**Streptomyces as Microbial Chassis for Heterologous Protein Expression**

Soonkyu Hwang1,2†, Yongjae Lee1,2†, Ji Hun Kim1,2†, Gahyeon Kim1,2†, Hyeseong Kim1,2†, Woori Kim1,2, Suhyung Cho1,2, Bernhard O. Palsson3,4,5 and Byung-Kwan Cho1,2,6*

1Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, South Korea, 2KAIST Institute for the BioCentury, Korea Advanced Institute of Science and Technology, Daejeon, South Korea, 3Department of Bioengineering, University of California, San Diego, La Jolla, CA, United States, 4Department of Pediatrics, University of California, San Diego, La Jolla, CA, United States, 5Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Lyngby, Denmark, 6Innovative Biomaterials Research Center, KAIST Institutes, Korea Advanced Institute of Science and Technology, Daejeon, South Korea

Heterologous production of recombinant proteins is gaining increasing interest in biotechnology with respect to productivity, scalability, and wide applicability. The members of genus *Streptomyces* have been proposed as remarkable hosts for heterologous production due to their versatile nature of expressing various secondary metabolite biosynthetic gene clusters and secretory enzymes. However, there are several issues that limit their use, including low yield, difficulty in genetic manipulation, and their complex cellular features. In this review, we summarize rational engineering approaches to optimizing the heterologous production of secondary metabolites and recombinant proteins in *Streptomyces* species in terms of genetic tool development and chassis construction. Further perspectives on the development of optimal *Streptomyces* chassis by the design-build-test-learn cycle in systems are suggested, which may increase the availability of secondary metabolites and recombinant proteins.

Keywords: *streptomyces*, heterologous expression, chassis, recombinant protein, secondary metabolite

**INTRODUCTION**

Many efforts have been made to produce recombinant proteins on a large industrial scale. Heterologous protein expression in the platform host by the introduction of the gene of interest is the most promising approach in several aspects. Heterologous expression hosts can overcome the complexities associated with native hosts, such as slow and fastidious growth, limited molecular biology tools, scarce genetic information, and low productivity (Park et al., 2020). Several representative heterologous expression hosts have been used according to their specialized characteristics and product types (Huo et al., 2019; Zhang et al., 2019; Kang and Kim, 2021; Pham et al., 2021). For example, *Escherichia coli* is the most extensively studied and used for heterologous protein expression, as it exhibits rapid growth, ease of genetic manipulation, and high productivity. *Bacillus subtilis* is another extensively used Gram-positive bacteria that acts as an efficient workhorse for the production of industrial enzymes and pharmaceuticals, having robust growth and good genetic tractability, along with many endogenous proteases. Beyond the microbial hosts, *Saccharomyces cerevisiae* is a commonly used eukaryotic host for recombinant protein production due to its recombinant DNA stability, easy genome engineering strategies, and the ability to provide post-translational modifications. Recently, a high-throughput platform for heterologous protein expression for fungal biosynthetic gene clusters (BGCs) in *S. cerevisiae*, named HEx, was established to successfully produce diverse fungal secondary metabolites (SMs)...
Chinese hamster ovary (CHO) cells are the mammalian cell line that dominates the other recombinant protein production hosts because of their capacity to express large and complex recombinant proteins, but genetic engineering and clonal selection are more difficult than microbial hosts.

Among the potential microbial chassis, the genus *Streptomyces*, a soil-derived Gram-positive bacterium with a high GC content genome, is an attractive microbial host for heterologous protein expression (Liu et al., 2018a). To survive under limited nutrient conditions and compete with various other microorganisms in soil, *Streptomyces* has a complex developmental cycle that sporulates after mycelial growth and produces diverse SMs (Van Wezel and Mcdowall, 2011; Barbuto Ferraiuolo et al., 2021). In addition, various enzymes are known to be produced and secreted to degrade complex nutrients, such as lignocellulose, and to perform various reactions on different substrates (Berini et al., 2020). SMs are synthesized by a series of reactions catalyzed by the biosynthetic enzymes encoded in the BGCs. For example, polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) are organized by multiple domains, and even by multiple modules in several types that constitute a large biosynthetic machinery (Hwang et al., 2020). In this sense, *Streptomyces* are likely to be more amenable to producing functional biosynthetic enzymes from BGCs relative to other microorganisms, such as *E. coli*. Although heterologous expression of BGC enzymes has been successful in *Streptomyces* platform hosts, several inadequacies and overcoming strategies have been identified.

In this review, the advantages and limitations of *Streptomyces* as a chassis for heterologous protein expression are discussed. Then, BGCs and recombinant protein examples of heterologous expression in *Streptomyces* are reviewed, particularly focusing on genetic tool development and chassis construction. In addition, future perspectives on effective strategies with respect to the design-build-test-learn (DBTL) cycle in synthetic biology are also proposed.

**STREPTOMYCES AS THE HETEROLOGOUS PROTEIN EXPRESSION HOST: ADVANTAGES AND LIMITATIONS**

*Streptomyces* as a Heterologous Protein Expression Host

The growth of several *Streptomyces* species is robust and scalable, as they have been intensively used for the industrial production of SMs (Berini et al., 2020). They are also favorable hosts for heterologous production of recombinant proteins. This is mainly due to their protein secretion systems in the extracellular milieu (Liu et al., 2013; Hamed et al., 2018; Berini et al., 2020). Secretion is beneficial in terms of protein folding, because the extracellular medium is an ordinarily oxidizing environment that promotes disulfide bond formation for correct folding, while the cytoplasm is a reducing environment that impedes the disulfide bonds (Anne et al., 2014). *Streptomyces* is a Gram-positive bacterium that has a cell wall layer without an outer membrane, and its secretion into the extracellular space is easier than that of Gram-negative bacteria. As the cell disruption is not required, the downstream protein purification from secreted proteins by *Streptomyces* is more comfortable without contamination by intracellular proteins (Sommers et al., 2009). In addition, *Streptomyces* exhibits low toxicity and does not contain lipopolysaccharides (LPSs), which may act as potent immunostimulatory endotoxins (Berini et al., 2020), allowing easy downstream purification processes. Moreover, *S. lividans*, the most frequently used *Streptomyces* host for recombinant protein production, is known to have low restriction enzyme and proteolytic activities, enabling efficient introduction of the recombinant DNA and high yield of the protein, respectively (Anne et al., 2014).

**Streptomyces as a Heterologous Expression Host of Secondary Metabolite BGCs**

The number of BGCs per *Streptomyces* genome was predicted to be 36.5 on average, by antiSMASH, reflecting the potential diversity of SMs and their biosynthetic enzymes (Lee et al., 2021). As these biosynthetic enzymes have specific and sophisticated structures, the cellular environment in the heterologous expression host to maintain their structures is important for their function (Hwang et al., 2020). Therefore, *Streptomyces* heterologous expression hosts are favorable for producing functional biosynthetic enzymes encoded in the BGCs of *Streptomyces* sources relative to other microorganisms, such as *E. coli*.

Correct folding is closely correlated with enzyme solubility. In particular, correct incorporation of multiple domains and modules is necessary for enzyme solubility. For example, exchanging the dehydratase domain or linker domain for combinatorial biosynthesis of polyketides (PKs) often generates insoluble aggregates of enzymes (Liew et al., 2012; Cai and Zhang, 2018). In addition, uncoupling transcription and translation may lead to insolubility due to misfolding, which requires optimization of the transcription and translation rate for heterologous expression (Skiba et al., 2018). Codon usage has a significant impact on the translation rate of GC-rich BGC sequences that are likely to be translated inefficiently at low-use codons in *E. coli* (Ke and Yoshikuni, 2020). However, *Streptomyces* hosts do not need additional codon optimization to improve the translation efficiency for BGC expression. Moreover, the cytoplasmic redox state of the heterologous expression host should be similar to that of the native host to stimulate correct disulfide bonds. For example, a large size of the main synthetase of the glycopeptide NRPS chloroeremomycin BGC CepA was expressed in *E. coli*, but it showed low activity, perhaps due to incorrect folding by the non-optimal redox state of *E. coli* (Trauger and Walsh, 2000; Skiba et al., 2018). Therefore, *Streptomyces* is a more convenient host for the expression of functional large BGC enzymes. Chaperone is also essential for the functioning of biosynthesis enzymes in several examples, including MbtH homologs within several NRPS BGCs and PqqD family chaperones within lasso peptide BGCs (Shao et al., 2019). Other considerations for the
heterologous expression of functional enzymes, including cDNA selection, expression system optimization, tagging, pH, temperature, and cofactor, were reviewed in a previous study (Skiba et al., 2018).

Metabolic background of *Streptomyces* plays a key role on successful heterologous production of SMs, especially in terms of substrate availability. As *Streptomyces* hosts produce a variety of SMs from native BGCs encoded in their genomes, they naturally possess diverse reactions to produce sufficient precursors such as propionyl-CoA, methylmalonyl-CoA, benzoyl-CoA, and others for PKs, and p-aminobenzoic acid, 3-amino-5-hydroxybenzoic acid, and various β-amino acids for NRPs, respectively (Pfeifer and Khosla, 2001; Luo et al., 2016; Sharma et al., 2021). Despite of the ability, sufficient precursor supply should be additionally optimized for the high yield by preventing the undesired flux to other pathways competing for the same precursor molecules to enhance the productivity.

Next, SM biosynthesis usually involves many diverse post-modifications at the final step by tailoring enzymes governing phosphorylation, methylation, acetylation, cyclization, farnesylation, and glycosylation (Pfeifer and Khosla, 2001; Liu et al., 2018a). As *Streptomyces* heterologous expression hosts have a high possibility of expressing functional tailoring enzymes, they would be better than other hosts. However, tailoring enzymes are distinct even between *Streptomyces* species that are carefully chosen for co-expression. For instance, a farnesyl transferase-coding gene was not found in the genome of *S. albus*, indicating the requirement for additional introduction of this enzyme to produce related SMs (Liu et al., 2018a). Lastly, high production of SMs, especially antibiotics, requires the transport and resistance genes to have self-tolerance against the products. *Streptomyces* heterologous expression hosts may have native transporters or resistance proteins with broad substrate specificity to have higher...
self-tolerance than other heterologous hosts. With the availability of precursors and the presence of other additional genes, such as regulatory, tailoring, transport, and resistance genes, *Streptomyces* heterologous expression hosts are a good choice because they are not very different from the native BGC hosts as compared to *E. coli*.

