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Research review paper

New strategies and approaches for engineering biosynthetic gene clusters of microbial natural products

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ABSTRACT

With the rapidly growing number of sequenced microbial (meta)genomes, enormous cryptic natural product (NP) biosynthetic gene clusters (BGCs) have been identified, which are regarded as a rich reservoir for novel drug discovery. A series of powerful tools for engineering BGCs has accelerated the discovery and development of pharmaceutically active NPs. Here, we describe recent advances in the strategies for BGCs manipulation, which are driven by emerging technologies, including efficient DNA recombination systems, versatile CRISPR/Cas9 genome editing tools and diverse DNA assembly methods. We further discuss how these approaches could be used for genome mining studies and industrial strain improvement.

Contents

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1. Introduction

Microbial natural products (NPs, also referred to as secondary metabolites) and their semisynthetic derivatives continue to be important sources of pharmaceutically active compounds for health care and plant crop protection (Butler et al., 2014; Katz and Baltz, 2016; Newman and Cragg, 2016). These valuable NPs exhibit diverse chemical structures with a broad spectrum of bioactivities, including antibiotic (penicillin), antitumor (bleomycin) and insecticide (avermectins) properties (Fig. 1). In microbial genomes, all of the genes responsible for the biosynthesis, regulation and resistance of NPs are generally compacted into biosynthetic gene clusters (BGCs) that vary in size from a few to >100 kb (Smanski et al., 2016) (Fig. 1). With the rapid advances in microbial (meta)genome sequencing, a large number of cryptic BGCs has been found in bacteria and fungi, which are regarded as an untapped treasure trove for the discovery of new medicines (Baltz, 2016a; Cimermancic et al., 2014; Doroghazi et al., 2014). For example, the most gifted actinomycete strains are capable of producing 30–50 chemical skeletons, approximately ten-fold more than previously reported (Baltz, 2016a). Over the past decade, the development of a variety of powerful computational approaches, such as antiSMASH and Pep2Path, has facilitated the rapid and high-throughput prediction of cryptic BGCs (Medema and Fischbach, 2015; Medema et al., 2014; Weber et al., 2015; Ziemert et al., 2016). More recently, a community standard for the annotation of BGCs (MiBiG) was proposed, providing a generally applicable reference dataset to promote the discovery and identification of novel NPs (Medema et al., 2015).

With the continuing accumulation of uncharacterized BGCs in public databases, the next key step is to rapidly express these cryptic BGCs in suitable hosts. Therefore, it is of great importance to develop a series of powerful tools for manipulating BGCs, including cloning, editing, amplification and deletion (Fig. 2). Except for the activation of silent BGCs, these tools will also facilitate the construction of reliable expression hosts as well as improve the yield of industrially important microbial drugs (Fig. 2). Over the past two decades, numerous DNA recombination-based approaches, including Red/ET recombineering, yeast transformation-associated recombination (TAR) and site-specific recombination (SSR), have been established for cloning and amplifying BGCs (Du et al., 2015; Fu et al., 2012; Kim et al., 2010; Murakami et al., 2011). On the other side, with the rapid development of synthetic biology, a wide range of enabling technologies has been extended for engineering microbial natural product BGCs (Cameron et al., 2014). First, DNA assembly methods, such as DNA assembler and Gibson assembly, can be conveniently used to assemble and refactor BGCs in a bottom-up manner (Gibson et al., 2009; Shao et al., 2011). Second, high-efficiency genome editing tools, particularly the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) 9 system, can facilitate direct cloning and multiplex modification of target BGCs as well as the construction of robust heterologous hosts by deleting unrelated endogenous BGCs (Hsu et al., 2014; Huang et al., 2015; Jiang et al., 2015; Lee et al., 2015; Liu et al., 2015). Finally, a series of standard synthetic parts, including promoters, ribosome binding sites (RBSs) and terminators, has been identified and exploited to refactor BGCs, which could completely decouple host regulation (Smanski et al., 2014; Temme et al., 2012).

In this review, we briefly summarize recent progress in the development of strategies and approaches for engineering BGCs to activate and enhance microbial NP biosynthesis. Special emphasis is placed on BGCs with high GC contents from actinomycetes, which produce over two-thirds of all known NPs and continue to be the main sources of new therapeutic drug leads (Barka et al., 2016; Doroghazi et al., 2014; Jensen et al., 2015). Several major challenges and future prospects for manipulating BGCs are also discussed.

