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Review

Synthetic Biology Tools for Novel Secondary Metabolite Discovery in *Streptomyces*

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Streptomyces are attractive microbial cell factories that have industrial capability to produce a wide array of bioactive secondary metabolites. However, the genetic potential of the *Streptomyces* species has not been fully utilized because most of their secondary metabolite biosynthetic gene clusters (SM-BGCs) are silent under laboratory culture conditions. In an effort to activate SM-BGCs encoded in *Streptomyces* genomes, synthetic biology has emerged as a robust strategy to understand, design, and engineer the biosynthetic capability of *Streptomyces* secondary metabolites. In this regard, diverse synthetic biology tools have been developed for *Streptomyces* species with technical advances in DNA synthesis, sequencing, and editing. Here, we review recent progress in the development of synthetic biology tools for the production of novel secondary metabolites in *Streptomyces*, including genomic elements and genome engineering tools for *Streptomyces*, the heterologous gene expression strategy of designed biosynthetic gene clusters in the *Streptomyces* chassis strain, and future directions to expand diversity of novel secondary metabolites.

Keywords: *Streptomyces*, secondary metabolites, biosynthetic gene cluster, antibiotics, synthetic biology, genome editing, CRISPR/Cas9, heterologous expression

Introduction

Streptomyces species are Gram-positive bacteria that produce more than two-thirds of the medically and agriculturally important secondary metabolites, including antibiotic, anticancer, antifungal, antiparasitic, and immunosuppressive compounds [1, 2]. Recent genome sequencing efforts have revealed that individual *Streptomyces* species have a wealth of genetic potential to produce novel secondary metabolites. However, most secondary metabolite biosynthetic gene clusters (SM-BGCs) are silent under laboratory culture conditions, limiting effective use of *Streptomyces* [3]. Therefore, activation of silent SM-BGCs is one of the most promising approaches to discover novel bioactive secondary metabolites from *Streptomyces*.

To activate silent SM-BGCs, various strategies have been applied, including culture media modifications, chemical or antibiotic treatments, heterologous gene expression in different hosts, and co-culture with cohabiting microbes [4]. However, these methods are untargeted, resulting in non-directed activation of silent SM-BGCs in *Streptomyces*. To overcome this limitation, a synthetic biology approach has been proposed to redesign and reconstruct target SM-BGCs [5, 6]. Generally, a main obstacle in activating cryptic SM-BGCs is the difficulty in bypassing the complex native regulation. Expression of SM-BGCs is tightly governed by multi-layered regulatory networks, which are often triggered by environmental signals [7]. Using synthetic genetic parts, such as promoters, ribosome binding sites (RBS), and terminators, which are not controlled by the

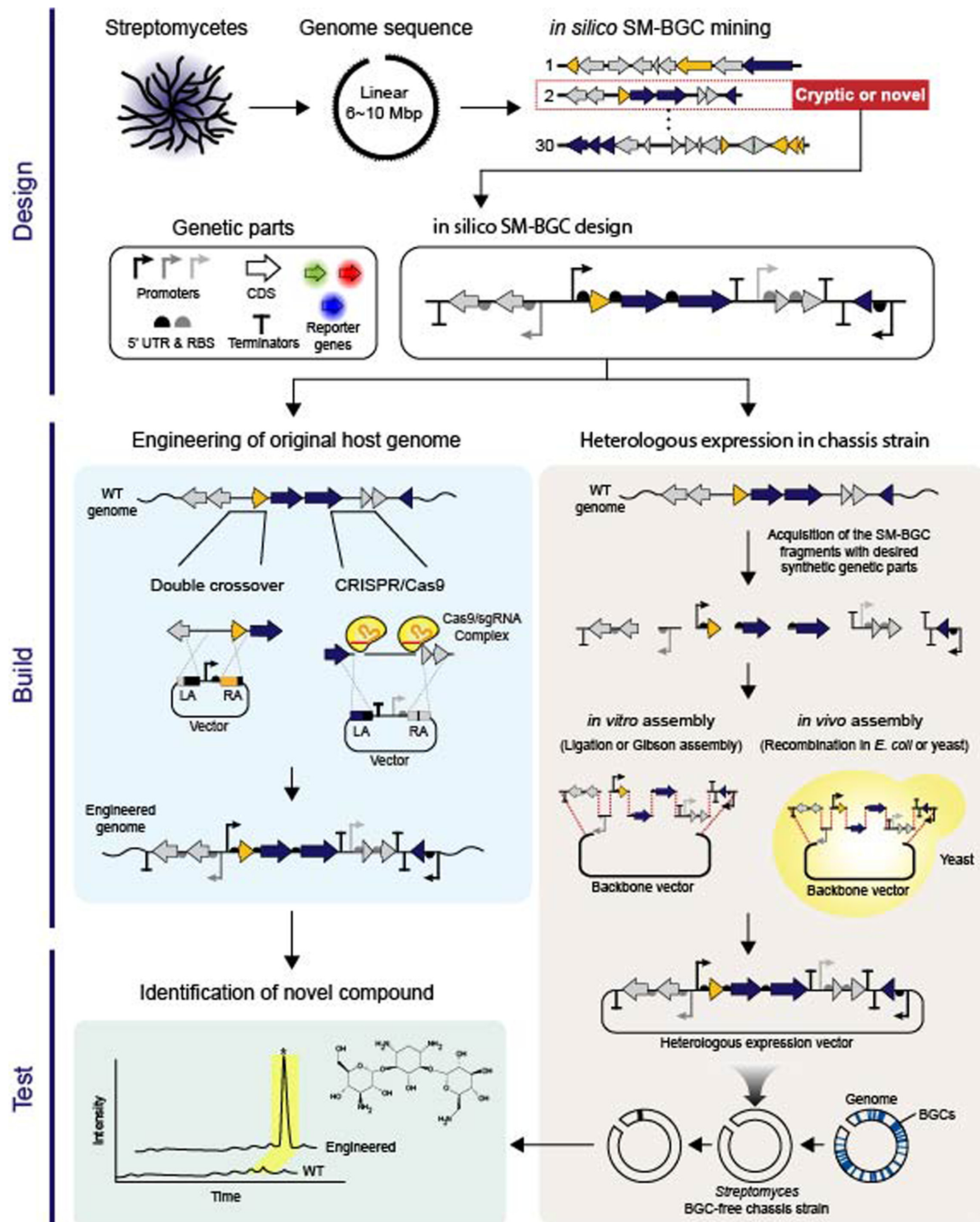

Fig. 1. Overview of synthetic biology strategy to produce novel secondary metabolite from *Streptomyces*.
Abbreviations: BGC, biosynthetic gene cluster; CDS, coding sequence; 5′ UTR, 5′ untranslated region; RBS, ribosome binding site; WT, wild type; LA, left homology arm; RA, right homology arm.

host's regulatory system, expression of target SM-BGC can be achieved through bypassing the native regulatory networks. In this respect, various synthetic genetic parts and genome engineering tools are required to regulate gene expression and redesign target SM-BGCs in *Streptomyces*. Even though high GC content and highly interconnected regulatory networks challenge the application of synthetic genetic parts that are widely used in other bacterial

systems to *Streptomyces*, enabling synthetic biology tools have been recently developed for *Streptomyces* engineering [8]. Here, we review synthetic biology strategies applied in the discovery of novel secondary metabolites in *Streptomyces*. As synthetic biology is defined by an iterative "design-build-test" cycle in engineering biology, we have classified the strategy into three steps (Fig. 1). First, for the design step, discovery of novel SM-BGCs via genome mining is described, followed by designing of SM-BGCs using various synthetic genetic parts developed for *Streptomyces*. Second, for the build step, the process of building the re-designed SM-BGCs is discussed, including genome engineering of original host and SM-BGC assembly into plasmids for heterologous expression. Further, *Streptomyces* chassis strains are suggested as optimal heterologous expression hosts for reconstructing target SM-BGCs. Third, for the test step, high-throughput testing methods are described to measure activation of target SM-BGCs for the next "design-build-test" cycle.