In summary, *Streptomyces* is a specialized host for BGC expression due to its functional biosynthetic enzyme expression, substrate availability, and presence of other accessory genes. Recombinant proteins are also effectively expressed in *Streptomyces*, mainly due to their secretion systems. However, there are also some drawbacks compared to other heterologous hosts in terms of the robustness of growth, genetic tools, and genetic information. To overcome these limitations, rational engineering approaches for heterologous protein production have been developed for *Streptomyces*, as presented in the following sections (Figure 1).

**GENETIC TOOLS FOR RATIONAL ENGINEERING OF BGC HETEROLOGOUS EXPRESSION IN *STREPTOMYCES***

Since a number of genes are included in a gene cluster, BGCs are generally large, ranging from 10 kb to over 200 kb in size. Cloning of large BGCs requires considerable time and effort and is limited in terms of efficiency. Therefore, many tools have been developed to improve the efficiency and fidelity of *Streptomyces* heterologous hosts. This section focuses on the cloning or assembly strategies that have been developed so far. The following section will provide the steps to improve heterologous protein expression via the engineering of genetic elements.

**Strategies for the Cloning and Assembly of BGCs**

The traditional cloning strategy is a library-based method in which genomic DNA is digested with restriction enzymes and ligated into vectors, such as cosmids, P1-derived atomic chromosome (PAC), and the bacterial atomic chromosome (BAC). Cosmid is a vector based on the *E. coli* λ bacteriophage, that can accommodate a small BGC of up to 42 kb (Jones et al., 2013), while BAC containing an F-factor or PAC vector can accommodate a relatively large BGC, up to 490 kb in BAC (Zimmer and Verrinder Gibbins, 1997) and 300 kb in PAC (Tu et al., 2018). The library-based cloning method was problematic in that it required numerous clones to be screened by polymerase chain reaction (PCR). Instead, direct cloning strategies for BGCs without library construction have emerged. Direct cloning strategies were further divided depending on whether the cloning was performed in vivo or in vitro (Table 1).

Plasmid *Streptomyces* bacterial artificial chromosome (pSBAC) is an *E. coli*-Streptomyces shuttle vector that can carry large BGCs. After insertion of the pSBAC vector into the restriction sites at the flanking regions of the BGC, the BGC could be separated from the genome by the restriction enzymes and captured into pSBAC (Nah et al., 2015). This vector can switch conveniently from single-copy to high-copy replication in *E. coli* and then integrate into heterologous *Streptomyces* host after intergenic conjugation (Liu et al., 2009). When integrated, pSBAC used phage ΦBT1 attP-int site-specific integration system instead of a F31 attP-int system, which has been reported to have detrimental effects on antibiotic production. Using this method, meridamycin (Liu et al., 2009), tautomycetin (Nah et al., 2015), and pikromycin (Pyeon et al., 2017), which are large BGCs, were successfully expressed. However, during the process, target BGCs must be free of restriction sites, and sometimes it is challenging to find unique restriction sites.

Both linear-to-circular homologous recombination (LCHR) and linear-to-linear HR (LLHR) are strategies in which homologous recombination occurs between the flanking sequences of the target BGC from digested genomic DNA and the vector containing homology arms after co-transformation into *E. coli* (Ma and Wang, 2019). LCHR occurs between the linear insert and the circular vector using the Red-α/β protein derived from the lambda red phage, whereas LLHR occurs between two linearized DNA fragments using RecE/T from a Rac prophage (Fu et al., 2012). These methods are rapid and highly efficient for cloning small- or medium-sized BGCs, such as gougerotin (Jiang et al., 2013), streptoketides (Qian et al., 2020), and oxytetracycline (Yin et al., 2016). However, this is limited because the linearized vector and BGC segment must be introduced simultaneously into *E. coli* before homologous recombination occurs. Recently, exonuclease combined with RecET recombination (ExoCET), an improved strategy from LLHR, was developed (Wang et al., 2018). Because of the activity of 3' exonuclease from *in vivo* T4 polymerase, approximately 80 bp single-strand homologous arms were generated from the flanking regions of a linear vector and a target BGC fragment. Two DNA fragments were annealed *in vitro* before transformation into *E. coli*. Owing to the *in vitro* annealing step, the efficiency of *in vivo* homologous recombination through the RecE/T system could be improved. Using ExoCET, 106 kb salinomycin was cloned without further assembly processes (Yin et al., 2015; Wang et al., 2018).

Unlike the above methods, transformation-associated recombination (TAR) cloning is a method in which the insert and vector are co-transformed into yeast. Yeast is known to have excellent recombination machinery, so it can clone large DNA fragments of up to 250 kb in size (Kouprina and Larionov, 2006). After cloning the linearized shuttle vector pCAPO1 and the DNA fragment through homologous recombination in yeast, the vector was transformed into *E. coli*, transformed with *Streptomyces* by conjugation, and inserted into the chromosome via the attP-int site. However, some isolated clones may be unstable in yeast or lost during the mitotic propagation of yeast, while some DNA fragments may not be fully recovered in yeast (Kouprina and Larionov, 2006).

The DNA assembler is another *in vivo* assembly method in yeast based on TAR cloning (Shao et al., 2012). All successive DNA fragments were designed to overlap with each other and amplified by PCR. When fragments and a linearized vector were
**TABLE 1 | Selected examples of strategies for cloning and assembly of secondary metabolite BGCs.**

| Strategies | Principle | NP (class, size (kb)) | Heterologous host | Advantage | Limitation | Reference |
|------------|-----------|-----------------------|-------------------|-----------|-----------|-----------|
| Genomic library | ● Restriction digestion and ligation | A54145 (NRP, 60) | S. ambofaciens BES2074, S. albus J1074 | Suitable for small to large size of fragments | Efficiency of ligation and transformation is low | (Alexander et al., 2010; Li et al., 2017b; Tan et al., 2017; D’agostino and Gulder, 2018; Liu et al., 2018b; Tu et al., 2018) |
| | ● Integrative into chromosome with prophage cassette | Kinamycin (PKS, >65) | S. coelicolor M1152, S. albus J1074, S. lividans TK24, S. coelicolor strains | Genome sequence data is not required | Screening colonies could be time consuming and laborious process | |
| | | Abyssomicin (PK, 74) | | | | |
| | | Spinosad (PK, 80) | | | | |
| | | FK506 (PKS, 120) | | | | |
| pSBAC | ● Restriction digestion and ligation | Pikromycin (PKS, 60) | S. lividans TK21, S. coelicolor M145 | ~200 kb gDNA fragments could be inserted into shuttle vector with high efficiency | Challenging to achieve unique restriction sites | (Liu et al., 2009; Nah et al., 2015; Luo et al., 2016; Pyeon et al., 2017) |
| | ● Homologous recombination and ligation | Taumorycetin (PKS, 80) | S. coelicolor M145 | | Fragments must be free of such restriction sites | |
| | | Meridamycin (PKS, 95) | S. lividans TK21, S. lividans TK24/ K4-114 | | | |
| LCHR, LLHR | ● RecA-mediated linear to circular homologous recombination | Gougerotin (other, 17.8) | S. coelicolor M1146 | Rapid (2–3 days) and do not rely on PCR amplification | Difficult to clone large size of DNA fragments | (Jiang et al., 2013; Yin et al., 2015; Yin et al., 2016; Qian et al., 2020) |
| | ● RecET-mediated linear to linear homologous recombination | Streptoketides (PKS, 21.7) | S. coelicolor M1152/M1154 | Suitable for small size of DNA fragments | | |
| | | Oxytetracycline (PK, 29) | S. venezuelae WVR2006 | Application of a wide range of fragment size and genome complexities | | |
| | | Salmomycin (PKS,106) | S. coelicolor A3 (2) | Efficiency is not so high | | Wang et al. (2018) |
| ExoCET | ● In vivo RecET mediated homologous recombination with in vitro activities of T4 polymerase | Enterocin (PK, 21) | S. lividans TK23, S. coelicolor M1146, S. albus J1074 | Suitable for cloning various size of DNA fragments | Some clones are unstable in yeast, so undergo deletions during mitotic propagation in yeast cells | (Yamanaka et al., 2014; Bonet et al., 2015; Tang et al., 2015; Bilyk et al., 2016; Novakova et al., 2018) |
| TAR | ● In vivo homologous recombination of S. cerevisiae | Greccycline (PKS, 36) | S. lividans TK24, S. coelicolor M145 | Highly efficient recombination system | | |
| | | Mithramycin A (PK, 45) | S. coelicolor M1146 | | | |
| | | Taromycin A (NRP, 67) | S. lividans TK24 | | | |
| DNA assembler | ● PCR-amplified small DNA fragments and vectors are co-transformed into S. cerevisiae and in vivo assembled | PTMs (PKS/ NRP, 18) | S. lividans 66 | Refactoring for cryptic gene clusters in a single-step manner with high efficiency | Difficult to clone large size of DNA fragment | (Luo et al., 2013; Shao et al., 2013; Shao and Zhao, 2013) |
| | | Aureothin (PK, 29) | S. lividans 66 | | | |
| | | Spectinamiblin (PK, 45) | S. lividans 66 | | | |
| Gibson assembly | ● Two adjacent DNA fragments with same terminal sequences overlap to become one fragment by 5’ exonuclease, DNA polymerase and DNA ligase | Bicyclomycin (other, 9) | S. coelicolor M1146/M1152 | Assembled DNA molecules up to 100 kb in E. coli with low error rates | Inefficiency for large size of DNA fragments | (Zhao et al., 2015a; Linares-Otaya et al., 2017; Vior et al., 2018) |
| | | | S. coelicolor M1146 | Not rely on restriction enzyme site | | |
| DIPAC | ● Long amplicon PCR and in vitro HIFI assembly with Q5 polymerase | Kocurin (RPP, 12) | S. coelicolor M1146 | Simultaneous cloning and refactoring of BGCs are possible | Time-consuming for cloning and costly cloning reagents | (D’agostino and Gulder, 2018; Greunke et al., 2018) |
| | | Hapalasin (PK/ NRP, 23) | S. coelicolor M1154 | | | |
| | | Anabaeonepterin NZ857 (NRP, 29) | E. coli BAP1 | | | |
| | | Erythromycin (PK, 54.6) | S. coelicolor M1152/M1154 | | | |
| | | Septacidin (other, 24) | S. albus J1074 | | | |
| | | Tu3010 (PK/ NRP, 27.4) | S. avermitilis MA-4680 | | | |
| CATCH | ● RNA-guided Cas9 nuclease excision and Gibson assembly | | Various size of any DNA fragments can be extracted and assembled in a single step | | Efficiency is not so high | (Tang et al., 2018; Tao et al., 2019a) |
co-transformed into yeast, they were assembled into a vector with in vivo homologous recombination (Shao et al., 2009). This strategy has the advantage of modularity, which makes it possible to refactor gene clusters. In particular, by refactoring the well-known promoter, RBS, and terminator regions with the gene cluster using overlap extension PCR, silent BGCs could be expressed (Shao et al., 2013; Joska et al., 2014).

