mcl*, *mch*, *mct*, *meh*, *pcs*	$Spec^R$, $Gent^R$	3-HPA bicycles	—	[36]
Tools							
Synechococcus 7942	Epi	RS1010	*flp*	$Spec^R$	FLP/FRT	—	[37]
	Chr	NS1, NS2	*araC*, *araBAD*	$Spec^R$, Cm^R	*araBAD* system	—	[38]
	Epi	pCas9, pSyn-1	*cas9*, *donor DNA*	Cm^R, $Spec^R$	CRISPR	—	[39]
	Chr	NS1, NS2	*dcas9*, *sgRNA*	Cm^R, $Spec^R$	CRISPRi	—	[40]
Synechococcus 7002	Chr	NS1, *AcsA*, *GlpK*	*dcas9*, *sgRNA*	Cm^R, $Spec^R$	CRISPRi	—	[41]
Synechocystis 6803	Chr	*ndhB*	*rhaS*	Kan^R	*rhaBAD* system	—	[42]
	Chr	*psbA1*, *slr2030-31*, *slr0168*	*dcas9*, *sgRNA*	Kan^R, Cm^R	CRISPRi	—	[43]
	Epi	RS1010	*cpf1*, *sgRNA*	Kan^R	CRISPR	—	[44]
Synechococcus 2973	Epi	RS1010, pCRISPomyces-2	*cas9*, *sgRNA*	Kan^R, Am^R	CRISPR	—	[45]
	Epi	RS1010	*cpf1*, *sgRNA*	Kan^R	CRISPR	—	[44]

[a]Expression strategies include Chr (chromosomal), Epi (episomal, including endogenous plasmids and self-replicating ones), and Chr + Epi (combined strategy);
[b]The genome loci in *Synechococcus* 7942 are denoted as NS. The basic information includes: NS1 (*Synepcc7942_2497-2498*), NS2 (*Synepcc7942_0084-0085*), NS3 (*Synepcc7942_1428-1429*), NS4 (*Synepcc7942_1428-1429*), and NS5 (*Synepcc7942_0893-0894*, denoted as NSIII and NS in the original papers). *Synechococcus* 7002 share the same NS with *Synechococcus* 7942. The plasmids are described in the original backbones rather than the names used in the publication. pCas9, pSyn-1, and pCRISPomyces-2 are used for transient expressions which cannot replicate in cyanobacteria;
[c]Markers include Amp^R (ampicillin resistance), $Spec^R$ (spectinomycin resistance), Kan^R (kanamycin resistance), Cm^R (chloramphenicol resistance), $Gent^R$ (gentamicin resistance), and Am^R (apramycin resistance);
[d]A2543, A1838, and A2842 are abbreviations of Synepcc7002_ A2543, Synepcc7002_ A1838, and Synepcc7002_ A2842, respectively;
[e]This study employed the knock-in/out markerless editing and the Kan^R marker was removed after the *tesA137* was integrated;
[f]The *nif* gene cluster contains 25 genes.

recent study showing a self-replicating plasmid carrying a *nif* gene cluster containing 35 genes from *Cyanothece* sp. ATCC 51142 facilitated the identification of the minimum *nif* genes required for nitrogen fixation.[9] Moreover, self-replicating plasmids can harbor helper genes, such as recombinase and nuclease, to facilitate strain engineering.[34,37,44,45]

To date, self-replicating vectors employed in cyanobacteria have been largely limited to RSF1010 derivatives, but novel shuttle vectors have been constructed by combining an endogenous cyanobacterial plasmid with another of *E. coli* origin[51] (Figure 1C). For example, a self-replicating shuttle vector based on the small endogenous plasmid pANS was designed specifically for *Synechococcus* 7942,[52] and two shuttle vectors were developed for *Synechocystis* 6803 based on the endogenous plasmids pCA2.4 and pCB2.4.[53] Recently, a comprehensive broad-host-range cyanobacterial vector system has been developed with in silico tools to aid design, assembly, and construction of customized vectors based on improved RSF1010 replicons and cyanobacterial replicons from endogenous plasmids, including pDU1, pFDA, pDC1, and pANS.[54] Such effort will promote the use of shuttle vectors for more cyanobacterial species by facilitating the creation of a wider variety of cyanobacterial shuttle vectors that may be compatible with different cyanobacteria species. However, although the inventory of self-replicating plasmids is expanding, propelled by the fast development of synthetic biology and metabolic engineering in cyanobacteria, examples of plasmid usage in cyanobacteria are still limited, especially in *Synechococcus*, which suggests challenges in developing exogenic vectors that can be stably maintained.[55]