Mining of Secondary Metabolite Biosynthetic Gene Clusters

In the pre-genome mining era, most of the secondary metabolites in *Streptomyces* were discovered via chemistry-based methods of changing culture conditions and mass spectrometry or NMR biosynthetic identification [9]. Due to the biochemical complexity of SM-BGCs, however, the discovery of novel secondary metabolites has been challenging. This limitation has been overcome through a genome mining approach, in which recent advances in DNA sequencing technology caused a rapid increase in the number of high-quality *Streptomyces* genome sequences [10, 11]. To date, about 500 scaffold-level and 84 complete-level genome sequences of *Streptomyces* strains are available in the NCBI database. These large numbers of genome sequences encode invaluable resources for novel secondary metabolite discovery [12]. To identify SM-BGCs from these genome sequences, several genome-mining tools have been developed, such as ClustSCAN, NP.searcher, GNP/PRISM, and antiSMASH [13−16]. Among them, antiSMASH is the most comprehensive and widely used software pipeline for genome mining, offering a user-friendly web interface and prediction of the broad spectrum of SM-BGCs [16]. antiSMASH has detected 45 different classes of SM-BGCs based on a rule-based cluster detection approach and further predicted the modular domain structures of genes within these SM-BGCs [17].

In general, each *Streptomyces* genome encodes approximately 30 SM-BGCs, which are diverse and differ between species, indicating that *Streptomyces* strains are an immeasurable source of novel secondary metabolites [18−20]. Information about SM-BGCs mined from genome sequences is not only essential data for novel secondary metabolite discovery, but also a resource to facilitate rational design of SM-BGCs based on the synthetic biology approach. In particular, polyketides (PK) and nonribosomal peptides (NRP) can be redesigned using this approach as they are synthesized by serially connected modular enzymes that recognize module-specific CoAs or amino acids, respectively [21]. For example, replacement of AveA1 and module 7 of AveA3 in avermectin BGC of *Streptomyces avermitilis* with MilA1 and MilA3 in milbemycin BGC of *S. hygroscopicus* resulted in milbemycin production in *S. avermitilis* [22]. Taken together, a genome mining approach can accelerate secondary metabolite discovery at an unprecedented rate with an ever-growing number of *Streptomyces* genome sequences.

Genetic Parts for *Streptomyces* Synthetic Biology

Genomic information on *Streptomyces* strains has revealed the great potential of *Streptomyces* to produce novel secondary metabolites. However, most SM-BGCs in *Streptomyces* are inactive under general laboratory culture conditions. For example, although *S. coelicolor*, *S. griseus*, and *S. avermitilis* genomes encode more than 30 SM-BGCs, respectively, only 3–5 secondary metabolites have been detected [1, 11, 23]. Although many efforts have been made to activate silent SM-BGCs in *Streptomyces* strains, including the One Strain-Many Compounds (OSMAC) strategy and co-cultivation methods [24, 25], these approaches resulted in non-directed activation of silent BGCs. For specific activation of targeted SM-BGCs, the following methods have been implemented: (1) promoter replacement, (2) overexpression or repression of regulatory genes, (3) heterologous expression in different hosts, and (4) refactoring of targeted SM-BGCs [26]. To this end, the number of genetic parts, such as promoters, ribosome-binding sites (RBS), and terminators available for *Streptomyces* species, has dramatically increased as summarized in Table 1.

Genetic Parts for Transcriptional Regulation in *Streptomyces*

In bacterial cells, a transcription unit is defined as a basic unit of regulation and is composed of several genes and accessory genetic elements, including promoters, transcription start sites (TSS), RBSs, and terminators. Design and

Table 1. Genetic parts for *Streptomyces*.

Genetic parts	Feature	Reference
Constitutive promoters		
*ermE** promoter	Mutation at the promoter of the erythromycin resistance gene of *Streptomyces erythraeus*	[31]
SF14P promoter	Genome of *Streptomyces ghanaensis* phage I19	[34]
kasOP promoter	Promoter of SARP family regulator in *Stretpomyces coelicolor* A3	[33]
gapdh promoter	Promoter of glyceraldehyde-3-phosphate dehydrogenase in *Streptomyces griseus*	[35]
rpsL promoter	Promoter of 30S ribosomal protein S12 in *Streptomyces griseus*	[35]
195 native or synthetic promoters	High-throughput screening in *S. venezueale*	[58]
32 native promoters	Transcriptome data-based selection in *S. albus*	[38]
166 native promoters	Transcriptome data-based selection in *S. coelicolor*	[39]
2 native promoters	Multi-omics data-based selection in *S. coelicolor*	[59]
Inducible promoters		
tipA promoter	Thiostrepton-induced promoter	[40]
nitA promoter	ε-caprolactam-induced promoter	[45]
xylA promoter	Xylose-induced promoter	[46]
tcp830	Tetracycline-induced promoter	[43]
PA3-rolO	Resorcinol-induced promoter	[44]
P21-cmt	Cumate-induced promoter	[44]
Terminators		
Fd	Bidirectional transcription termination originated from *E. coli* phage fd	[48]
TD1	Bidirectional transcription termination originated from *Bacillus subtilis* phage Φ29	[49]
RBS		
AAAGGAGG	Typical RBS sequence of *S. coelicolor*	[134]
192 native or synthetic RBSs	High-throughput screening in *S. venezueale*	[58]
4 native RBSs	Multi-omics data-based selection in *S. coelicolor*	[59]
Reporter genes		
luxAB cassette	n-Decanal as substrate; absorbance at 490 nm wavelength	[61]
amy gene	Soluble starch with 3,5-dinitrosalycilic acid (DNS) as substrate; absorbance at 540 nm wavelength	[64]
xylE gene	Catecol as substrate; absorbance at 375 nm wavelength	[62]
gusA gene	p-Nitrophenyl-β-D-glucuronide as substrate; absorbance at 415 nm wavelength	[63]
eGFP	Green fluorescent protein; excitation wavelength 470-490 nm and emission wavelength 515 nm	[65]
sfGFP	Green fluorescent protein; excitation wavelength 488 nm and emission wavelength 500~550 nm	[58]
mRFP	Red fluorescent protein; excitation wavelength 584 nm and emission wavelength 607 nm	[67]
mCherry	Red fluorescent protein; excitation wavelength 587 nm and emission wavelength 610 nm	[68]

utilization of these genetic parts, at appropriate strengths, is critical for precise transcriptional and translational regulation of targeted gene expression. In transcriptional regulation, the most important genetic part is the promoter, which is the binding site of RNA polymerase to initiate transcription. However, widely used promoters for bacterial genetic engineering, such as *lacZ* and T7 promoters, are not directly applicable in *Streptomyces* [27, 28], thus only a few promoters have been used for gene expression in *Streptomyces*. The terminator is also important in preventing transcriptional read-through to downstream genes [29]. Particularly, when redesigning SM-BGCs in S*treptomyces*, due to their operon-like genomic structure, precise transcriptional termination is required between transcription units. In this section, we described genetic parts that have been developed and used to regulate the transcription of *Streptomyces*.