In addition to in vivo cloning, BGCs can also be cloned in vitro. In vitro cloning involves DNA assembly, which is mainly based on Gibson Assembly. Gibson Assembly assembles several fragments into one fragment by overlapping adjacent DNA fragments with the same terminal sequences using a 5′ exonuclease, DNA polymerase, and ligase (Gibson et al., 2009). Using this method, short- and medium-sized BGCs were efficiently cloned (Zhou et al., 2015a; Linares-Otoya et al., 2017). Recently, to compensate for the decrease in efficiency due to high GC content, a modified Gibson Assembly was developed, and 67 kb of BGC was successfully cloned (Li et al., 2015; Li et al., 2018).

Direct pathway cloning (DiPaC) is an in vitro cloning method based on Gibson Assembly. After making a long amplicon PCR of approximately 10 kb per each using Q5 high-fidelity polymerase, the fragments were assembled into a BGC using HiFi DNA assembly and captured as a PET28 vector (Greunke et al., 2018). This strategy is suitable for small- or medium-sized BGCs; however, this is a time-consuming process and the reagent is expensive. Recently, a strategy has been introduced to overcome the limitations of DiPaC with sequence- and ligation-independent cloning (SLIC), using T4 polymerase instead of HiFi DNA assembly (D’agostino and Gulder, 2018).

The Cas9-assisted targeting of chromosome segments (CATCH) method is also an in vitro cloning method based on Gibson Assembly. All or partial fragments of a BGC are directly cleaved using PCR-based amplified single-guide RNA (sgRNA) and Cas9 protein and then captured into a vector using Gibson Assembly, the whole process being performed at once on agarose gels (Jiang and Zhu, 2016). Since only the target region is cleaved using the Cas9 protein, intact fragments without an off-target could be captured into vectors. In addition, the risk of DNA shearing is low compared to other methods because chromosomes are protected by the agarose matrix. Thus, even very large fragments of approximately 100 kb can be cloned (Jiang et al., 2015).

In vivo cloning strategies can clone large fragments with high efficiency, but since cloning has to be done in bacteria or yeast, the process is slow because it has to wait for cells to grow. Furthermore, extraction from organisms is necessary to achieve the vector, which is then transformed into E. coli to transfer them to Streptomyces by conjugation. On the other hand, since in vitro cloning is not performed in cells, the process proceeds easily and quickly. However, the efficiency of cloning large DNA fragments is not yet high. If in vivo and in vitro strategies can be used together to compensate for their respective disadvantages, large BGCs can be cloned more efficiently and maintained in stable conditions.

Genetic Parts

The development of efficient cloning tools has made it possible to introduce target BGCs into heterologous hosts. However, after being introduced into a heterologous host, only trace amounts of target SMs were detected. This is because the expression of BGCs is regulated by complex regulatory networks (Xia et al., 2020). Therefore, attempts have been made to engineer additional genetic parts to express target BGCs (Table 2).

Vectors can be divided into two types depending on whether they self-replicate in the host cell or integrate into the host genome. First, replicative vectors are mainly derivatives based on the pRM5 vector containing the SCP2 replicon (Marti et al., 2000). This backbone vector is maintained in Streptomyces in a low-copy state and can be stably replicated up to a size of 31 kb or more (Fong et al., 2007). Meanwhile, pKC1139 is mainly used to improve the productivity of target BGCs while maintaining 20–50 copy numbers in Streptomyces (Du et al., 2015). Since BGCs are large in size, low-copy replicative vectors were used to maintain stability within Streptomyces (Marti et al., 2000). However, as these vectors also have some limitations, such as difficulties in genetic manipulation and reduced DNA purity, due to a low level of DNA recovery, the strategy was changed from a replicative vector to an integrative vector (Liu et al., 2009; Heidorn et al., 2011). φC31 integrase is commonly used, but the problems of detrimental effects on the production of BGCs and entering a pseudo position have been reported in some strains. Therefore, φBT1 replaced φC31. In addition, several other integrases were identified (Table 2). Recently, a modular and integrative vector that is easily compatible with vectors for cloning and assembly methods has been developed (Aubry et al., 2019). The antibiotic resistance cassette module and the integration system cassette module can be easily replaced with other cassettes by unique restriction sites, so multiple vector types can be generated from one backbone. Because it has the advantage of being able to add a module, this method can contribute to resolving the difference in efficiency depending on the type of host or cloning strategy (Aubry et al., 2019).

One of the most critical factors for expressing target BGCs is the promoter element. First, constitutive promoters are commonly used for overexpression of heterologous genes. In particular, the ermE promoter, which contains two regions, ermEp1 and ermEp2, is widely used as a strong constitutive promoter. ermEp* is a variant that is upregulated by the deletion of trinucleotides from the ermEp1 region (Alexander et al., 2010). In addition to the ermE series, there are several strong constitutive promoters, SF14, rpsLp, gapdhp, and kasOp, each of which is known to have similar or stronger intensities to ermEp* (Labes et al., 1997; Shao et al., 2013; Wang et al., 2013). kasOp was engineered by removing two binding sites of regulators and then using random mutation. Finally, kasOp* was developed, which showed the strongest strength in S. coelicolor and S. avermitilis compared to SF14 and ermEp*, because its structure can be recognized more readily by sigma factor, HrdB, than the other two promoters (Wang et al., 2013).

Promoters have different strengths depending on the species. To overcome this limitation, a method was developed to screen strong constitutive promoters among synthetic promoter libraries by randomizing the consensus sequences of known promoters (Seghezzi et al., 2011; Siegl et al., 2013; Bai et al., 2015). For example, to acquire stronger promoters than kasOp*, a library...
TABLE 2 | Examples of genetic parts for gene expression.  

| Vector | Strategies       | Name                  | Reference                                                                 |
|--------|-----------------|-----------------------|---------------------------------------------------------------------------|
| Replicative | Low-copy       | pRMS, pHU204, pOJ446, pJJ101 | (Marti et al., 2000; Medema et al., 2011; Carrillo Rincon et al., 2018)     |
|         | High-copy       | pUC119, pKCI139       | Du et al. (2015)                                                           |
| Integrative | ΦCS1 attP-int locus | pSET152, pOJ486, pJU6902, pJU10702 (cosmid), pHL931 (BAC), pESAC13 (BAC), pCAP01 (TAR cloning) | (Huang et al., 2005; Jones et al., 2013; Bonet et al., 2015; Yin et al., 2016; Horbal et al., 2018a; Liu et al., 2018b) |
|          | ΦBT1 attP-int locus | pMS82, pJU10257, pSBAC | (Gregory et al., 2003; Hong et al., 2005; Liu et al., 2009)                |
|          | VWB attP-int locus | pCLY10 (TAR cloning)  | Bilyk et al. (2016)                                                        |