2.3. Chromosomal Versus Episomal Expression

Chromosomal and episomal gene expressions both have their strengths and weaknesses. The reliability of chromosomal expression in cyanobacteria inherently surpasses episomal expression for long-term cultivation and applications due to the possibility of plasmid loss.[56] Therefore, chromosomal pathway expression is particularly important for bioproduction of biofuels and biochemicals because the lengthy bioprocess and exposure to molecular stressors and inhibitors may increase plasmid instability. However, it is difficult to concurrently employ multiple genetic sites for strain engineering with current integration approaches, thus chromosomal integration for expression of heterologous genes to assemble synthetic pathways is hampered. One reason is the limited choice of antibiotic markers, which is necessary for selecting transformants, and the other is the inhibition of cellular metabolism upon accumulation of markers from integrated cassettes, which is deleterious to cell viability as antibiotics are typically required during cultivation to ensure maintenance of the integrated genes.[25,57,58] Therefore, methods for curing the markers or markerless integration techniques are necessary to enable efficient chromosomal expression and will be discussed in the following section.

Tools for episomal expression are critical for advancing cyanobacteria synthetic biology because even in polyploid cyanobacteria, the copy number of chromosomes is lower than that of exogenous plasmids and the expression levels are consequently lower from chromosomes than plasmids,[16,31,52] which results in lower yield and productivity when synthetic pathways are integrated chromosomally. Although this issue has been partially resolved by employing endogenous plasmids for gene expression, insertion of genes into endogenous plasmids is fundamentally similar to chromosomal integration; thus, the abovementioned obstacles in multiplexing integration remain. Therefore, it is imperative to develop exogenous plasmids for cyanobacteria. The higher copy number of exogenous plasmids per chromosome, as reported for RSF1010 and pANS derivatives,[52,54] can increase the gene expression level. Importantly, the availability of stable and fine-tuned plasmids will facilitate the utilization of the genetic toolkits by, for example, carrying "helper genes" (e.g., recombinases and nucleases) to perform "one-time" tasks, such as recombination and DNA cleavage.[37,59,60] Moreover, exogenous plasmids are more convenient for "proof-of-principle" studies because they eliminate the segregation process and make strain engineering less time-consuming.

However, developing exogenous self-replicating plasmids is challenging. One hurdle is that current shuttle vectors derived from endogenous plasmids may cause incompatibility between the exogenous and endogenous plasmids, leading to unexpected physiologies.[56] Another challenge is the limited genetic information for plasmid replication in cyanobacteria, which hinders the creation of stable plasmids other than the RSF1010 derivatives. Therefore, it is necessary to further investigate the mystery of DNA replication in cyanobacteria to develop more applicable plasmids for facilitating metabolic engineering and synthetic biology in these autotrophic hosts.

3. Genome Editing

Genome editing possesses the capability of adding genetic information and deleting competing pathways through insertions, deletions, and single-nucleotide mutations.[10,11] Basic editing methodologies are fairly established in cyanobacteria, as exemplified by the aforementioned chromosomal-gene-integration strategies. However, techniques for efficient and multiloci editing of cyanobacteria are lacking, hence impeding optimization of metabolic pathways and hampering advancement of metabolic engineering in cyanobacteria. In this section, we review and discuss the progress made and hurdles present in markerless and scarless genome editing of cyanobacteria, particularly the "knock-in/out" editing method and clustered regularly interspaced short palindromic repeats (CRISPR)-based systems.

3.1. Knock-In/Out Markerless Genome Editing

Currently, genome editing is achieved by introducing the desired functional genes along with selection markers. Such methods have been employed for metabolic engineering to understand and improve biosynthetic pathways. For instance, Kanno et al.[57] constructed six single-gene deletion strains with the replacement of essential genes in relevant pathways and discovered the key genes for 2,3-butanediol production, leading to an engineered strain with supreme productivity. However, to facilitate multiloci editing, the markers need to be removed to prevent accumulation