First, constitutive promoters, which generate constant gene expression levels regardless of growth phases, are extensively used for SM-BGC expression in *Streptomyces*. In particular, *ermE** promoter is the most commonly used

strong constitutive promoter in *Streptomyces*, which is a derivative of the *ermE* promoter, containing a trinucleotide deletion in the ermEp1 region of the erythromycin resistance gene in *S. erythraeus* [30−32]. Further, *SF14P* and *kasOP*, discovered from the *S. ghanaensis* phage I19 genome and promoter of the SARP family regulator in *S. coelicolor* A3, respectively, constitutively transcribe gene expression similarly or more strongly than the *ermE** promoter [33, 34]. Additionally, two strong constitutive promoters, *gapdhP* and *rpsLP*, which have higher activity than the *ermE** promoter, were obtained from the promoter region of housekeeping genes in the *S. griseus* genome [35]. In addition, several strategies have been applied to develop strong constitutive promoters. One approach screens strong synthetic promoters from a randomized promoter library. For example, the randomized sequence library of the *kasO* promoter was used to generate synthetic promoters with promoter strength ranging from 0.95% to 187.5%, compared to the parental *kasO* promoter [33]. Similar approaches have been conducted on the *ermE* and *actII-orf4* promoters of *S. coelicolor* [36]. Several strong promoters were screened through this approach; however, all promoters were weaker than the *ermE** promoter [37]. Another rational strategy identifies strong promoters using gene expression data. In *S. albus*, promoter sequences of highly expressed genes were selected based on transcriptional profiling data, and the strength of each promoter was tested, resulting in the selection of ten promoters that were stronger than the *ermE** promoter [38]. A similar study was performed on *S. coelicolor*, which identified 166 potentially constitutive promoters across the *Streptomyces* genus [39].

Second, expression of SM-BGC genes under constitutive promoters sometimes causes growth retardation. Therefore, it is desirable to establish a controllable gene expression system in *Streptomyces*. To date, the most widely used inducible promoter in *Streptomyces* is the *tipA* promoter, which is induced by thiostrepton treatment [40, 41]. The basal expression level of the *tipA* promoter is considerable, which limits precise regulation of the targeted gene expression. However, basal expression of the *tipA* promoter is sometimes used to maintain low expression levels of toxic genes [42]. The tetracycline-induced strong promoter, *tcp830,* was constructed by combining conserved sequences of the *ermE* promoter and Tn*10 tetR/tetO* systems [43]. However, like *tipA* promoter, a major disadvantage of this promoter is its basal expression level. To overcome this limitation, inducible promoters with low leaky expression, such as *PA3-rolO* and *P21-cmt* promoters, have been synthesized. The *PA3-rolO* promoter is a resorcinol-induced promoter synthesized by combining the *rolO* operator and synthetic promoter *PA3*. The *P21-cmt* promoter is a cumate-induced expression system synthesized by fusing the operator of the *Pseudomonas putida* F1 cumate degradation operon to the *P21* synthetic promoter [44]. Other inducible systems used in *Streptomyces* are *nitA* and *xylA* promoters. The *nitA* promoter, which originates from the nitrilase promoter of *Rhodococcus rhodochrous*, is induced by a complex of ε-caprolactam and the transcription regulator NitR [45]. The recently developed *xylA* promoter is a strictly regulated xylose-induced expression promoter [46]. Additionally, several glycerol-inducible systems have been developed for *Streptomyces*; however, these systems have not been utilized because glycerol treatment may alter intrinsic cellular metabolism [47].

Further, only a limited number of terminator sequences is available in *Streptomyces* strains. Two bidirectional transcription terminators, Fd originated from *Escherichia coli* phage fd and TD1 originated from *Bacillus subtilis* phage φ29, are efficiently recognized in *Streptomyces* [48, 49]. Although lambda T0 and T7 terminators have been used in several *Streptomyces* vectors, these terminators have not been systematically validated in *Streptomyces* for their effects on gene expression levels [50]. While most studies have focused on promoter strength as a determinant of gene expression levels, transcription terminators also have crucial roles in recycling transcription complexes and ultimately gene expression level [51, 52]. To expand the repertoire of transcription terminators in *Streptomyces*, understanding transcription termination and identifying native terminator sequences in *Streptomyces* are required. In this regard, Term-seq, which is a recent RNA sequencing method that enables genome-wide determination of transcript 3′ end positions, is a suitable technique for screening terminator sequences in *Streptomyces* genomes [53].

Taken together, many efforts in molecular biology of *Streptomyces* have provided genetic parts to control gene expression, including constitutive promoters, inducible promoters, and terminators. However, to flexibly and precisely control secondary metabolite production in *Streptomyces*, more generalized and validated genetic parts for transcription, which can be used in various *Streptomyces* species and at different strengths, have to be developed.

Genetic Parts for Translational Regulation in *Streptomyces*

Because cellular protein level does not directly correlate with mRNA abundance, yet depends on translational

efficiency, transcriptional regulation alone is not sufficient to design an efficient gene expression system in *Streptomyces* [54, 55]. Translational efficiency is primarily determined by 5′ untranslated regions (5′-UTR), RBS, and codon usage of target genes [56]. For example, the RBS contains the Shine Dalgarno (SD) sequence, which includes a complementary sequence with the 3′ end of the 16s rRNA region of the 30S ribosomal subunit. Sequence diversity and accessibility of the SD sequence influence binding affinity with the ribosome, determining translational efficiency [57]. In this regard, several studies have measured the strength of the 5′-UTR and RBS in *Streptomyces* species to expand genetic parts for *Streptomyces* engineering. In this section, we will provide two examples of translational regulatory genetic part screenings in *Streptomyces* genomes.

First, in *S. venezuelae*, sequences of the strongest RBS among 15 native RBSs were selected and randomized. After comparing the RBS strength, 177 synthetic RBSs with activity over 200-fold compared to their parental RBSs were collected. Furthermore, seven promoters were combined with nine RBSs in a pairwise manner to screen the most optimal promoter-RBS set for gene expression [58]. Second, two promoters and four 5′-UTR sequences were selected from *S. coelicolor* based on multi-omics data, including TSS-seq, RNA-seq, and Ribo-seq. Pairwise sets of promoters and 5′-UTR sequences showed strength in a range of 0.03- to 2.4-fold, compared to the *ermE** promoter with the SD sequence of the *nitA* gene [59]. Although development of a translational regulatory genetic part is at the beginning stage, as compared to transcription, ultimately transcriptional and translational genetic parts with various strengths have to be combined and utilized to design and control enzyme stoichiometry in SM-BGCs for enhancing secondary metabolite production.

Reporter Systems for High-Throughput Screening

For high-throughput characterization of genetic parts developed for *Streptomyces*, reporter systems that rapidly represent gene expression level with minimal influence to the cell physiology are required. Although many antibiotic resistance genes have been used as conventional markers for gene expression, their effects on cellular metabolism and narrow dynamic range limit their suitability for gene expression quantification [32, 60]. To quantify gene expression, colorimetric methods are more appropriate because gene expression levels can be quantified by measuring the absorbance of a specific wavelength of light. To date, various colorimetric methods, including *luxAB*, *amy*, *xylE*, and *gusA*, have been employed in *Streptomyces* strains [61−64]. In particular, *gusA* is the most widely used colorimetric reporter system in *Streptomyces* strains [44, 59]. However, colorimetric reporter systems are based on an enzymatic reaction that requires an additional substrate treatment, which may affect cellular metabolism (Table 1). For example, catechol dioxygenase, encoded by *xylE*, produces hydroxymuconic semialdehyde with a yellow color from catechol as a substrate [62]. In contrast, fluorescent proteins do not require any supplemental reagents and thus are suitable for high-throughput screening using fluorescence-activated cell sorting (FACS). However, since *Streptomyces* have relatively high levels of autofluorescence, several studies have argued that fluorescent proteins are not appropriate for high-throughput screening of *Streptomyces* strains [63]. Despite this limitation, several efforts have been made to apply fluorescent proteins in *Streptomyces* [65]. Among the fluorescent proteins, GFP derivatives, such as eGFP and sfGFP, are widely used in *Streptomyces,* and mRFP has been implemented to enable multi-color fluorescence-based studies [58, 65−67]. To test the available fluorescent proteins in *S. venezuelae*, seven fluorescent proteins (mTagBFP, mCerulean, mTFP, sfGFP, mCherry, mKate, and mCardinal) were expressed, and the fluorescence intensity of each fluorescent protein was compared. Among the seven fluorescent proteins, mCherry protein showed the most significant reduction in signal-to-background noise level and was used for further studies to characterize genetic parts of *S. venezuelae* [68].