| Genetic parts | Features | Reference |
|---------------|----------|-----------|
| Integrase     | ΦCS1     | Derived from Streptomyces phage ΦCS1 | Khaleel et al. (2011)                                                       |
|               | ΦBT1     | Derived from Streptomyces phage ΦBT1 | (Saltz, 2012)                                                               |
|               | VWB      | Derived from bacteriophage VWB, introduced to a tRNA^AGG (AGG) gene in several Streptomyces species | Van Mellaert et al. (1998)                                                  |
|               | RP3      | Derived from Streptomyces phage RP3, introduced to a tRNA^AGG (AGG) gene in S. nivosus | Van Mellaert et al. (1998)                                                  |
|               | R4       | Derived from S. parvulus phage R4, site-specifically introduced into the chromosome of S. parvulus | Foor et al. (1985)                                                          |
|               | TG1      | Derived from Streptomyces phage TG1, site-specifically introduced in S. castellae | Foor et al. (1985)                                                          |
|               | Bxb1     | Derived from Mycobacteriophage Bxb1 | Ghosh et al. (2006)                                                         |
|               | SV1      | Derived from S. venezuelae phage SV1 | Fayed et al. (2014)                                                         |
|               | Φ1/6     | Derived from prophage Φ1/6, introduced to chromosome of tetracycline producing strains, S. aureofaciens | Farkasovska and Godany, (2012)                                              |
|               | ΦO2J     | Derived from actinophage OzzyJ | Ko et al. (2020)                                                             |
| Constitutive  | ermEp    | Derived from S. erythraeus, promoter of erythromycin resistance gene | Alexander et al. (2010)                                                    |
| Promoter      | ermEp*   | Trinucleotide deletion in the ermEp1 region of ermEp | Alexander et al. (2010)                                                    |
|               | SF14     | Derived from phage 119 isolated from S. ghanaensis | Labes et al. (1997)                                                        |
|               | gapdhP   | Derived from S. griseus, promoter of gyceraldehyde-3-phosphate dehydrogenase | Shao et al. (2013)                                                          |
|               | rpsLp    | Derived from S. griseus, promoter of 3OS ribosomal protein | Tan et al. (2017)                                                           |
|               | kasOp    | Derived from S. coelicolor A3, promoter of SARP family regulator | Wang et al. (2013)                                                          |
|               | kasOp*   | Engineered by removing the binding sites of ScbR and ScbR2 | Wang et al. (2013)                                                          |
|               | thrM4p   | Derived from S. chattanoogensis L10, promoter of peptidase M4 thermolysin | Wang et al. (2019a)                                                         |
| Synthetic     | ermEp1   | Randomized to construct synthetic promoter library in S. lividans, 38 synthetic promoters sequenced | Seghezzi et al. (2011)                                                     |
| promoter      | library  | Randomized synthetic promoter library in S. venezuelae. Among 180, six showed stronger strength than kasOp* | Bai et al. (2015)                                                           |
|               |          | Synthetic promoter library based on ermEp1 promoter. Among 56, one promoter shows stronger strength than ermEp1 | Siegl et al. (2013)                                                         |
| Inducible     | tipAp    | Thiostrepton inducible promoter from S. lividans 66 | Chiu et al. (1999)                                                          |
| promoter      | tcp820p  | Tetracycline inducible promoter | Rodriguez-Garcia et al. (2006)                                             |
|               | rolAp    | Isovaleronitrile or ε-caprolactam inducible promoter from Rhodococcus rhodochrous J1 | Komeda et al. (1996)                                                       |
|               | xylAp    | Xylose inducible promoter | Noguchi et al. (2018)                                                      |
|               | crtBp    | Oxytetracycline inducible promoter | Yang et al. (2019)                                                          |
|               | PA3-rolA-RolR | Resorcinol inducible promoter | Horbal et al. (2014)                                                        |
|               | P21-cmt-CymR | Cymate inducible promoter | Horbal et al. (2018b)                                                       |
| Terminator    | TD1      | Derived from Bacillus subtilis phage F29 | Pulido et al. (1987)                                                        |
|               | Fd       | Derived from E. coli phage fd | Ward et al. (1986)                                                          |
|               | tsabB    | Heterologous expression level of glucuronidase in Mycobacteria | Horbal et al. (2018b)                                                       |
| Riboswitch    |          | Synthetic theophylline-dependent riboswitches | Eyles et al. (2018)                                                         |

was constructed by mutating the spacer sequence between the -35 and -10 regions of kasOp* in S. venezuelae, and their strengths were measured by GFP fluorescence. Among the 180 synthetic promoters, six synthetic promoters were stronger than kasOp* (Bai et al., 2015). Alternatively, an approach to discover strong native promoters within the host has been developed. For
example, to expand a panel of strong native promoters, 32 candidates that may be strong promoters were screened using RNA-seq data from \textit{S. albicus}. Then, through \textit{xylE} activity assay with time course analysis, 10 native promoters were validated to have stronger strength than \textit{kasOp+} (Luo et al., 2015).

The disadvantage of constitutive promoters is that sustained expression of BGCs can generate negative effects on host bacteria, such as products becoming toxic or unable to maintain the bacterial growth rate. Inducible promoters have the advantage of being able to turn on gene expression only at a desired point. However, some inducible promoters have the disadvantage of leaky expression (Huang et al., 2015), such as the commonly used \textit{tipA} promoter (Chiu et al., 1999) induced by thiostrepton and \textit{tcp830} strong promoter (Rodriguez-Garcia et al., 2005) induced by tetracycline. To overcome the problem of leaky expression of promoters, synthetic inducible systems have been developed. One is the PA3-rollo-RolR system, which consists of a codon-optimized RolR regulator and synthetic promoter A3 with operater rolO from \textit{C. glutamicum}, induced by resorcinol. The other is the P21-\textit{cnt}-CymR system, similar to the resorcinol inducible system, induced by the presence of cumate, by binding CymR to the \textit{cnt} operator fusing with the synthetic promoter 21 (Horbal et al., 2014).

The transcriptional terminator is also an essential genetic component for gene expression to prevent readthrough problems. Terminators mainly consist of inverted repeat sequences, which are known to contribute to stability by forming a stem-loop structure in the mRNA state. However, only a limited number of terminators are known to contribute to stability by forming a stem-loop structure in the bacterial growth rate. Inducible promoters have the advantage of being able to turn on gene expression only at a desired point. However, some inducible promoters have the disadvantage of leaky expression (Huang et al., 2015), such as the commonly used \textit{tipA} promoter (Chiu et al., 1999) induced by thiostrepton and \textit{tcp830} strong promoter (Rodriguez-Garcia et al., 2005) induced by tetracycline. To overcome the problem of leaky expression of promoters, synthetic inducible systems have been developed. One is the PA3-rollo-RolR system, which consists of a codon-optimized RolR regulator and synthetic promoter A3 with operater rolO from \textit{C. glutamicum}, induced by resorcinol. The other is the P21-\textit{cnt}-CymR system, similar to the resorcinol inducible system, induced by the presence of cumate, by binding CymR to the \textit{cnt} operator fusing with the synthetic promoter 21 (Horbal et al., 2014).

The transcriptional terminator is also an essential genetic component for gene expression to prevent readthrough problems. Terminators mainly consist of inverted repeat sequences, which are known to contribute to stability by forming a stem-loop structure in the mRNA state. However, only a limited number of terminators are efficient in \textit{Streptomyces}. The TD1 terminator, derived from \textit{B. subtilis} phage \textit{Φ29}, successfully recognized the termination of \textit{S. lividans} and was active as an \textit{in vivo} terminator (Pulido et al., 1987). Likewise, the Fd terminator originating from \textit{E. coli} phage \textit{fd} can also be used as an \textit{in vivo} terminator (Ward et al., 1986). Another example is the synthetic bi-directional transcriptional terminator \textit{B} (ttsb\textit{BB}) is a highly efficient terminator in \textit{S. lividans} in the test of heterologous glucuronidase activity (Huff et al., 2010; Horbal et al., 2018b).

The riboswitch is composed of an aptamer region that detects a compound and a gene expression control region that can change the structure after the aptamer binds. By redesigning sequences that interact with aptamers, a synthetic riboswitch was developed that can initiate the transcription when a specific compound enters (Rudolph et al., 2013). Based on this principle, a theophylline-dependent riboswitch introduced in \textit{S. coelicolor} increased heterologous bottromycin production by 120-fold (Eyles et al., 2018). In addition, efforts have been made to increase or control the efficiency of gene expression by using genetic parts for translational regulation, such as RBS and codon usage (Makrides, 1996). Nevertheless, since many genetic parts still show different efficiencies depending on \textit{Streptomyces} species, continuous development of genetic parts is required.

**CHASSIS DEVELOPMENT FOR RATIONAL ENGINEERING BGC HETEROLOGOUS EXPRESSION IN \textit{STREPTOMYCES}**

The expression of BGCs in native hosts might be difficult in genetic engineering, have a complex metabolic background, and their endogenous BGCs are often cryptic. Then, the producers showed no expression of BGCs under laboratory conditions. Therefore, BGC expression using a heterologous production platform is a solution. The key characteristics for robust heterologous expression chassis are as follows: 1) fast growth, 2) genetic amenability and well-established genetic toolkits, 3) clean metabolic background to supply precursors for building a variety of SMs and express diverse biosynthetic classes with minimal interruption from host SMs; 4) providing all of the genes of heterologous biosynthetic pathways regarding transcription, translation, and post-translational modifications, 5) resistance to SMs, effective efflux pump system, 6) efficient nutrient and oxygen utilization, and 7) optimization of downstream processes. Based on these principles, many chassis strains have been constructed in diverse species (Table 3).

**\textit{S. coelicolor}**

\textit{S. coelicolor} is a genetically well-characterized \textit{Streptomyces} species. The 8.6 Mb genome has been reported, including over 20 BGCs (Bentley et al., 2002). \textit{S. coelicolor} is the genetically best-studied species as the regulatory systems of endogenous BGCs has been well-understood. Genetic tools, including replicative and integrative vectors and well-established genetic parts, can be applied in \textit{S. coelicolor}. Several mutant strains have been developed for successful heterologous expression of BGCs for the production of SMs. In addition to the deletion of the main antibiotic gene clusters (\textit{S. coelicolor} M1146), point mutations in \textit{rpoB} encoding RNA polymerase β-subunit (\textit{S. coelicolor} M1152), and \textit{rpsL} encoding ribosomal protein S12 were introduced to regulate the strain at both the transcriptional and translational levels (\textit{S. coelicolor} M1154) (Hu et al., 2002; Ochi, 2007; Gomez-Escibano and Bibb, 2011). As a result, transcriptional and translational fidelity increased BGC expression, and SM productivity was enhanced without growth impairment. These strains successfully expressed 18 different heterologous BGCs, leading to increased SM yields ranging from 0.4 to 160 mg/L. Moreover, the genome-minimized strain was constructed by deleting all the PKS and NRPS BGCs and also 900 kb subtelomeric sequence (\textit{S. coelicolor} ZM12) (Zhou et al., 2012). Galbonolide BGC was successfully expressed in heterologous \textit{S. coelicolor} ZM12 with a clean background, which verified the essential role of core genes in the biosynthesis of galbonolides (Liu et al., 2015).