of antibiotic-resistant genes and enable recycling of the markers. Several methods have been developed for markerless genome editing. In general, they commence with "knock-in" integration of a cassette containing an antibiotic resistance marker and a killer gene. After an initial antibiotic resistance-based selection, the cells are counterselected for those that have concomitantly removed the selection marker along with the killer gene via "knock-out," which enables marker-rescued cells to survive and manifest. One example is the "hit-and-run" allele replacement approach in *Synechococcus* 7942 that used *sacB* from *Bacillus subtilis*, which encodes a levansucrase that produces a lethal product from sucrose, as the killer gene.[11] Similarly, two other methods utilizing an *rps12* mutant[11] and the endoribonuclease *mazF*[59] as killer genes for the counterselection were developed. In contrast, Tan et al.[37] employed the flippase (FLP)/FLP recognition target (FRT) recombination system for markerless editing. This method expresses the FLP in the second round of selection to recombine FRT sequences flanking the antibiotic resistance gene, thus eliminating the marker from the deletion cassette.[37,61] Analogous to the FLP/FRT system, the Cre/Lox recombination system was adopted in *Synechocystis* 6803 and *Anabaena* sp. PCC7120 to demonstrate markerless genome editing.[62]

Although markerless editing strategies have been developed, they have not been extensively used or reported to promote metabolic engineering of cyanobacteria. Moreover, engineered cyanobacterial strains are often edited only at a single locus.[19,33,57] One major obstacle is the long time required for markerless editing. Based on the protocols,[11] at least 4 weeks in total are required to achieve one markerless editing. To overcome this bottleneck, a major challenge is the variable chromosome copy number, which doubles the genome editing time due to the need for segregation. In cyanobacteria, the chromosome copy number ranges from 1 to >200.[8,56] Although it would be an evolutionary advantage, the variable chromosome copy number impedes efficient genome editing because the gene fragment along with the selection marker needs to be integrated into every chromosome to achieve a homozygous population, yet a single copy of the marker gene is sufficient for a strain to meet the selection criteria. Without a homogeneous population, the integrated genes will soon be diluted by the wild-type sequence on unmodified chromosomes through HR,[8] hence significantly hindering strain engineering.

3.2. CRISPR-Based Genome Editing

CRISPR-based systems provide a "dead or alive" selection to facilitate genome editing and its use has been thriving in bacterial, fungal, and mammalian cells.[63,64] In the Class II Type II CRISPR system, the CRISPR-associated protein 9 (Cas9) nuclease cuts double-stranded DNA at a site targeted by a single guide RNA ([sgRNA] with a chimeric structure of CRISPR RNA and *trans*-acting CRISPR RNA). The resulting cleavage in the genome increases the HR efficiency and editing occurs when the DNA breakage is repaired by a donor DNA. This "dead or alive" selection has inherent advantages over marker-based selection methods by offering one-step, markerless, and scarless editing. Moreover, CRISPR-based systems possess immense potential for cyanobacterial genome editing, as the CRISPR nuclease cuts the targeted sequences on every chromosome all at once, thereby bypassing the time-consuming segregation process (Figure 1D). Li et al.[39] reported the first success of CRISPR-based system in *Synechococcus* 7942 using transiently expressed *Streptococcus pyogenes* Cas9. However, the repairing donor DNA used still contained an antibiotic resistance marker. To illustrate the full potential of the CRISPR-based system for markerless editing, Wendt et al.[45] expressed Cas9 transiently in *Synechococcus* 2973 using a plasmid initially designed for *Streptomyces* species and used a donor DNA carrying only the homologous sequences to achieve 100% editing efficiency in a single round of selection. The feasibility of employing Cpf1, a Class II Type-V CRISPR nuclease with lower toxicity than Cas9, for editing the genomes of *Synechococcus* 2973, *Synechocystis* 6803, and *Anabaena* 7102 was demonstrated by Ungerer et al.[44] Although only 25% of the colonies contained the desired modification in *Synechococcus* 2973, the genome could be edited with just a single round of selection, and the plasmid harboring *cpf1*, sgRNA, and the donor DNA could be easily cured. These examples show the importance of CRISPR-based techniques for reducing the time needed for cyanobacterial genome editing.