CRISPR/Cas-Based Genome-Engineering Tools for *Streptomyces*

To utilize genetic parts and reconstruct metabolic pathways for secondary metabolite production, efficient genome engineering tools are required. Conventional genome engineering of *Streptomyces* is heavily dependent on either single or double crossover of a plasmid, which is laborious and time-consuming to obtain the desired clones [32]. Furthermor, the use of selection markers, such as antibiotics resistance genes, is often required to avoid reversion of the engineered genotype to wild type and may confer undesired effects, including polar effects. To overcome this limitation, site-specific recombination strategies, including Cre/*loxP*, Dre/*rox* and Flp/*FRT*, have been exploited in *Streptomyces* [69−72]. However, these approaches retain recombinase recognition sites in the chromosome and may limit successive applications for multiple genetic manipulations.

Recently, CRISPR/Cas9 (clustered regularly interspaced

short palindromic repeats and CRISPR-associated protein 9) has emerged as a promising tool for genome engineering of *Streptomyces* strains [50, 73−78]. Briefly, the Cas9 endonuclease forms a complex with guide RNA (gRNA) and is guided to a protospacer sequence, which is complementary to a spacer sequence of gRNA [79]. The guided Cas9 induces a double-strand break (DSB) in the genome, followed by repairing the DSB via native non-homologous end joining repair (NHEJ) or homology directed repair (HDR) mechanisms which facilitate genome engineering. CRISPR/Cas9 systems are superior to simple homologous recombination or site-specific recombination systems as they facilitate unmarked genome engineering with reduced time and labor. Even though other genome engineering strategies, such as zinc finger nuclease and transcription activator-like effector nuclease, have been developed, the protein-mediated recognition of target DNA sequence requires design of appropriate proteins for individual target sequences and thus limits their applications [80−82]. Therefore, the CRISPR/Cas9 system has become a dominant genome engineering tool, outpacing their performance (Table 2) [83].

Editing *Streptomyces* Genomes and Secondary Metabolite Biosynthetic Gene Clusters

The DSB by Cas9 occurs only if a specific sequence motif, called a protospacer adjacent motif (PAM), is present next to the protospacer [84]. All applications of the CRISPR/Cas9 system in *Streptomyces* strains utilize Cas9 from *Streptococcus pyogenes* (SpCas9), whose cognate PAM sequence is 5′-NGG [85]. As a large portion of *Streptomyces* genome is typically composed of G and C, SpCas9 offers plenty of potential target sites for CRISPR/Cas9-based genome engineering. Using the CRISPR/Cas9 system, genetic manipulations, including deletion, insertion, and point mutation, have been executed in various *Streptomyces* species [18, 42, 50, 86−94].

CRISPR/Cas9-based approaches can be divided by the type of DSB repair, NHEJ and HDR. NHEJ-mediated genome engineering generates random mutation, insertion or deletion of a few nucleotides, to disrupt a gene of interest [86, 88, 93]. Further, it has been applied to *S. coelicolor* and *S. rimosus* to disrupt actinorhodin synthesis and the pentose phosphate pathway, respectively. However, this technology was inefficient since the NHEJ system of most *Streptomyces* species is incomplete [86]. To enhance NHEJ-based genome engineering efficiency, LigD, the lacking component of NHEJ, was simultaneously introduced into *S. coelicolor* with CRISPR/Cas9 [86]. However, to avoid this limitation, CRISPR/Cas9-mediated genome engineering approaches in *Streptomyces* utilize HDR. For HDR-mediated genome engineering, template DNA for homologous recombination is introduced with CRISPR/Cas9 to enhance production of secondary metabolites and activate cryptic SM-BGCs in *Streptomyces* [87, 89, 90, 93]. For example, oxytetracycline production was increased in *S. rimosus* by deleting *zwf2* and *devB*, and redirecting oxygen and NADPH to oxytetracycline biosynthesis [93]. In addition, knock-in of a strong promoter upstream of pathway-specific transcriptional activators or secondary metabolite biosynthesis genes increased secondary metabolite production and activated silent SM-BGCs [87].

In addition to the in vivo genome engineering, CRISPR/Cas9 can be utilized for cloning and refactoring of large SM-BGCs [89, 95−99]. Restriction enzymes or PCR-based cloning strategies are not suitable for manipulation of large-sized DNA fragments due to the limited restriction sites and DNA amplification errors. The high-resolution site-specific cleavage activity of the CRISPR/Cas9 system enables efficient in vitro manipulation of large SM-BGCs, up to 100 kb [96]. In addition, multiplexed refactoring of promoters in a SM-BGC has been facilitated by using a CRISPR/Cas9 system with transformation-associated recombination (TAR) in yeast [97]. Therefore, these in vitro SM-BGC engineering tools will provide an efficient strategy for cloning and repurposing SM-BGCs to enhance secondary metabolite production and activate silent SM-BGCs.

CRISPR/Cas9-Based Transcriptional Repression and Activation

Cas9 contains two nuclease domains, RuvC1 and HNH, which are responsible for DSB formation at the target DNA sequence [79]. Introduction of two silencing mutations to the RuvC1 and HNH nuclease domains (D10A and H840A) generates catalytically dead Cas9 (dCas9), which lacks nuclease activity yet retains DNA binding activity. By guiding dCas9 to the promoter region or coding region of the target gene, transcription initiation or transcription elongation can be blocked, respectively [100]. The CRISPR/dCas9-based transcriptional repression system, called CRISPR interference (CRISPRi), has been exploited for transcriptional repression of genes in *S. coelicolor* [86, 101]. Further, transcriptional repression of genes within SM-BGC by the CRISPRi resulted in decreased production of secondary metabolites [86, 101]. In addition, transcriptional repression by CRISPRi system can be utilized to screen functional genes [101]. Although a high-throughput functional gene screening system based on transposon sequencing (Tn-seq) has been implemented in *Streptomyces*, the

Table 2. Application of CRISPR/Cas9-mediated engineering in *Streptomyces*.