**\textit{S. lividans}**

\textit{S. lividans} lacks anendonuclease restriction system, whereas \textit{S. coelicolor} and \textit{S. avermitilis} restrict methylated DNA, making it highly acceptable to foreign DNA. Notably, \textit{S. lividans} presents high conjugation efficiency, and thus, this species is applicable for high-throughput transfer of the libraries (Martinez et al., 2004). Indeed, \textit{S. lividans} TK24-derived strains have been used as heterologous hosts for library expression and function-directed screening systems (LEXAS) (Xu et al., 2016). \textit{S. lividans} SBT5 was developed by the deletion of Act, Red, and CDA BGCs from \textit{S. lividans} TK24 (Shima et al., 1996). For high-throughput heterologous expression and screening of genomic libraries to express cryptic BGCs and to mine bioactive compounds, SBT5
### TABLE 3 | Examples of Streptomyces heterologous expression chassis.

| Chassis | Genetic manipulation | Natural product | Product type | Effect | Reference |
|---------|----------------------|-----------------|--------------|--------|-----------|
| S. coelicolor M1146 | Deletion of four endogenous BGCs (Act, Red, Cpk, and CDA) from the genome of S. coelicolor M145, a derivative of S. coelicolor A3 (2) strain lacking two plasmids | Chloramphenicol, congocidine, cypemycin, griseomycin, actagardine, planosporin, GE3746B, napismycin, clorobiocin, coumervomycin A1, caprazamycin, FK506/FK520 (tarcolimus), meroclorin, gougerotin, endophenazine, roseoflavin, holomycin, and tunicamycin | NRP, PK, Inaridin, RIP, oligopyrrole, aminocoumarine, and other | Reduced competition for precursor | [Gomez-Escritiano and Bibb, 2011; Gomez-Escritiano and Bibb, 2014; Bekiesch et al., 2016] |
| S. coelicolor M1152 | Introduction of point mutations in rpoB [S433L] in S. coelicolor M1146 | Flavulin | PK | Higher transcriptional and translational fidelity | [Wang et al., 2018; Liu et al., 2015] |
| S. coelicolor M1154 | Introduction of point mutations in rpsL [K98E] in S. coelicolor M1152 | Murayauquinone, hybrubins, and Whole genome BAC library from S. rochei | PK and PKS-NRPS hybrid | Positive regulation of afsRS gene on cryptic BGC gene expression | [Xu et al., 2016] |
| S. lividans SBT5 | Deletion of Act, Red, and CDA BGCs from S. lividans TK24 | Murayauquinone, hybrubins, and Whole genome BAC library from S. rochei | PK and PKS-NRPS hybrid | Superior host for high-throughput heterologous expression of BGCs and LEXAS screening | [Peng et al., 2018] |
| S. lividans GX28 | Stepwise integration of two global regulatory genes (afsRS<sub>up</sub> and afsRS<sub>down</sub>) and two codon-optimized multi-drug efflux pump genes (hmrA, mdaA) into S. lividans SBT5 | Murayauquinone, hybrubins, dehydrodromelotomycin, pienicidin A1, and actinomycin D | PK, PKS-NRPS, hybrid, and NRP | Increased heterologous production of PKs, NRPs, and hybrid antibiotics | [Ahmed et al., 2020] |
| S. lividans LJ1018 | Deletion of negative regulatory gene (wbiA<sup>+</sup>) from S. lividans SBT5 | S. albus subsp. chlorinus NRRL B-24108 genomic library | Library | Increased success rate in isolation of novel bioactive NPs originating from eDNA | [Ahmed et al., 2020] |
| S. lividans YA9 | Deletion of 9 endogenous BGCs (178.5 kb) within the chromosome of S. lividans TK24 using iterative marker excision system (IMES) | Tunicamycins, deoxycoformycin, deoxycnymycin, and 7 new compounds | Nucleoside antibiotic, lanthipeptide, and aromatic polycyclotetide PK, RIP, and new compound | Used in the production of amino acid-based natural products | [Myronovskyi et al., 2018] |
| S. lividans YA11 | Deletion of 11 endogenous BGCs (228.5 kb) | Cinnamycin, tunicamycin, didemethylmensacarcin, fralnimycin, bhimamycin A, aloeasiporarin II, abicudin, ciliotin, and S. albus subsp. chlorinus NRRL B-24108 genomic library | Tunicamycin B2, monomycin M, didemethyl meniscaracin, demethoxyaranciamycione, griseorhodin, and cinnamycin | Clean metabolic background | [Ye et al., 2016] |
| S. albus Def14 | Deletion of 15 endogenous BGCs using IMES from S. albus J1074, defective in an active SaI3 restriction-modification system (500 kb) (Cluster-free J1074) | Tunicamycin B2, monomycin M, didemethyl meniscaracin, demethoxyaranciamycione, griseorhodin, and cinnamycin | PK, saccharide, and lanthipeptide peptide | Reduced competition for precursor | [Ahmed et al., 2020] |
| S. albus B4 | Introduction of two additional phage phiC31 attB loci into the chromosome for stability (total four attB sites) | S. albus subsp. chlorinus NRRL B-24108 genomic library | Library | Multi-copy integration | [Ahmed et al., 2020] |

(Continued on following page)
TABLE 3 | (Continued) Examples of Streptomyces heterologous expression chassis.

| Chassis       | Genetic manipulation                                                                                                                                                                                                 | Natural product                                                                 | Product type          | Effect                                                                                       | Reference                  |
|---------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-----------------------|-----------------------------------------------------------------------------------------------|-----------------------------|
| S. albus ZXJ-6 | - Introduction of a three-gene cassette for the biosynthesis of ethylmalonyl-CoA and salinomycin  
- Subsequent deletion of the salinomycin BGC                                                                                                              | Actinorhodin                                                                   | PK                    | - Host for heterologous production of PK                                                    | Zhang et al. (2017b)        |
| S. avermitilis SUKA5 | - Deletion of 1.51 Mb left arm (two major endogenous BGCs) and oligomycin BGC from S. avermitilis WT (82.11%)                                                                                                         | Pladienolid                                                                     | PK                    | - Rich supplies of common PK precursors including malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA  
- Enhanced intracellular energy (ATP) and reducing power (NADPH/NADP+)                                                                                     | Komatsu et al. (2010)                                                          |
| S. avermitilis SUKA17 | - Deletion of three terpene compound BGCs (geosmin, neosenthalenolactone, and carotenoid) from S. avermitilis SUKA5 (81.46%)                                                                                          | Cephamycin, and amorpha-1,11-diene                                               | NRP and plant terpenoid intermediate | - Clean metabolic background  
- Increase in genetic stability  
- Reduced competition for precursor  
- Large amount of cell mass  
- Functionalization of positive regulator gene  
- Capable of expressing diverse BGCs                                                                 | (Komatsu et al., 2013; Bekiesch et al., 2016)                                  |
| S. avermitilis SUKA22 | - Isogenic to SUKA17, the right side of the deletion region of SUKA17 was replaced by a mutant-type loxP sequence to prevent undesired recombination                                                                 | Shinorine, porphyra-334, 17-hydroxyslabadan A, raimonol, pholopomycin, resistomycin, baliformycin B1, and nemadectin | NRP, PK, terpene, and other | - Positive morphological change                                                                 | (Komatsu et al., 2013; Bekiesch et al., 2016) |
| S. chattanoogensis L321 | - Deletion of 0.7 Mb non-essential genomic region (7 putative BGCs and complete fatamycin BGC)                                                                                                                                                 | eGFP, indigoidine, and actinorhodin                                              | NRP and PK            | - Enhanced ATP and reducing power  
- Improved productivity of protein and secondary metabolite  
- Positive morphological change  
- Clean metabolic background  
- Increase in genetic stability  
- Promising platform cell to produce PK                                                                 | Bu et al. (2019)                                                               |
| S. venezuelae YJ003 | - Deletion of all des gene clusters (desI/desF/II and desR) from the wild type                                                                                                                             | Gentamicin A2, kanamycin                                                        | Aminoglycoside antibiotics | - Used in the production of polypeptides and aminoglycosides                                  | Hong et al. (2004)          |
| S. venezuelae YJ028 | - Deletion of both pkA PKS genes and des genes                                                                                                                                                                                               | Doxorubicin                                                                    | PK                    | - Used in the sugar engineering                                                              | Jung et al. (2007)          |
| S. venezuelae DHS2001 | - Deletion of pkA PKS gene from wild type                                                                                                                                                                                                  | Tylosin polyketide synthase, epothilones, flavonoid, stilbene, flavones, flavonols, barbamide, naringenin, pinocembrin, and 4-O-demethylbarbamide | PKS, plant-specific (PK and lipopeptide (hybrid NRPS-PKS)) | - Diverse precursors for PKs  
- Used in the production of PKS, NRPS, and PKS-NRPS hybrid                                                                 | (Jung et al., 2006; Park et al., 2009; Park et al., 2010; Park et al., 2011; Kim et al., 2015) |
| S. venezuelae WVR2006 | - Deletion of jadomycin biosynthetic gene cluster  
- Downstream of Pik PKS (pakAV, pakC, pakD) remains intact                                                                                                                             | Oxytetracycline                                                                | PK                    | - Normal growth, differentiation  
- Cleaner metabolite profiles  
- Improved oxytetracycline production up to 430 mg/ L in 48 h                                                                                     | (Fan et al., 2012; Yin et al., 2016)                                           |
| S. rimosus SR0 | - Deletion of whole oxytetracycline gene cluster of S. rimosus 461  
- Introduction of constitutively expressed cluster-situated activator gene cltcB                                                                                              | Chlortetracycline                                                              | PK                    | - Several grams level of titer with industrial grade, one of the highest titer reports of heterologous antibiotics production | Wang et al. (2019b)         |
was further developed by elevating conjugation efficiency and the positive effects of global activators (Edgar and Bibi, 1997; Poelarends et al., 2002; Behnken et al., 2012; Peng et al., 2018). For example, S. lividans GX28 was used as a library expression host (Peng et al., 2018; Nah et al., 2021). As a result, high-throughput LEXAS of one BAC library and two cosmid libraries from three different Streptomyces strains successfully screened three antibiotic BGCs (Peng et al., 2018).