Despite the successes in applying CRISPR-based methods on cyanobacteria, there are two main challenges to be resolved before the techniques can be routinely applied for metabolic engineering. One is the toxicity of the nuclease. Wendt et al.[45] discovered that Cas9 expression in *Synechococcus* 2973, using a self-replicating plasmid, results in extremely low survival rates even in the absence of a targeting sgRNA. This issue could be circumvented either by tuning the expression level of Cas9[45] or using the less toxic nuclease Cpf1.[44] However, there remains another critical challenge, which is the lack of suitable episomal expression methods for the CRISPR nuclease. As discussed above, the expression of heterologous genes in cyanobacteria relies much on chromosomal integration, while the utility of Cas9 or Cpf1 requires independent expression on exogenous plasmids.[65,66] This may explain the lack of reports on CRISPR-based editing in *Synechococcus* 7942 and *Synechococcus* 7002, in which chromosomal expression dominates (Table 1). The hurdles in Cas9 expression may be overcome by developing self-replicating plasmids with tightly regulated expression levels or approaches that fine-tune transient expression, akin to those performed in mammalian or plant cells.[67,68] By successfully addressing these issues, CRISPR-based technology will remove the need for marker rescue and dramatically reduce the complexity and time required for genome editing, thus accelerating the improvement of cyanobacteria hosts for biochemical production.

4. Programmable Regulations

In metabolic engineering and synthetic biology, the genes of interest need to be programmably controlled to optimize the metabolic flux in synthetic pathways for enhanced production of biofuels and biochemicals.[69] Regulatory parts and modules, including promoters, RBSs, CRISPR interference (CRISPRi), and RNA-structure-based modules, have been developed in cyanobacteria and are used to construct complex genetic circuits. Most of the regulatory designs have been well-established in cyanobacteria, and autonomous programmable multigene regulation has exhibited great potential for the

production of biofuels and biochemicals. However, cyanobacteria have characteristics that hamper implementation of programmable regulation. Here, the progress and bottlenecks in the development of programmable regulatory parts and modules in cyanobacteria are reviewed.

4.1. Promoters and RBSs

Available promoters for cyanobacteria have been comprehensively reviewed by Berla et al.[7] and Sun et al.[55] The inducible systems frequently used in cyanobacteria employ adapted DNA-binding repressor proteins, such as TetR and LacI, and their respective responsive promoters, such as P_{Tet} and P_{Trc}, to enable tuned gene regulation (**Figure 2**A). Notably, systems inducible by sugars, including L-arabinose[38] and L-rhamnose,[42] have been introduced to cyanobacteria. In addition, cyanobacteria have been engineered to respond to environmental signals including darkness, O_2, and nitrogen sources.[70,71] These environmental signal- and sugar-based induction methods are nontoxic and economical, hence making them particularly industrially relevant for biofuel and biochemical production in cultivation lagoons or bioreactors.

In addition to the above "transplanted" regulatory components, cyanobacteria promoters have also been studied. Ruffing