Cas	Target	Strategy	Vector	Repair	Organism	Related secondary metabolite	Remark	Ref
SpCas9	actI-orf1	Disruption	pCRISPR-Cas9	NHEJ	*S. coelicolor*	ACT	Reconstituted NHEJ with ligD expression	[86]
	actVB	Disruption	pCRISPR-Cas9	NHEJ	*S. coelicolor*	ACT	Reconstituted NHEJ with ligD expression	
	actI-orf2	Disruption	pWHU	NHEJ	*S. coelicolor*	ACT	codA(sm)-based screening system for plasmid-cured strain	[88]
	zwf2	Disruption	pCRISPomyces	NHEJ	*S. rimosus*	-	Oxytetracycline production enhancement by disruption of competitive gene	[93]
	devB	Disruption	pCRISPomyces	NHEJ	*S. rimosus*	-	Oxytetracycline production enhancement by disruption of competitive gene	
	sshg_00040 - sshg_00050	Deletion	pCRISPomyces	HDR	*S. albus*	Lanthipeptide	-	[50]
	sshg_05713	Deletion	pCRISPomyces	HDR	*S. albus*	Polycylic tetramic acid macrolactam	-	
	Formicamycin cluster	Deletion	pCRISPomyces	HDR	*S. formicae*	Formicamycin	-	[91]
	forV	Deletion	pCRISPomyces	HDR	*S. formicae*	Formicamycin	-	
	actVA-orf5	Deletion	pCRISPomyces	HDR	*S. lividans*	ACT	-	[50]
	redD - redF	Deletion	pCRISPomyces	HDR	*S. lividans*	RED	-	
	redN	Deletion	pCRISPomyces	HDR	*S. lividans*	RED	-	
	actVA-orf5 and redN	Deletion	pCRISPomyces	HDR	*S. lividans*	ACT and RED	Multiplexed editing	
	devB	Deletion	pCRISPomyces	HDR	*S. rimosus*	-	Oxytetracycline production enhancement by disruption of competitive gene	[93]
	zwf2	Deletion	pCRISPomyces	HDR	*S. rimosus*	-	Oxytetracycline production enhancement by disruption of competitive gene	
	phpD	Deletion	pCRISPomyces	HDR	*S. viridochromogenes*	Phosphinothricin tripeptide	-	[50]
	phpM	Deletion	pCRISPomyces	HDR	*S. viridochromogenes*	Phosphinothricin tripeptide	-	
	sceN	Deletion	pCRISPR-Cas9	HDR	*Streptomyces* sp.SD85	BGC11 (sceliphrolactam)	-	[18]
	sceQ-sceR fusion	Deletion	pCRISPR-Cas9	HDR	*Streptomyces* sp.SD85	BGC11 (sceliphrolactam)	Fusion of sceQ and sce R by deleting stop codon of sceQ, intergenic region between sceQ and sceR, and start codon of sceR	
	actI-orf1	Deletion	pCRISPR-Cas9	HDR	*S. coelicolor*	ACT	-	[86]
	actVB	Deletion	pCRISPR-Cas9	HDR	*S. coelicolor*	ACT	-	

Table 2. Continued.

Cas	Target	Strategy	Vector	Repair	Organism	Related secondary metabolite	Remark	Ref
SpCas9	ACT cluster	Deletion	pKCCas9	HDR	*S. coelicolor*	ACT	-	[42]
	actII-orf4	Deletion	pKCCas9	HDR	*S. coelicolor*	ACT	-	
	actII-orf4 and redD	Deletion	pKCCas9	HDR	*S. coelicolor*	ACT and RED	Multiplexed editing	
	CDA cluster	Deletion	pKCCas9	HDR	*S. coelicolor*	CDA	-	
	glnR	Deletion	pKCCas9	HDR	*S. coelicolor*	-	-	
	RED cluster	Deletion	pKCCas9	HDR	*S. coelicolor*	RED	-	
	redD	Deletion	pKCCas9	HDR	*S. coelicolor*	RED	-	
	papR3	Deletion	pKCCas9	HDR	*S. pristinaespiralis*	pristinamycin	-	[90]
	snaE1 and snaE2	Deletion	pKCCas9	HDR	*S. pristinaespiralis*	pristinamycin	-	
	actI-orf2	Deletion	pWHU	HDR	*S. coelicolor*	ACT	Development of codA(sm)-based selection system for screening plasmid-cured strain	[88]
	rpsL	Point mutation	pKCCas9	HDR	*S. coelicolor*	-	Lys88Glu mutation	[42]
	ACT, CDA, CPK, RED deleted region	Replacement	pKCCas9	HDR	*S. coelicolor* M1146, M1152	-	ΦC31 attB integration	[89]
	Non-target BGCs	Replacement	pKCCas9	HDR	*S. pristinaespiralis*	BGC2, 3, 5, 13, and 15	Non-target BGC replacement with ΦC31 *attB* or ΦBT1 attB site	
	indC-like indigoidine synthase	Insertion	pCRISPomyces	HDR	*S. albus*	Indigoidine	*KasO** promoter knock-in to activate silent BGCs	[87]
	redD	Insertion	pCRISPomyces	HDR	*S. lividans*	RED	*KasO** promoter knock-in to activate silent BGCs	
	actII-orf4	Insertion	pCRISPomyces	HDR	*S. lividans*	ACT	*KasO** promoter knock-in to activate silent BGCs	
	frbD operon and frbC homolog	Insertion	pCRISPomyces	HDR	*S. roseosporus*	FR-900098	*KasO** promoter knock-in to activate silent BGCs	
	main synthase gene	Insertion	pCRISPomyces	HDR	*S. roseosporus*	BGC3 (T1pks)	*KasO** promoter knock-in to activate silent BGCs	
	luxR-type regulator	Insertion	pCRISPomyces	HDR	*S. roseosporus*	BGC18 (T1pks)	*KasO** promoter knock-in to activate silent BGCs	
	SSGG_RS0133915	Insertion	pCRISPomyces	HDR	*S. roseosporus*	BGC24 (Nrps-t1pks)	*KasO** promoter knock-in to activate silent BGCs	
	rppA and cytochrome P450	Insertion	pCRISPomyces	HDR	*S. venezuelae*	BGC16 (T3pks)	*KasO** promoter knock-in to activate silent BGCs	
	SSQG_RS26895-RS26920 operon	Insertion	pCRISPomyces	HDR	*S. viridochromogenes*	BGC22 (T2pks)	*KasO** promoter knock-in to activate silent BGCs	
	rkD	Cloning	-	-	-	RK-682	ICE	[95]
	homE	Cloning	-	-	-	Holomycin	ICE	
	stuE~stuF2	Cloning	-	-	-	Tü 3010	ICE	[98]
	stuD1, stuD2	Cloning	-	-	-	Tü 3010	ICE	

Table 2. Continued.