In addition, a genome-minimized strain was constructed for cleaner and simpler metabolite profiles than the parental strain, in which 11 endogenous BGCs were deleted and two additional phage ΦC31 attB sites were introduced (S. lividans ΔYA11) (Ahmed et al., 2020). The benefit of adding additional integration sites was validated by expressing heterologous gene clusters in both parental S. lividans TK24 and ΔYA11, whose production levels were elevated in S. lividans ΔYA11 by approximately two-fold.

**S. albus**

*S. albus* is one of the most widely used heterologous expression hosts in *Streptomyces*. It provides successful heterologous expression of diverse BGCs for the production of PKs, NRPs, terpenes, and saccharides with high productivity (Wendt-Pienkowski et al., 2005; Gullon et al., 2006; Lombo et al., 2006; Winter et al., 2007; Feng et al., 2009; Makitynskyy et al., 2010; Bilyk and Luzhetsky, 2014). The versatility of *S. albus* is highly related to its relatively small genome (6.8 Mb) and the availability of efficient genetic toolkits (Zaburannyi et al., 2014). *S. albus* J1074 is a derivative of the *S. albus* G1 defective in an active Sall restriction-modification system; thus, heterologous BGCs can be easily transformed. *S. albus* J1074 mostly showed the best performance in isomigrastatin (PK production), which was 2 to 10-fold higher than that of other *Streptomyces* chassis strains (Yang et al., 2011). The remarkable production capacity was also demonstrated by discovering novel SMs from cryptic gene clusters of the metagenome, which did not yield on other chassis hosts. From *S. albus* J1074, all dispensable BGCs, including PKS, NRPS, lanthipeptide, and glycoside antibiotic clusters, were gradually deleted and marker-free; thus, an extremely clean metabolite profile was achieved, named *S. albus* Del 14. Additionally, ΦC31 attB sites were introduced for multi-copy integration (*S. albus* B4). This large deletion did not influence growth, morphological characteristics, or fitness.

**S. avermitilis**

*S. avermitilis* is an industrial microorganism that produces important anthelmintic agent avermectins (Miller et al., 1979; Gao et al., 2010). *S. avermitilis* SUKA5, SUKA17, and SUKA22 strains were genome-reduced derivatives from the wild-type. *S. avermitilis* has an intrinsically stable genome because it has the shortest terminal inverted repeats (TIR) (Komatsu et al., 2013). Such genetic properties are strengthened by systematic large-scale deletions; thus, the strain is more suitable for the expression of exogenous BGCs (Komatsu et al., 2010; Komatsu et al., 2013). The difference in pladienolide production between wild-type and SUKA5 is more than 20-fold, which appears to be due to the competition for the common acyl-CoA precursor for pladienolide biosynthesis and avermectin biosynthesis in the wild-type strain, which demonstrates the extended precursor availability in mutants. In addition, morphological differentiation, growth rate, and biomass in the stationary phase were enhanced compared to the wild-type strain (Komatsu et al., 2013). The engineered host can produce heterologous PKs, NRPs, aminoglycosides, shikimate-derived compounds, and terpenes.

**Others**

*S. chattanoogensis* is an industrial microorganism used for the production of natamycin. Through the rational deletion of non-essential genomic regions based on systematic analysis, a genome-reduced chassis strain was constructed (*S. chattanoogensis* L321) (Zhou et al., 2015b). Unlike wild-type, the engineered strain does not have an endogenous CRISPR/Cas system; therefore, several CRISPR/Cas9 systems were successfully introduced without any interference and improved the efficiency of genome editing. Heterologous production level of SMs in wild-type and the chassis was investigated during serial passages of the culture, resulting in the constant level in the chassis, while the reduced level in the wild-type. This is because the undesired mutations were generated and accumulated in the wild-type genome according to the serial passages, while the removal of mobile genetic element in the chassis genome showed a positive effect on genetic stability. Also, the rational deletion of non-essential genomic regions might pleiotropically influence the engineered strain, resulting in highly efficient expression of BGCs. Indeed, the *S. chattanoogensis* chassis strain showed great performance as a heterologous host, especially in PKs (Zhou et al., 2015b).

*S. venezuelae* ATCC 15439 has a fast growth rate, which enables a large accumulation of cell mass and metabolites (Xue and Sherman, 2001). In addition, ease of genetic manipulation, liquid sporulation in a dispersed manner, and abundant building blocks for SMs are other advantages. Three major chassis strains of *S. venezuelae* are 15,439, DHS 2001, YJ003, and YJ28. These chassis strains are promising heterologous hosts that successfully express diverse heterologous BGCs from different sources.

*S. rimosus* 461 is a high-yielding industrial producer of oxytetracycline. Therefore, the construction of a heterologous host from *S. rimosus* 461 is worthwhile for expression of BGCs for the production of other tetracycline antibiotics and type II PK, validated by the heterologous expression of chlorotetracycline BGC in SR0 chassis, which is 38-fold higher than that of the original producer, and 68-fold higher than that of the *S. lividans* strain (Wang et al., 2019b).

Furthermore, a rational study using computational approaches, such as comparative genome analysis, should be widely used for the prediction of dispensable genetic elements, such as mobile genetic elements, genomic islands, and BGCs, to carefully engineer the strains for heterologous expression of BGCs. In addition to BGC expression, elimination of the competing precursor sinks greatly facilitates the identification of exogenous bioactive compounds, improves production yield with increased precursor pool, and streamlines downstream processing.
RATIONAL ENGINEERING APPROACHES FOR HETERLOGOUS PRODUCTION OF RECOMBINANT PROTEINS IN STREPTOMYCES

As a decomposer in natural habitats, Streptomyces secretes multiple enzymes to degrade saprophytic compounds, and its secretion capacity makes Streptomyces attractive as a host for recombinant protein production (Crawford, 1978). In this section, the secretion pathways of Streptomyces and approaches to increase recombinant protein yield are discussed.

Secretion Pathway of Streptomyces

The Sec-pathway is the dominant bacterial protein export pathway, comprising approximately 96% of exported proteins in E. coli (Orfanoudaki and Economou, 2014). On the other hand, for Streptomyces, especially S. lividans, twin-arginine translocation (Tat-pathway) is exploited for approximately 21% of the secreted proteins (Tsolis et al., 2018). Utilization of the Tat-pathway, which secretes proteins in the folded state, can be advantageous over the Sec-pathway, since cytoplasmic folding is crucial for the activity of some proteins (Weiner et al., 1998; Feilmeier et al., 2000; Thomas et al., 2001). However, the Sec-pathway is generally superior to the Tat-pathway for heterologous protein production in terms of production titer and applicability. For example, the production yield of streptokinase from Streptococcus equisimilis in S. lividans was 30 times higher when the Sec-pathway was utilized (Kim et al., 2010). In addition, the overproduction of Sec-pathway dependent protein (α-amylase) using Tat-pathway was unsuccessful in S. lividans (Gullon et al., 2015b). Since the Tat-pathway exports folded proteins, the secretion efficiency may be highly dependent on the structure of the protein (Fisher et al., 2008). To demonstrate the lower product yield via the Tat-pathway, a comparative transcriptomics approach was applied to S. lividans overexpressing proteins via either Sec-pathway or Tat-pathway to identify possible bottlenecks for protein production, and a stringent response was induced when the Tat-pathway was exploited for protein secretion (Gullon et al., 2015a). However, the Tat-pathway is still worth investigating for proper intracellular folding of proteins.

Approaches to Increase the Protein Yield

Streptomyces would not be a preferred host over E. coli for heterologous protein production, with few exceptions, including proteins from Streptomyces, due to the lower product yield (Kim et al., 2010). Many efforts have been made to improve protein production, and representative examples are presented in Table 4.

Table 4

| Approach to Increase Protein Yield | Description | Example |
|----------------------------------|-------------|---------|
| Strong Inducible Promoters       | Ensure strong induction for high expression | XylE from Pseudomonas putida |
| Regulatory Engineering           | Modulate expression levels | csnR deletion to improve chitosanase production |
| Signal Peptide Optimization      | Enhance cleavage and secretion efficiency | xysA signal peptide optimization |

The most frequently used approach for improved protein production is to increase the gene expression level, mainly by adopting strong promoters. One of the frequently used promoters is ermE*, which has been widely adopted as a strong constitutive promoter in Streptomyces (Bibb et al., 1985). While many of the promoters from relatively close species are functional in the production host, regulatory elements for the promoters would also be conserved, resulting in transcriptional inhibition by negative regulators. To overcome this drawback, the promoter sequence or Streptomyces host was engineered to avoid negative regulation. For example, the activity of the strong inducible promoter xysAp from S. halstedii was further increased by deleting the homologs of negative regulators, BxR and XlnR, in S. lividans, resulting in up to 70% higher production of a heterologous protein (Sevillano et al., 2016). Another example is the production of chitosanase from Kitasatospora sp. N106, an actinomycete in S. lividans (Dubéau et al., 2011). To improve protein production, the negative regulatory gene csnR was deleted and/or two base pairs of the palindromic negative regulatory sequences in the promoter were mutated. The approach for increased gene expression level still holds more potential for improvement, since only a small number of promoters have been exploited (Table 5). Accumulation of transcriptomic data enables the identification and utilization of strong endogenous promoters, and a synthetic promoter library has been demonstrated for Streptomyces (Wang et al., 2013; Luo et al., 2015). In addition to transcription activation-related factors, transcriptional terminators are worth investigating to improve gene expression. Streptomyces lacks an extremely strong expression system, such as the T7 expression system of E. coli BL21 (DE3) (although T7 RNA polymerase has been demonstrated in S. lividans); increasing the half-life of transcripts by exploiting strong transcriptional terminators may serve as an efficient tool for higher gene expression (Lussier et al., 2010; Lee et al., 2019; Hwang et al., 2021).