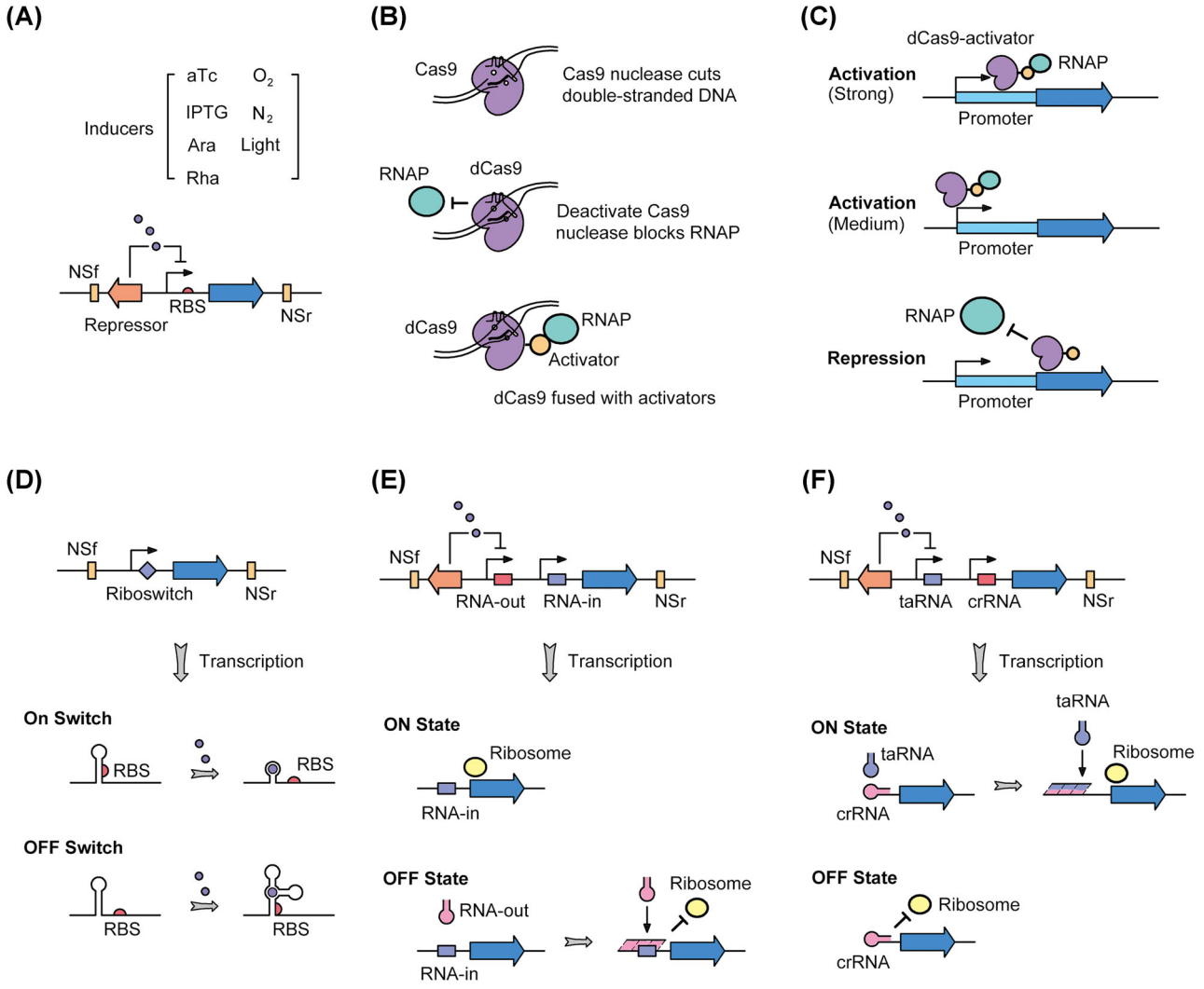

Figure 2. Programmable regulations in cyanobacteria. A) Frequently used DNA-binding strategy to control gene expression at the transcription level. It usually contains a repressor and a cognate promoter, such as *tetR* and P_{Tet}, *lacI* and P_{Trc}, *araC* and P_{BAD} as well as *rhaS* and P_{Rha}. B) CRISPR-based regulation modules. dCas9 is derived from Cas9 via the D10A and H840A mutations in the HNH and RuvC domains, respectively, and dCas9 is fused with activators, such as ω subunit of RNAP in *E. coli* or KRAB and VP64 in mammalian cells, to enable programmable activation of genes. C) Graded regulation of gene expression from activation to repression by dCas9 activator. Activation or repression is achieved by targeting different loci of the promoter and coding sequence. D) Scheme of ON and OFF riboswitches in cyanobacteria. E) RNA-in/out systems. Without the RNA-out, the gene is expressed. When the RNA-out is transcribed, it binds the RNA-in sequence to prevent the binding of ribosomes. F) Scheme of riboregulator. Without taRNA, crRNA forms a loop and inhibits translation. When taRNA is transcribed, the taRNA binds the crRNA to allow the binding of ribosome.

et al.[72] investigated 25 native promoters in *Synechococcus* 7002 and determined different strengths of the promoters. Liu and Pakrasi[53] systematically evaluated the elements in the endogenous plasmids of *Synechocystis* 6803 and developed various "plug-in" tools including 12 promoters, 20 RBSs, and 8 terminators.[53] Due to these advances, a broad range of induction could be attained by combining different promoters and RBSs to achieve an approximately 8000-fold difference in the expression level between the strongest and weakest systems. Interestingly, incompatibilities have been identified between some promoters and the noncognate RBSs,[53] indicating the presence of a specific post-transcriptional mechanism. To facilitate RBS development, Wang et al.[73] introduced a rational RBS design strategy for *Synechocystis* 6803 based on the discovery that efficient RBSs for cyanobacteria contain a disrupted AT-rich region, a mutated Shine–Dalgarno sequence and a spacer upstream of the start codon. By using current calculation approaches and taking into consideration post-transcriptional factors, the design algorithm of RBS for cyanobacteria was improved.[73] The array of new regulatory components will greatly aid the modulation of metabolic flux by allowing large numbers of promoter–RBS gene combinations to be constructed and investigated to identify the optimum pathway design in cyanobacteria.