2. Strategies for acquiring target BGCs—the starting point of NP research

Methods for acquiring BGCs of interest are among the most important tools for NP research and development. Conventional cloning techniques involving library construction and screening still hold promise for bioprospecting novel small molecules from difficult-to-culture or uncultivated microorganisms. However, this approach is laborious and time-consuming, and many BGCs are too large to be intactly cloned into a single cosmid or bacterial artificial chromosome (BAC). With the advent of recombinant DNA and synthetic biology technologies, a large number of advanced approaches for cloning or assembling target BGCs have been developed over the past two decades. For instance, full-length RecE/T-mediated linear-linear homologous recombination (LLHR) and yeast TAR system can be conveniently used to directly clone target BGCs (Fu et al., 2012; Kim et al., 2010). Meanwhile, several synthetic biology-based methods, such as MASTER ligation, DNA assembler and Gibson assembly, have also been established for assembling large DNA constructs (Chen et al., 2013; Li et al., 2015; Shao et al., 2011) (Fig. 3 and Table 1). It is worth noting that, due to the relatively high cost of gene synthesis, the initial DNA fragments for assembling BGCs still require multiple PCR amplification steps, which could introduce random mutations. When capturing large BGCs (>50 kb), it is suggested that a direct cloning strategy may be utilized to obtain small portions of a BGC and then the entire BGC be reconstituted by DNA assembler or Gibson assembly.

2.1. Direct cloning

2.1.1. LLHR-mediated cloning

Based on the proteins expressed by Redαβ from λ coliophage or its counterpart RecET from Rac prophage, Red/ET recombineering has been established as an efficient *in vivo* homologous recombination strategy in *E. coli* (Muyrers et al., 1999; Wang et al., 2016; Yu et al., 2000; Zhang et al., 2000). In 2005, the Müller group first used this technology to reconstitute an entire BGC (43 kb, responsible for the biosynthesis of myxochromide) from two overlapping cosmids (Wenzel et al., 2005). Afterwards, this recombineering technology has been widely applied to the heterologous expression of a variety of natural product BGCs from different bacteria, including *Streptomyces*, *Sorangium* and *Cystobacter* (Binz et al., 2008; Chai et al., 2012; Fu et al., 2008). In these cases, the reconstitution process was mediated by very short homologous regions (usually 40–50 bp) between a replicative circular vector and a linear DNA molecule, which was therefore termed "linear-circular homologous recombination (LCHR)". However, the approach