Enhancing protein secretion efficiency is another important approach that drastically increases the protein yield. The native signal peptides are generally functional for protein secretion in the Streptomyces host; however, optimization of signal peptides improves protein production and secretion in terms of both the proportion of secretion and product yield (Snajder et al., 2019). For example, various signal peptides were tested for the production of XylE, the catechol 2,3-dioxygenase, from Pseudomonas putida (Bibb et al., 1985). For a more general solution, modulating the secretion pathway, rather than

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### TABLE 4 | Selected examples of optimization of heterologous protein production in *Streptomyces* since 2010.

| Optimization | Product | Native host | Expression host | Plasmid | Promoter | Promoter host | Signal peptide | Signal peptidase host | Host engineering | Reference |
|-------------|---------|-------------|----------------|---------|----------|--------------|---------------|----------------------|------------------|-----------|
| Gene expression | DagA (agarase) | *S. coelicolor* A3 (2) | *S. lividans* TK24 | pWMM3-TR1R2 | spc7p | *S. griseus* | Native | Native | | Temuujin et al. (2011) |
| | SCO3H7 (β-agarase) | *S. coelicolor* A3 (2) | *S. lividans* TK24 | pULM201 PV | ermEp | Saccharopolyspora erythraea | Native | Native | | Temuujin et al. (2012) |
| | PVA (penicillin V acylase) | *S. lividans* ATCC 13664 | *S. lividans* TK24 | pEM4 | ermEp | Saccharopolyspora erythraea | Native | Native | | Torres-Baceta et al. (2015) |
| | ETA hydrolase | *Thermobifida fusca* YX | *S. rimosus* R7 | pIJ8600 | tipAp | *S. lividans* | Native | Native | | Sinsereekul et al. (2010) |
| | Transglutaminase | *S. hygroscopicus* | *S. lividans* TK24 | pHSEV-1 tipA | Native | Native | Native; codon-optimized | Native | | Liu et al. (2016) |
| | Lipase | Metagenomic | *S. lividans* 10-164 | pAC109 | C109lp | - | Native | | Cote and Sherek (2012) |
| | CrtB (root glucanase) | *Thermobifida fusca* YX | *S. lividans* 1,326 | pSRU02 | xylose isomerase promoter | Actinoplanes missouriensis | Native | | U et al. (2013) |
| | Phospholipase D | *Streptomyces* | *S. lividans* TK24 | pIJ12719 | spc/ermEp dual promoter | *S. griseus* | saccharopolyspora erythraea | Native | Tao et al. (2019b) |
| | Chitosanase | *Kitasatospora* sp. N106 | *S. lividans* TK24 | pHM8BαM | Native | Native | Native | Native | | Dubeau et al. (2011) |
| | Chitinase | Metagenomic | *S. lividans* TK24 | pPL6 | ermEp | Saccharopolyspora erythraea | Native | | | Berini et al. (2013) |
| | Glutenase | Actinomadurium A8 | *S. lividans* TK24 | pL86 | ermEp | Saccharopolyspora erythraea | Native | | | Cavaletti et al. (2019) |
| | Chitinase | *S. avermitilis* MA-4880 | *S. lividans* 1,326 | p3350 | xylAp | *S. avermitilis* MA-4880 | Native | | | Noguchi et al. (2018) |
| | Sfp2 (keratinase) | *S. fradiae* var. k11 | *S. lividans* 1,326 | pJTU4881 | xylAp | Actinoplanes missouriensis | Native | | | Li et al. (2013) |
| | O-glycoprotein | *Mycobacterium tuberculosis* | *S. lividans* 1,326 | pIJ6021 | tipA | *S. lividans* | Native | | | Gamboa-Suasnavart et al. (2011) |
| | Secretion system | DagA (agarase) | *S. coelicolor* | *S. lividans* TK21 | pUG86 | Native | Native | | | Gabarró et al. (2017) |
| | DagA (agarase) | *S. coelicolor* | *S. lividans* TK21 | pAG401 | Native | Native | | | Gabarró et al. (2015) |
| | Transglutaminase | *S. mobaraensis* | *S. lividans* TK21 | pTOA4 | Native | Native | | | | Gabarró et al. (2017) |
| | Parinolase | Aeropyrum pernix K1 | *S. lividans* TK21 | pWPM | S. cinemamus | puf + 3 codons of the mature amylase | Native | | Noda et al. (2013) |
| | Xyl1 (xylanase) | *S. halstedii* IMI8 | *S. lividans* TK21 | pN702GEM3 | Native | S. griseus IMI 3570 | Native | | Sefviano et al. (2014) |

(Continued on following page)
| Optimization | Product | Native host | Expression host | Plasmid | Promoter host | Promoter | Signal peptide | Signal peptid host | Host engineering | Reference |
|-------------|---------|-------------|----------------|---------|---------------|----------|---------------|------------------|-----------------|-----------|
| α-amylase  | S. griseus IMI 3570 | | | | | | | | | ssA overexpression |
| Small laccase | S. coelicolor | | | | | | | | | strR deletion |
| XyE (catalase 2,3-dioxygenase) | Pseudomonas putida | S. rimosus M4018 | | | | | | | | Carrillo Rincon et al. (2018) |
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| AppA (phosphatase) | Escherichia coli | | | | | | | | | |
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diversifying signal peptides, has been demonstrated. Deletion of the sipY gene, which encodes a major signal peptidase, led to increased heterologous production of agarase from *S. coelicolor* in *S. lividans* (Gabarró et al., 2017). The secretion of agarase in the SipY-deficient strain can be complemented by other signal peptidases, such as SipW, SipX, and SipZ, while extracellular protease activity is reduced (Parro et al., 1999; Escutia et al., 2006). Although the agarase of *S. coelicolor* is the only example of
production improvement utilizing the SipY-deficient strain compared to the wild-type strain, the low extracellular protease activity would be favorable for the secretion of other proteins, and deletion of extracellular proteases may further elicit an increase in protein production.

In addition to increasing gene expression and optimizing the secretion system, cell morphology is related to protein secretion, and formation of clumps during culture is generally unfavorable. For example, overexpression of the ssgA gene, which is related to cell division and morphogenesis, led to improved protein yield. For other bacteria, codon optimization may be necessary for successful protein production. The genome of Streptomyces is GC-rich (approximately 70%), and thus, GC-rich codons are preferred (Ruckert et al., 2015). In particular, the use of the rare leucine codon TTA would have to be avoided, since the codon is decoded by a dedicated tRNA species encoded by the bldA gene, and bldA is temporally regulated (Hesketh et al., 2007). For this reason, even proteins of Streptomyces origin are often codon-optimized to eliminate TTA when the production host is Streptomyces (Guan et al., 2015).

PERSPECTIVES ON FUTURE ENGINEERING APPROACHES FOR HETEROLOGOUS PROTEIN EXPRESSION IN STREPTOMYCES

Although rational engineering approaches to improve the yield of heterologous production of recombinant proteins in Streptomyces have been introduced as described above, some challenges remain. First, genetic tools should be more efficient for cloning large BGCs (Nah et al., 2017). Technical advances in the preparation of intact large-size BGC sequences for in vitro cloning are needed, which may be accomplished by the optimization of genomic DNA extraction to minimize shearing. In addition, the improvement of TAR cloning efficiency in terms of a large number of small- or medium-sized BGC fragments should be considered. The development of a new bacterial TAR cloning hosts, instead of yeast, may be one of the solutions. Alternatively, each fragment can be de novo synthesized by a gene synthesis technique, which is beneficial for refactoring the standardized genetic parts, but may pose an issue with respect to length. BGC expression by the integrative vector system seems to be more stable and controllable than the replicative vector system. Comparative studies of integrative attachment sites and their genomic positions would assist in the optimization of BGC expression. Moreover, available synthetic parts for Streptomyces, including promoters, RBSs, riboswitches, and terminators for refactoring BGC genes should be expanded to tightly control gene expression in terms of strength and timing.

To determine the performance of expression systems and select the optimal clone with high yield among the libraries, high-throughput screening techniques are indispensable. Fluorescence-based reporter genes, such as the superfolder green fluorescent protein (sfGFP), have been applied in Streptomyces, which can lead to high-throughput screening of clones using fluorescence-activated cell sorting (FACS) (Bai et al., 2015). Streptomyces based cell-free protein synthesis (CFPS) is also noteworthy for high-throughput screening. Efforts have been made to improve CFPS systems (Li et al., 2017a; Moore et al., 2021). Since DNA can be directly added to the production environment, extremely large BGCs can be expressed easily compared to in vivo systems, owing to the low transformation efficiency of large-sized DNA. In addition, it can be performed on a multi-well plate scale that can easily facilitate the automation device application.

The main challenge in yield improvement by rational engineering after heterologous expression of BGC in Streptomyces is the lack of genetic information. In other words, finding engineering targets when the yield of heterologous expression is low is not simple. This is because heterologous BGC may interact with complex endogenous factors in the host which is the unpredictable interference hindering the orthogonal heterologous expression system (Beites and Mendes, 2015). The effects of heterologous host factors on the native host expression elements may be predicted by homology-based search, and it was the most common approach used in previous studies. However, BGC expression in the heterologous host phylogenetically close to the native host is not always better than that in the distant host. For example, a heterologous expression study of violacin BGC from Pseudoalteromonas luteoviolacea 2ta16 in the three different hosts revealed that violacin yield was higher in the phylogenetically distant host, Agrobacterium tumefaciens LBA4404, than in E. coli, because of the difference between PviR activator homologs (Zhang et al., 2017a). Therefore, high-throughput approaches using systems and synthetic biology to design, build, and test all possible individual factors, followed by learning from the data for the positive feedback to the next DBTL cycle would be an effective strategy.