4.2. CRISPR Interference

CRISPRi is an RNA-guided transcriptional inhibition method that is able to accomplish genome-scale regulation in a programmable manner. Qi et al.[74] repurposed the CRISPR/Cas9 system by mutating the active sites of Cas9 to generate a deactivated Cas9 (dCas9) without nuclease activity. Guided by sgRNAs, the dCas9 still binds to the targeted sites but does not cut the DNAs. Instead, when targeted to coding sequences of genes of interest, the bound dCas9 blocks the RNA polymerase (RNAP), hence interfering with the transcription of the target genes (Figure 2B). CRISPRi has been introduced to various cyanobacteria strains.[40,41,43] Huang et al.[40] integrated dCas9 into *Synechococcus* 7942 and successfully repressed expression levels of *glgC*, *sdhA*, or *sdhB* by >90%, leading to an increased yield of succinate. Yao et al.[43] installed CRISPRi in *Synechocystis* 6803 and four genes were simultaneously knocked down by 50–95%, further demonstrating the capability of CRISPRi for multiloci repression in cyanobacteria. Recently, an application of CRISPRi for enhanced production of fatty alcohol has been reported.[32] Six genes encoding the enzymes in acyl–acyl carrier protein-consuming pathways were knocked down simultaneously and the role of an essential phosphate acyltransferase was discovered, resulting in the highest reported titer of fatty alcohol in cyanobacteria. These examples demonstrate that the multiplex capability of CRISPRi can drastically reduce the time required for genome editing of cyanobacteria and will expedite engineering of cyanobacteria for chemical bioproduction.

dCas9 can also function as an activator when fused to activating factors, such as ω subunit of RNAP in *E. coli* or KRAB and VP64 in mammalian cells.[75] The resulting dCas9-activator complex enables graded control of gene expression, from repression to upregulation, by targeting different regions of the promoter and coding sequences (Figure 2C),[76] illustrating great capability for multiloci targeting and versatile regulatory control. Due to the success of dCas9 in cyanobacteria, this dCas9-activator module could also be adopted in cyanobacteria for programmable regulation of genes of interests to enable enhanced bioproduction via genome-wide regulation without needing conventional deletion or overexpression approaches.

4.3. RNA-Structure-Based Modules

RNA-based regulation systems rely on the secondary structures of or interactions between messenger RNAs (mRNAs) to control gene expressions. Since they do not depend on host-specific regulatory elements to function, most of the tools developed can be easily transferred to cyanobacteria for tight control of gene expressions at post-transcriptional levels. Thus far, RNA-based regulation systems established in cyanobacteria include riboswitch, RNA-in/out, and riboregulator.

Riboswitches have been used to enable dosage-dependent ON/OFF control of gene expressions (Figure 2D). Nakahira et al.[77] introduced a theophylline ON riboswitch into *Synechococcus* 7942 to control the gene expression level. Without theophylline, the aptamers that form the riboswitch assume a conformation that prevents the binding of ribosomes. When theophylline reaches the threshold concentration, it binds to the aptamers to expose the RBS, thus allowing ribosomes to express the gene; a 190-fold difference in the expression level between the ON and OFF states was achieved. Ma et al.[78] characterized six theophylline ON/OFF riboswitches in four different strains and demonstrated the utility of this post-translational system in cyanobacteria.

Another RNA-structure-dependent method implemented in cyanobacteria is the RNA-in/out-based system. In *Synechococcus* 7002, a *trans*-acting small RNA was transcribed to serve as RNA-out, which forms a hairpin and interacts with an RNA-in sequence to block the binding of ribosomes to the RBS for regulating gene expressions (Figure 2E).[79] Additionally, a riboregulator system based on *cis*-repressing (crRNA) and *trans*-activating RNA (taRNA) interaction was also transplanted from *E. coli* and optimized for *Synechocystis* 6803.[28,80,81] In brief, when the *trans*-activating RNA is not expressed, the transcribed crRNA will form a loop structure at the 5′-UTR of the mRNA and prevent binding of ribosomes. If the taRNA is transcribed, it binds the crRNA and hence releases the RBS to allow translation (Figure 2F).[82] Given the success of riboswitches in cyanobacteria and high portability of riboswitches between microorganisms, they hold promise for strain engineering and pathway optimization, as demonstrated in conventional hosts.[83]