Cas	Target	Strategy	Vector	Repair	Organism	Related secondary metabolite	Remark	Ref
SpCas9	Tetarimycin BGC	Cloning	-	-	-	Tetarimycin	mCRISTAR	[97]
	spr1 region (pglE - snbC)	Cloning	-	-	-	Pristinamycin	mCRISTAR	[90]
	5-oxomilbemycin BGC	Cloning	-	-	-	5-oxomilbemycin	mCRISTAR	[99]
	Jadomycin and chlortetracycline BGC	Cloning	-	-	-	Jadomycin, and chlortetracycline	CATCH	[96]
	Chloramphenicol, YM-216391, and pristinamycin II BGCs	Cloning	-	-	-	Chloramphenicol, YM-216391, and pristinamycin	CRISPR/Cas9 cleavage and Gibson assembly	[89]
SpdCas9	actI-orf1	CRISPRi	pCRISPR-dCas9	-	*S. coelicolor*	ACT	-	[86]
	actI-orf1	CRISPRi	pSET-dCas9	-	*S. coelicolor*	ACT	-	[101]
	actII-orf4	CRISPRi	pSET-dCas9	-	*S. coelicolor*	ACT	-	
	cdaPS1	CRISPRi	pSET-dCas9	-	*S. coelicolor*	CDA	-	
	cpkA	CRISPRi	pSET-dCas9	-	*S. coelicolor*	CPK	-	
	redQ	CRISPRi	pSET-dCas9	-	*S. coelicolor*	RED	-	
	actI-orf1 and cdaPS1	CRISPRi	pSET-dCas9	-	*S. coelicolor*	ACT, CDA	Multiplexed editing	
	actI-orf1 and cdaPS1, cpkA	CRISPRi	pSET-dCas9	-	*S. coelicolor*	ACT, CDA, and CPK	Multiplexed editing	
	actI-orf1, cdaPS1, and cpkA, redQ	CRISPRi	pSET-dCas9	-	*S. coelicolor*	ACT, RED, CDA, and CPK	Multiplexed editing	
	Proteins with AmiR and NasR Transcriptional Antiterminator Regulator domain (ANTAR)	CRISPRi	pSET-dCas9	-	*S. coelicolor*	-	Gene essentiality test	
FnCpf1	actI-orf1	Disruption	pKCCpf1	NHEJ	*S. coelicolor*	ACT	-	[106]
	actI-orf1	Disruption	pKCCpf1	NHEJ	*S. coelicolor*	ACT	Reconstituted NHEJ with ligD and Ku expression	
	redX	Disruption	pKCCpf1	NHEJ	*S. coelicolor*	RED	-	
	redX	Disruption	pKCCpf1	NHEJ	*S. coelicolor*	RED	Reconstituted NHEJ with ligD and Ku expression	
	redX, redG	Deletion	pKCCpf1	NHEJ	*S. coelicolor*	RED	Deletion by reconstituted NHEJ with ligD and Ku expression at two cleavage sites	
	actI-orfI	Deletion	pKCCpf1	HDR	*S. coelicolor*	ACT	-	
	redX	Deletion	pKCCpf1	HDR	*S. coelicolor*	RED	-	
	actI-orf1, redX	Deletion	pKCCpf1	HDR	*S. coelicolor*	ACT and RED	Multiplexed editing	
	SBI00792	Deletion	pKCCpf1	HDR	*S. hygroscopicus*	Adjacent to 5-oxomilbemycin	-	
FnddCpf1	actI-orf1	CRISPRi	pSETddCpf1	-	*S. coelicolor*	ACT	-	
	redX	CRISPRi	pSETddCpf1	-	*S. coelicolor*	RED	-	
	cpkA	CRISPRi	pSETddCpf1	-	*S. coelicolor*	CPK	-	
	redX, actI-orf1, and cpkA	CRISPRi	pSETddCpf1	-	*S. coelicolor*	RED, ACT, and CPK	Multiplexed editing	

resolution of Tn mutagenesis is relatively low, and thus insufficient for screening of essential *Streptomyces* genes [102]. The CRISPRi-based screening will enable high-throughput identification of essential genes and provide invaluable resources for the construction of genome-reduced chassis for efficient production of secondary metabolites via heterologous expression [103, 104].

Cpf1 as an Alternative to Cas9

Since SpCas9 recognizes 5′-NGG as the PAM sequence, there are considerable target sites in the GC-rich *Streptomyces* genome [85]. However, AT-rich regions in the *Streptomyces* genome are not targetable by the CRISPR/SpCas9 system, limiting precise engineering of the desired genomic locus. To overcome this limitation, another Cas enzyme from *Francisella novicida*, FnCpf1, has been introduced to *Streptomyces* as an alternative genome engineering tool. FnCpf1 recognizes 5′-TTV as the PAM sequence, and thus is suitable for targeting AT-rich regions, further elevating the potential of CRISPR/Cas-based genome engineering in *Streptomyces* [105]. Based on CRISPR/Cpf1, successful applications, including NHEJ, reconstituted NHEJ and HDR-mediated genome editing, have been made in *Streptomyces* [106]. In addition to genome editing, transcriptional repression based on DNase-deactivated Cpf1 (ddCpf1) has been applied in *Streptomyces*, successfully covering all applications of CRISPR/Cas9 for *Streptomyces* [106]. Cpf1 not only expands the potential targets for CRISPR/Cas mediated genome engineering, but it also broadens the range of CRISPR/Cas applicable organisms with less cellular toxicity compared to Cas9 [106]. However, *in vitro* applications of the CRISPR/Cpf1 system have not been described for SM-BGC cloning in *Streptomyces*. Unlike Cas9, Cpf1 generates a DSB with sticky ends, which may enable efficient directional cloning with simple ligation [105].

Heterologous Expression of Secondary Metabolite Biosynthetic Gene Clusters

Compared to secondary metabolite production in the native host, heterologous expression has several advantages, including: (1) it enables SM-BGCs expression of unculturable or slow-growing native host strains, (2) it overcomes the difficult genetic manipulation of the native host, and (3) it bypasses the innate regulatory network of the native host [107]. In fact, the *Streptomyces* species is the most suitable host for heterologous expression of SM-BGCs, as compared to other organisms such as *E. coli*, bacillus, or yeast, because of its (1) abundant precursors, cofactors, and enzymes for secondary metabolite biosynthesis; (2) sophisticated post-modification system for secondary metabolites such as phosphorylation, acetylation, farnesylation, and glycosylation; (3) broad antibiotic resistance and tolerance; (4) proper protein folding for the functionality of multi-enzyme complexes; and (5) other cellular environments, including pH and redox potential [108]. Over the past few decades, approximately 100 SM-BGCs have been heterologously expressed in *Streptomyces*, particularly in *S. coelicolor*, *S. lividans*, *S. avermitilis*, and *S. albus* [109]. Although heterologous expression has been successfully used for secondary metabolite production, several limitations still remain. First, the large size of SM-BGC hampers the efficiency of genetic manipulation [110]. Further, even if the transfer of SM-BGCs to the heterologous expression host was successful, their expression may be insignificant due to differences in the precursor pool from that of the native host and metabolic competitions between the target SM-BGC and other endogenous SM-BGCs in the expression host [108]. In this subsection, we discuss cloning strategies for large-size SM-BGCs and optimization of the heterologous expression host.

There are four steps to express the SM-BGC in the heterologous expression host, which include (1) acquisition of the target SM-BGC from the native host genome, (2) ligation or assembly of the SM-BGC to the vector, (3) transfer of the SM-BGC-encoded vector to the heterologous expression host, and (4) target of secondary metabolite production (Table 3).

The most frequently used method for acquisition of the target SM-BGC is the genomic library construction using cosmid, fosmid, BAC, and PAC vectors [109]. This method can be applied to a broad range of *Streptomyces* genomes, in which the full sequence is unknown. It is particularly effective to discover novel secondary metabolites from the metagenome, including unculturable bacteria [111]. The second method is to cut both ends of the target SM-BGC from the genomic DNA. However, restriction sites are not generally available at both ends of target SM-BGCs. Thus, unique restriction sites can be introduced using a suicide plasmid, which contains a homologous sequence of one of the end sites, and integrated into both ends of target SM-BGCs by single crossover. *Streptomyces* bacterial artificial chromosome system (pSBAC) is a successful example for some SM-BGCs, such as tautomycetin (80 kb) and pikromycin (60 kb) [112, 113]. Another strategy is the integrase-mediated recombination (IR) system that introduces integration sites, such as $attB_6$ and $attB_9$, at both ends by single crossover

Table 3. Different strategy for BGC cloning.