To realize a high-throughput DBTL approach, an optimal Streptomyces chassis system is urgently needed. However, it has been suggested that the “universal chassis” is difficult to be constructed because individual host factors differentially affect the expression of each BGC and protein of interest (Liu et al., 2018a; Ke and Yoshikuni, 2020). As introduced in the chassis development section above, Streptomyces chassis with reduced genome such as S. coelicolor M1146, S. avermitilis SUKA, S. lividans ΔYA9, and S. albus Del14 are generally efficient for the heterologous expression of BGCs due to their genome simplicity, but certain BGCs resulted in different expression levels among them. For example, heterologous expression screening of the BAC library of S. albus subsp. chlorinus NRRL B-24108 resulted that some BGCs were expressed in only one of S. albus Del14 and S. lividans ΔYA9 (Ahmed et al., 2020). For another example, the heterologous production yields of cephamycin C BGC of S. clavuligerus in S. coelicolor M1146 and S. avermitilis SUKA22 were both lower than the yield of the native host (Komatsu et al., 2013; Martinez-Burgo et al., 2014). The remaining biological complexity of these strains is likely to hamper them to be the “universal chassis” (Beites and Mendes, 2015). Instead, a “specialized chassis library” could be an alternative option. As a
proof of concept, a “specialized Streptomyces chassis library” is demonstrated in this review (Figure 2). Several Streptomyces species previously used for heterologous hosts due to their general and specific advantages would be the starting strains, and their genomes will be minimized by removing all of their native BGCs and genes with negative effects on heterologous expression, such as insertion sequence (IS) elements, resulting in the “minimized Streptomyces chassis library.” These strains are expected to have robust growth, sufficient cellular energy, clear metabolic profiles, and genetic stability. Then, the combination of additional genes that govern precursor supply, transport, tailoring, and regulations will be determined, and each combination will be introduced to the minimized chassis library, resulting in the “specialized Streptomyces chassis library.” Precursor supply gene sets for specific BGC types might be predicted by a pan-genome model containing all biosynthetic reactions of known SMs, which integrates the information of all genome-scale models of reported Streptomyces species. For example, the biosynthetic gene sets for representative PK precursors, such as malonyl-CoA, methyllumononyl-CoA, ethylmalonyl-CoA, and methoxymalonyl-ACP, will be co-expressed in minimal chassis, resulting in a PK-specialized chassis. Accessory gene sets, including transport, resistance and tailoring genes, might be determined from their specificity, but related studies on this are scarce. Regulatory gene sets might be determined by high-throughput clustered regularly interspaced short palindromic repeats interference (CRISPR) library-based approaches to screen regulatory genes with positive or negative effects on the expression of each BGC. As a result, the best clone with the highest yield of each BGC could be selected by introducing BGCs to the “specialized Streptomyces chassis library” and screening in a high-throughput manner. Learning from the systematic analysis of the best clone will aid in continuously optimizing the rational engineering design to improve the yield. This “specialized Streptomyces chassis library” will also be appropriate for testing uncharacterized BGCs to discover novel SMs.

As the production of recombinant proteins is less diverse than that of BGC products, the development of an optimal Streptomyces recombinant protein chassis may be a better choice than constructing a chassis library. Possible considerations of this chassis include the secretion pathway as the yield of functional recombinant proteins by either the Sec-pathway or Tat-pathway may be different according to their folding nature. In addition, comparative analysis of signal peptides may aid in determining the specific peptidases and controlling their expression for yield improvement. Also, more empirically, the further development of downstream processes such as purification steps will aid the improvement of the recombinant protein yield (Tripathi and Shrivastava, 2019).

CONCLUSION

This review focuses on rational engineering examples and perspectives of heterologous expression of BGCs and recombinant proteins in Streptomyces. Heterologous expression is an effective strategy for overcoming the native host in terms of growth, ease of genetic manipulation, and production yield. Streptomyces is an attractive heterologous expression host for BGCs and recombinant protein genes because of its functional biosynthetic enzyme expression, substrate availability, secretion systems, and other accessory genes. Rational engineering approaches for the yield improvement of heterologous expression in Streptomyces have been facilitated by the development of genetic tools, chassis construction, and additional genetic engineering strategies, emphasizing further demand for vigorous systems and synthetic biology approaches. Employing the high-throughput DBTL cycle using the “Streptomyces chassis library” or “Streptomyces chassis” for heterologous expression will open new horizons, expanding the availability and diversity of SMs and recombinant proteins.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

B-KC designed and supervised the project. SH, YL, JK, GK, HK, and WK wrote the manuscript. SH, SC, BP, and B-KC revised the manuscript. All authors read and approved the final manuscript.

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REFERENCES

Ahmed, Y., Rebets, Y., Estévez, M. R., Zapp, J., Myronovskiy, M., and Luzhetskyy, A. (2020). Engineering of Streptomyces Lividans for Heterologous Expression of Secondary Metabolite Gene Clusters. Microb. Cel Fact 19, 5. doi:10.1186/s12934-020-1277-8

Alexander, D. C., Rock, J., He, X., Brian, P., Miao, V., and Baltz, R. H. (2010). Development of a Genetic System for Combinatorial Biosynthesis of Lipopeptides in Streptomyces Fradiae and Heterologous Expression of the
through Heterologous Expression of a Whole-Genome Bacterial Artificial Chromosome Library in Streptomyces spp. Appl. Environ. Microbiol. 82, 5795–5805. doi:10.1128/aem.01383-16

Xue, Y., and Sherman, D. H. (2001). Biosynthesis and Combinatorial Biosynthesis of Pikromycin-Related Macrolides in Streptomyces Venezuela. Metab. Eng. 3, 15–26. doi:10.1006/mben.2000.0167

Yamanaka, K., Reynolds, K. A., Kersten, R. D., Ryan, K. S., Gonzalez, D. J., Nizet, V., et al. (2014). Direct Cloning and Refactoring of a Silent Lipopeptide Biosynthetic Gene Cluster Yields the Antibiotic Taromycin A. Proc. Natl. Acad. Sci. U S A. 111, 1957–1962. doi:10.1073/pnas.1319584111

Yang, D., Zhu, X., Wu, X., Feng, Z., Huang, L., Shen, B., et al. (2011). Titer Improvement of Iso-Migrastatin in Selected Heterologous Streptomyces Hosts and Related Analysis of mRNA Expression by Quantitative RT-PCR. Appl. Microbiol. Biotechnol. 89, 1709–1719. doi:10.1007/s00253-010-3025-1

Yang, T., Yang, K., Chen, Y., and Fan, K. (2019). Characterization of a Bi-directional Promoter OtrRp Involved in Oxytetracycline Biosynthesis. Curr. Microbiol. 76, 1264–1269. doi:10.1007/s00284-019-01753-1

Yin, J., Hoffmann, M., Bian, X., Tu, Q., Yan, F., Xia, L., et al. (2015). Direct Cloning and Heterologous Expression of the Salinomycin Biosynthetic Gene Cluster from Streptomyces Albus DSM41398 in Streptomyces Coelicolor A3(2). Sci. Rep. 5, 15081. doi:10.1038/srep15081

Yin, S., Li, Z., Wang, X., Wang, H., Jia, X., Ai, G., et al. (2016). Heterologous Expression of Oxytetracycline Biosynthetic Gene Cluster in Streptomyces Venezuela WVR2006 to Improve Production Level and to Alter Fermentation Process. Appl. Microbiol. Biotechnol. 100, 10563–10572. doi:10.1007/s00253-016-7873-1

Zaburannyi, N., Rabyk, M., Ostash, B., Fedorenko, V., and Luzhetskyi, A. (2014). Insights into Naturally Minimised Streptomyces Albus J1074 Genome. BMC Genomics 15, 97. doi:10.1186/1471-2164-15-97

Zhang, J. J., Tang, X., and Moore, B. S. (2019). Genetic Platforms for Heterologous Expression of Microbial Natural Products. Nat. Prod. Rep. 36, 1313–1332. doi:10.1039/c9np00025a

Zhang, J. J., Tang, X., Zhang, M., Nguyen, D., and Moore, B. S. (2017a). Broad-Host-Range Expression Reveals Native and Host Regulatory Elements that Influence Heterologous Antibiotic Production in Gram-Negative Bacteria. mBio 8. doi:10.1128/mBio.01291-17

Zhang, L., Zhu, B., Dai, R., Zhao, G., and Ding, X. (2013). Control of Directionality in Streptomyces Phage phiBT1 Integrase-Mediated Site-specific Recombination. PLoS One 8, e80434. doi:10.1371/journal.pone.0080434

Zhang, X., Lu, C., and Bai, L. (2017b). Conversion of the High-Yield Salinomycin Producer Streptomyces Albus Bk3-25 into a Surrogate Host for Polyketide Production. Sci. China Life Sci. 60, 1000–1009. doi:10.1007/s11427-017-9122-8

Zhou, M., Jing, X., Xie, P., Chen, W., Wang, T., Xia, H., et al. (2012). Sequential Deletion of All the Polyketide Synthase and Nonribosomal Peptide Synthetase Biosynthetic Gene Clusters and a 900-kb Subtelomeric Sequence of the Linear Chromosome of Streptomyces Coelicolor. FEMS Microbiol. Lett. 333, 169–179. doi:10.1111/j.1574-6968.2012.02609.x

Zhou, Y., Murphy, A. C., Samborsky, M., Prediger, P., Dias, L. C., and Leadlay, P. F. (2015a). Iterative Mechanism of Macrolide Formation in the Anticancer Compound Conglobatin. Chem. Biol. 22, 745–754. doi:10.1016/j.chembiol.2015.05.010

Zhou, Z., Xu, Q., Bu, Q., Guo, Y., Liu, S., Liu, Y., et al. (2015b). Genome Mining-Directed Activation of a Silent Angucycline Biosynthetic Gene Cluster in Streptomyces Chattanoogensis. Chembiochem 16, 496–502. doi:10.1002/chbc.201402577

Zhu, Y., Wang, L., Du, Y., Wang, S., Yu, T., and Hong, B. (2011). Heterologous Expression of Human Interleukin-6 in Streptomyces Lividans TK24 Using Novel Secretory Expression Vectors. Biotechnol. Lett. 33, 253–261. doi:10.1007/s10529-010-0428-0

Zimmer, R., and Verrinder Gibbins, A. M. (1997). Construction and Characterization of a Large-Fragment Chicken Bacterial Artificial Chromosome Library. Genomics 42, 217–226. doi:10.1006/geno.1997.4738

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