4.4. Genetic Circuits

Based on the regulatory tools developed, genetic circuits have been constructed in cyanobacteria to enable complex programmable regulation. Higo et al.[84] developed a positive feedback loop in *Anabaena* 7120 to achieve better expression of a reporter protein using a combination of DNA-binding proteins and

antisense RNAs. Immethun et al.[70] reported the first genetic logic gate in cyanobacteria. A two-input AND gate was built in *Synechocystis* 6803 using anhydrotetracycline (aTc) and O_2 as signals to control the *Salmonella* pathogenicity island 1 type III secretion and SicA chaperone systems, respectively, to express the reporter only when both signals are present. Subsequently, Taton et al.[85] designed four NOT gates using a combination of DNA-binding proteins and theophylline ON riboswitches in five cyanobacteria strains, including *Synechococcus* 7942, *Synechocystis* 6803, *Anabaena* 7120, *Leptolyngbya* BL0902, and *Synechocystis* WHSyn.[85] These designs enable cyanobacteria to process multiple signals, such as environmental stimuli or metabolic intermediates, and in response regulate genes of interests, making dynamic modulation of synthetic pathways possible.

The above reports demonstrated the possibilities to build complex circuits in cyanobacteria by assembling basic regulatory modules, such as chaperone systems, RNA-structure-based systems and CRISPRi. For instance, a two-input NOR gate can be constructed using the reported RNA-in/out module or CRISPRi based on circuit design principles.[86] Based on the same types of inputs and outputs, these circuits may be layered as demonstrated in model strains such as *E. coli*. Notably, the recent advances of genetic circuits in cyanobacteria are based on genome integration, hence illustrating genetic stability of the circuits. However, it would be challenging to simply transplant genetic circuit design from *E. coli* to cyanobacteria, as circuits behave significantly different on multicopy plasmids and chromosomes.[87] Moreover, the variable number of chromosomes may hinder precise regulation of circuits introduced, although its influence may be minimal, as Zheng et al.[88] reported that cyanobacteria maintain similar protein concentrations regardless of the chromosome copy number. However, much effort is necessary to examine the expression levels mediated by different regulatory modules to accomplish sophisticated control by genetic circuits. One potential engineering solution may be to embed a negative feedback loop to counter the effects of variable chromosome numbers, which has been demonstrated by engineering an incoherent feedforward loop to promoters in *E. coli*.[89] Furthermore, the metabolism of cyanobacteria is slower than *E. coli* and *S. cerevisiae*, and hence the kinetics of each circuit layer needs to be well-characterized and manipulated to achieve the designed response in reasonable time frames. While the application of genetic circuits for optimizing biochemical production has been demonstrated in conventional hosts,[69] further work has to be done to realize this in cyanobacteria given the greater biological complexity.

5. Conclusions

Cyanobacteria have been considered as an ideal autotrophic platform for chemical production.[28] They are also model organisms for studying protein assembly[90] and circadian rhythm, and for conducting geochemical, environmental, and ecological research and applications. Synthetic biology grants various nonnatural functionalities to cyanobacteria, especially for enhanced biofuel and biochemical production directly from CO_2 by introducing genetic modules. In the last two decades, the inventory of molecules produced from engineered cyanobacteria has vastly expanded and the performance has been dramatically improved due to the development of synthetic biology tools in gene expression, genome editing, and programmable regulations.

Despite the progress that has been made in developing synthetic biology toolkits for cyanobacteria, the tools available for manipulating cyanobacteria still lag behind those of conventional hosts such as *E. coli* and *S. cerevisiae*.[8] Therefore, we also highlighted the challenges in synthetic biology toolkits for cyanobacteria that hinder a revolution in cyanobacteria-based production of biofuels and biochemicals. To further the endeavor, fundamental innovations in synthetic biology toolkits are necessary and urgent, especially the toolset of plasmids for fine-tuned episomal expression, CRISPR-based system for precision genome editing, and programmable regulatory modules for dynamic and multiplex modulation of synthetic pathways. We believe these advancements will significantly accelerate the development of cyanobacteria into a mainstream microbial chassis for sustainable biochemical production.

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Conflict of Interest

The authors declare no conflict of interest.

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