BGC cloning steps	Strategies	Representative examples	Ref
Acquisition of the target BGC from the native host genome	Genomic library	Cosmid, fosmid, BAC, and PAC	[135]
	Cut off both ends of target BGC	Restriction: pSBAC	[113]
		Integrase: IR	[114]
		CRISPR: CATCH, mCRISTAR, and CRISPR-TAR	[96, 97, 120]
	PCR amplification	DNA assembler	[116]
Ligation or assembly of the target BGC to the vector	In vitro	Sticky end ligation: pSBAC	[113]
		Blunt end ligation: ICE	[95]
		Gibson assembly: CATCH and MSGE	[89, 96]
	In vivo	Recombination in native host: IR	[114]
		Recombination in *E. coli*: LLHR	[117]
		Recombination in yeast: TAR, DNA assembler, DiPac, and mCRISTAR	[97, 115, 116, 118]
Transferring BGC vector to the expression host	Conjugation	pUWLcre	[136]
	Protoplast transformation	pSKC2 and pOJ446	[137]
Target secondary metabolite production by expression of the BGC vector	Integrative	pSET152, pCAP01, and pESAC	[118, 138]
	Replicative	pSKC2 and pUWL201	[139]

homologous recombination of the vector, such as pKC1139 [114]. Φ-integrase-mediated excision of the target SM-BGC is subsequently performed to obtain the SM-BGC vector in vivo. Recently, in vitro site-specific digestion of genomic DNA using the CRISPR/Cas9 system was used as an alternative strategy for the acquisition of target SM-BGCs [94−97, 115]. The third method for acquisition of target SM-BGCs is PCR amplification [116], in which each fragment has homologous arms at both ends added by PCR primers to self-assemble or assemble together with genetic parts such as promoters and RBSs. As all three SM-BGC acquisition methods have their own advantages and limitations, they should be applied according to specific situations.

Ligation or assembly methods of target SM-BGC to the heterologous expression vector can be largely divided into two groups, in vitro and in vivo. In vitro cloning of the target SM-BGCs involves (1) ligation of cognate sticky ends formed by restriction enzyme digestion or two CRISPR/Cas9 digestion using T4 DNA ligase [95, 109] and (2) Gibson assembly using 5′ exonuclease [89, 96]. Ligation of two fragments by DNA ligase is simple and efficient, but preparation of cognate ends for ligation is required. Conversely, the Gibson assembly method is theoretically universal for any fragments by introduction of homologous sequence at their ends through PCR amplification. In vivo cloning of SM-BGCs exploits the homologous recombination system in the native host [114], *E. coli* [117], and yeast [118]. For example, integrase-mediated recombination (IR) directly obtains the SM-BGC-containing vector from the genomic DNA of the native host [114]. Linear-linear homologous recombination (LLHR) using λ-Red system or RecET system in *E. coli* is one of the most widely used in vivo recombination systems with high cloning efficiency, but their reported size limit is about 50 kb [107, 109, 117]. Unlike bacteria, yeast have their own efficient homologous recombination system, such that more than two large fragments can be assembled by transformation of all fragments simultaneously into yeast, which is named as transformation-associated recombination (TAR) [119]. The TAR cloning method has shown relatively higher efficiency, size capacity, and number of fragments in many strategies such as CRISPR-TAR, mCRISTAR, and DNA assembler, compared to other in vivo cloning methods [97, 116, 120]. Thus, TAR and in vitro Gibson assembly are considered as the most high-throughput and efficient strategies for the preparation of *Streptomyces* SM-BGCs vectors.

In most cases, constructed SM-BGC vectors have been transformed to the heterologous expression host through conjugation between *E. coli* and *Streptomyces* strains [109]. Otherwise, the vector can be directly transformed to the heterologous expression host by the protoplast method [32], which depends on the vector components such as *oriT* (essential for conjugation) and the *Streptomyces* species. After transfer of the SM-BGC vector to the expression host, the SM-BGC vector can be integrated into the host genome or remain as a replicative plasmid. Most heterologous

expression vectors in previous studies were integrative vectors, which are more stable after serial generations [109]. Genetic stability is very important for the further fermentation process of secondary metabolites [121]. However, the copy number of the integrative SM-BGC vector is only one compared to multi-copy replicative vectors. Therefore, multi-copy integration of the SM-BGC vector into genome or promoter refactoring of SM-BGC genes should be additionally performed to increase productivity [89]. Thus far, many SM-BGC cloning strategies for heterologous expression have been developed. However, cloning of a large size and number of SM-BGC fragments is a common limitation. Therefore, a high-throughput, stepwise, systematic strategy for efficient cloning of SM-BGCs for heterologous expression should be considered.

Streptomyces Chassis Strains for Heterologous Gene Expression

To improve yields of secondary metabolites, *Streptomyces* hosts were genetically modified by removing endogenous SM-BGCs, nonessential genes, and genomic regions and engineering genes with pleiotropic functions. Representative examples of optimized *Streptomyces* as heterologous expression hosts are shown in Table 4. Removing SM-BGCs resulted in a "reduced *Streptomyces* genome," which can conserve energy and other building blocks in addition to the specific precursor pool. In other words, the nucleotide and energy for replication of the reduced genome will be decreased, and this redundant energy can be used for the target metabolite production. Indeed, several engineered strains of *S. coelicolor* (4 SM-BGCs deletion), *S. lividans* (3 SM-BGCs deletion), and *S. albus* (15 SM-BGCs deletion) have shown improved target secondary metabolite production and reduced background chemical profiles [104, 122–124].

Nonessential genomic regions (NGR) are usually located at the ends of linear chromosomes, which are not conserved in all species and dispensable for cell growth. They include genomic islands (GI), IS elements, and endogenous CRISPR array regions that decrease genomic stability. For example, NGRs in *S. avermitilis* (1.48 Mb) and *S. chattanoogenisis* (0.7 Mb) were selected, based on comparative genomics of *Streptomyces* genomes, and deleted by λ-Red system or Cre/loxP recombination system [121, 125]. As expected, deletion of NGRs increased the fitness level of the engineered strain relative to the wild-type strain with beneficial effects on morphology, ATP level, NADPH level, transformation efficiency, and genetic stability in *S. chattanoogenisis* [121]. However, large deletion of NGRs may cause undesired deleterious effects on cell growth due to the unknown essential function of genes and synthetic lethality of more than two abundant essential genes. Therefore, the systems level of functional genomic studies should be followed to determine the nonessential regions more precisely. Integration of these functional studies with multi-omics data and experimental validations might allow construction of a highly efficient *Streptomyces* chassis strain for heterologous expression of SM-BGCs [126, 127].

Genes with pleiotropic functions can be additionally engineered to improve productivity of target secondary metabolites. For instance, deletion of phosphofructokinase gene *pfk* and global transcriptional regulator gene *wblA* and overexpression of global transcriptional regulator gene *crp* in *S. albus* increased secondary metabolite production, redox potential, and fitness by changing the global transcriptional status [128]. Further, point mutations of RNA polymerase gene *rpoB* and ribosomal protein gene *rpsL* in *S. coelicolor* showed increased fitness and secondary metabolite production by altering regulation at both the transcriptional and translational levels [104]. As pleiotropic functions usually include undesired phenotypes for secondary metabolite production, the engineering target should be selected carefully through rational design based on systematic information.

Future Perspective

In this review, we summarized a synthetic biology strategy to produce novel secondary metabolites in *Streptomyces*. Accumulation of genetic information and SM-BGCs aided by recent advances in Next-Generation Sequencing (NGS) has revealed the enormous potential of *Streptomyces* as a reservoir for novel bioactive compounds and is far outpacing our capacity to explore SM-BGCs and their products [129]. To fully harness *Streptomyces*' ability to produce valuable secondary metabolites, rational design and efficient synthetic biology tools for *Streptomyces* are essential. To date, however, in silico SM-BGC prediction tools, such as antiSMASH, still need to be optimized for precise mining capability. Further, synthetic biology tools for *Streptomyces* are limited to fulfill the precise designs for novel secondary metabolite production. To this end, integration of massive omics data and vigorous functional studies can elevate the fidelity of SM-BGC mining and increase genetic parts for *Streptomyces* engineering. Specifically, construction of genetic part libraries based on transcriptome and translatome data by screening using a

Table 4. Representative examples of *Streptomyces* chassis strain for optimal heterologous expression.

Heterologous host	Engineering	Target genes or regions	Deletion method	Expressed BGC	BGC vector	Effect	Limitation	Ref
Streptomyces coelicolor M145	BGC deletion and Pleiotropic gene engineering	Deletion of four BGCs (ACT, RED, CPK, and CDA) Point mutations of *rpoB* and *rpsL*.	Homologous recombination by double crossover of the plasmid	Shloramphenieocol and congocidine	Cosmid	Improved production, clean profile of background metabolites	Low fitness	[104]
Streptomyces sp. FR-008	BGC deletion	Deletion of three BGCs (candicidin, type III PKS, and type I PKS)	Homologous recombination by double crossover of the plasmid	None	None	Improved fitness, sporulation, and clean profile of background metabolites	Heterologous expression was not tested	[124]
Streptomyces lividans TK24	BGC deletion	Deletion of three BGCs (ACT, RED, and CDA) One copy integration of AfsRS by attB integrase	Homologous recombination by double crossover of the plasmid	Streptothiricins, borrelidin, and linear lipopeptides	BAC	High-throughput functional genome mining of *Streptomyces rochei*	Low fitness, laborious screening of BAC libraries	[123]
Streptomyces lividans TK24	BGC deletion	Deletion of three BGCs (ACT, RED, and CDA) Additional copies integration of AfsRS by attB integrase	Homologous recombination by double crossover of the plasmid	Hybrubins	BAC	High-throughput functional genome mining of *Streptomyces variabilis* Pathway crosstalk between incompletely deleted RED cluster.	Low fitness	[140]
Streptomyces albus J1074	BGC deletion	Deletion of fifteen BGCs (Frontalamide, Paulomycin, Geosmin, Lantibiotic, carotenoid, flaviolin, candicidin, antimycin, 2 PKS-NRPS, and 4 NRPS)	Homologous recombination by double crossover of the plasmid using λ-red system	Tunicamycin B2, moenomycin M, griseorhodin A, pyridinopyrone A, bhimamycin A, didesmethylmensacarcin, didemethoxyaranciamycinone, aloesaponarin II, and cinnamycin, fralnimycin	Fosmid and BAC	Improved production, clean profile of background metabolites	Moenomycin M productivity was reduced.	[122]
Streptomyces avermitilis	Nonessential region deletion and BGC deletion	Deletion of 1.48 Mb left arm determined by comparative genomics	Homologous recombination by double crossover of the plasmid using λ-red system Cre/loxP system	Streptomycin, cephamycin C, and pladienolide	Cosmidand BAC	Improved production by additional introduction of regulatory gene and optimization of codon usage	Low conjugation efficiency	[103]
Streptomyces avermitilis	Nonessential region deletion	Deletion of 1.48 Mb left arm and some regions determined by comparative genomics	Homologous recombination by double crossover of the plasmid using λ-red system Cre/loxP system	Streptomycin, ribostamycin, kasugamycin, pholipomycin, oxytetracycline, resistomycin, pladienolide B, erythromycin A, bafilimycin B1, nemadectin α, aureothin, leptomycin, cephamycin C, holomycin, lactacystin, clavulanic acid, rebeccamycin, novobiocin, chloramphenicol, 2-methylisoborneol, pentalenolactone, amorpha-1,4-diene, taxa-4,11-diene, levopimaradiene, and abietatriene	Cosmid and BAC	Improved production, fitness, clean profile of background metabolites. Broad precursor capacity (sugar, polyketide, peptide, shikimate, and MVA or MEP)	Ribostamycin, oxytetracycline productivity were reduced	[125]

Table 4. Continued.

Heterologous host	Engineering	Target genes or regions	Deletion method	Expressed BGC	BGC vector	Effect	Limitation	Ref
Streptomyces chattanoogensis L10	Nonessential region deletion	Deletion of 1.3 Mb and 0.7 Mb nonessential arms determined by comparative genomics and prediction tools	Cre/loxP recombination	ACT	pMM1	Improved production, fitness, ATP, NADPH, transformation efficiency, and genetic stability. Dispersed morphology.	1.3 Mb deleted strain was detrimental due to deletion of some unknown genes	[121]
Streptomyces albus J1074	Pleiotropic gene engineering and BGC deletion	Deletion of *pfk*, *wblA*, overexpression of *cpk*, and deletion of one BGC (paulomycin)	Homologous recombination by double crossover of the plasmid using λ-red system	ACT	Fosmid	Improved production, fitness, and NADPH.	Undesirable effects might be incurred due to the global change of transcriptome	[128]

high-throughput reporter system will deliver a universal set of genetic parts for *Streptomyces*.

Although genetic tools based on the CRISPR/Cas system have offered diverse strategies to enhance secondary metabolite production and activate silent SM-BGCs [87, 89, 90, 93, 99], further optimization of the CRISPR/Cas system for *Streptomyces* is still required. An example is that the toxicity of Cas nucleases is disadvantageous for multiplexed applications and CRISPR/Cas bearing systems. In addition, expansion of PAM recognition is required to enable efficient genome engineering. These limitations can be overcome by modulating Cas expression and exploiting Cas variants [106, 130, 131]. Particularly, CRISPR activation (CRISPRa) has not been applied to *Streptomyces* [132]. The CRISPRa approach can serve as an efficient strategy to investigate silent SM-BGCs without labor-intensive genome editing efforts. Multiplexing the precise transcriptional regulation through integration of both CRISPRa and CRISPRi is expected to reconstruct the metabolic network to enhance the precursor supply, reducing flux toward competing pathways or unwanted by-products, bypassing gene expression regulation, and expressing the SM-BGCs. Recently, toehold-gated gRNA was developed, which links endogenous signals to activation of the CRISPR/Cas system [133]. As the production of secondary metabolites requires sufficient accumulation of precursors and activation of biosynthetic genes, the combination of toehold-gated gRNA and CRISPRa and CRISPRi strategies will suggest a new metabolic engineering approach, linking the production of precursors to production of secondary metabolites.

Development of synthetic biology tools can also be exploited to construct the *Streptomyces* chassis for heterologous expression of novel SM-BGCs. So far, *S. albus* J1074 with deletion of 15 BGCs and *S. chattanoogensis* L10 with deletion of 0.7 Mb of a nonessential arm seem to be the best *Streptomyces* chassis for heterologous expression [121, 122]. A further challenge might be the construction of the "superhost *Streptomyces* chassis" by removing all endogenous SM-BGCs, nonessential genes, and genomic regions and adding all precursor synthetic genes. By heterologous expression of the target SM-BGC in this superhost chassis, productivity of target secondary metabolites will be further improved. In addition, novel secondary metabolites will be discovered from a wide array of silent SM-BGCs in a high-throughput manner. However, construction of a superhost for all secondary metabolites might not be feasible due to precursor differences in *Streptomyces* species. Indeed, all five types of secondary metabolites were produced in the *S. albus* J1074 strain; however, some metabolites were not produced significantly [122]. Therefore, construction of several "specialized *Streptomyces* chassis hosts" for each type of secondary metabolite might be a better choice [108]. This "design-build-test (DBT)" cycle, which is a rational design based on *in silico* SM-BGC mining, the build of a SM-BGC expression in *Streptomyces* chassis, and the high-throughput test of secondary metabolite production will be iterated to learn and optimize production of novel secondary metabolites (Fig. 1). This synthetic biology strategy will ultimately expand the productivity and diversity of available novel secondary metabolites as potential biopharmaceuticals.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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