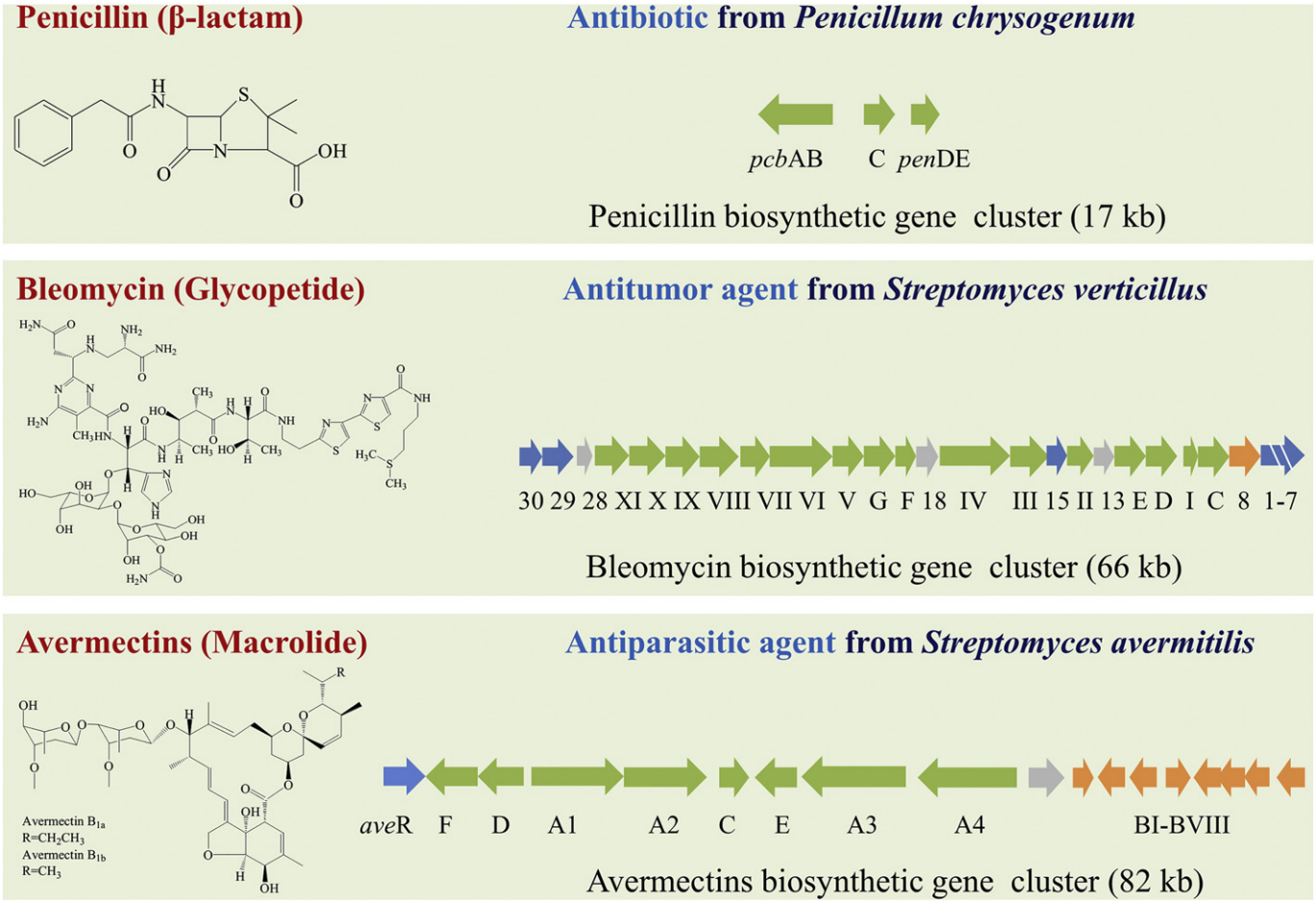

Fig. 1. Representation of the complex structures and diverse bioactivities of microbial natural products and their respective biosynthetic gene clusters: penicillin, bleomycin and avermectins.

using Redαβ or the truncated version of RecET is inefficient at mediating homologous recombination between two linear DNA molecules, hampering its use for direct cloning of target BGCs (Fu et al., 2012).

In 2012, the Müller group surprisingly discovered that the full-length RecE and its partner RecT could mediate high-efficiency linear-linear homologous recombination (LLHR), allowing for the direct capture of large genome regions (Fu et al., 2012) (Fig. 3A). Using this approach, nine polyketide synthase (PKS) and non-ribosomal polypeptide synthetase (NRPS) BGCs (with sizes from 10 to 37 kb) from the gram-negative bacterium *Photorhabdus luminescens* were directly cloned into linear expression vectors. Two new molecules, luminmycin A and luminmide A/B, were successfully identified in the heterologous *E. coli* host. However, they failed to clone a large *plu*2670 gene cluster (52 kb). To address this limitation, a two-step cloning strategy was developed: LLHR was first used to capture target BGCs, and LCHR was then used to introduce a selection marker to reduce background. It is worth noting that, to insure a high efficiency for the LLHR-mediated cloning method, genomic DNA must be cleaved by unique restriction enzymes near the 5′ and 3′ ends of target BGCs. However, it is not always easy to find suitable restriction enzyme cutting sites (Wang et al., 2016). With the advent of the programmable CRISPR/Cas9 system, which is able to recognize and cut DNA sequences near target BGCs easily, this bottleneck could be overcome (Jinek et al., 2012; Lee et al., 2015).

2.1.2. TAR cloning

Transformation-associated recombination (TAR) cloning, which is based on the native recombination capacity of *Saccharomyces cerevisiae*, was originally developed to isolate large pieces of mammalian genomic DNA in the 1990s (Kouprina and Larionov, 2016; Larionov et al., 1996; Larionov et al., 1997). In 2010, the Brady group extended TAR cloning to the capture of microbial natural product BGCs (Kim et al., 2010) (Fig. 3A). To facilitate the rapid expression of target BGCs in *Streptomyces*, the yeast-*E.coli*-*Streptomyces* shuttle vector pTARa was constructed containing three elements: a CEN6/ARS4 sequence and a selection marker allowing for single-copy maintenance in yeast, bacterial artificial chromosome (BAC) elements for plasmid enrichment and verification in *E. coli* and shuttle/integration components for expression in *Streptomyces*. Using pTARa, multiple BGCs (>50 kb in size) were directly cloned or reassembled from environmental DNA (eDNA) libraries (Chan et al., 2016; Katz et al., 2016). Afterwards, the Moore group created a similar cloning vector pCAP01, which is derived from the SuperCos1 cosmid equipped with a pUC *ori*. A 67-kb silent non-ribosomal peptide (NRP) gene cluster from the marine actinomycete *Saccharomonospora* sp. *CNQ-490* was captured and successfully activated in the heterologous host *Streptomyces coelicolor* M1146 by remodeling the cluster-situated regulatory genes (Yamanaka et al., 2014).

Although TAR cloning can be used to directly clone BGCs of interest, the method exhibits a very low cloning efficiency (0.5–2%) due to vector recircularization *via* non-homologous end joining (NHEJ), which leads to time-consuming screening of hundreds of clones. Recently, two different strategies were introduced to increase positive rates in the TAR-mediated cloning method (Lee et al., 2015; Tang et al., 2015). The first strategy is to use a counter-selection marker for colony selection. The *URA3* gene, which encodes orotidine 5′-phosphate decarboxylase and is under control of the strong promoter pADH1, is introduced to pCAP01, generating pCAP03. Because pADH1 can tolerate an insertion of up to 130 bp in length between the transcription initiation site and

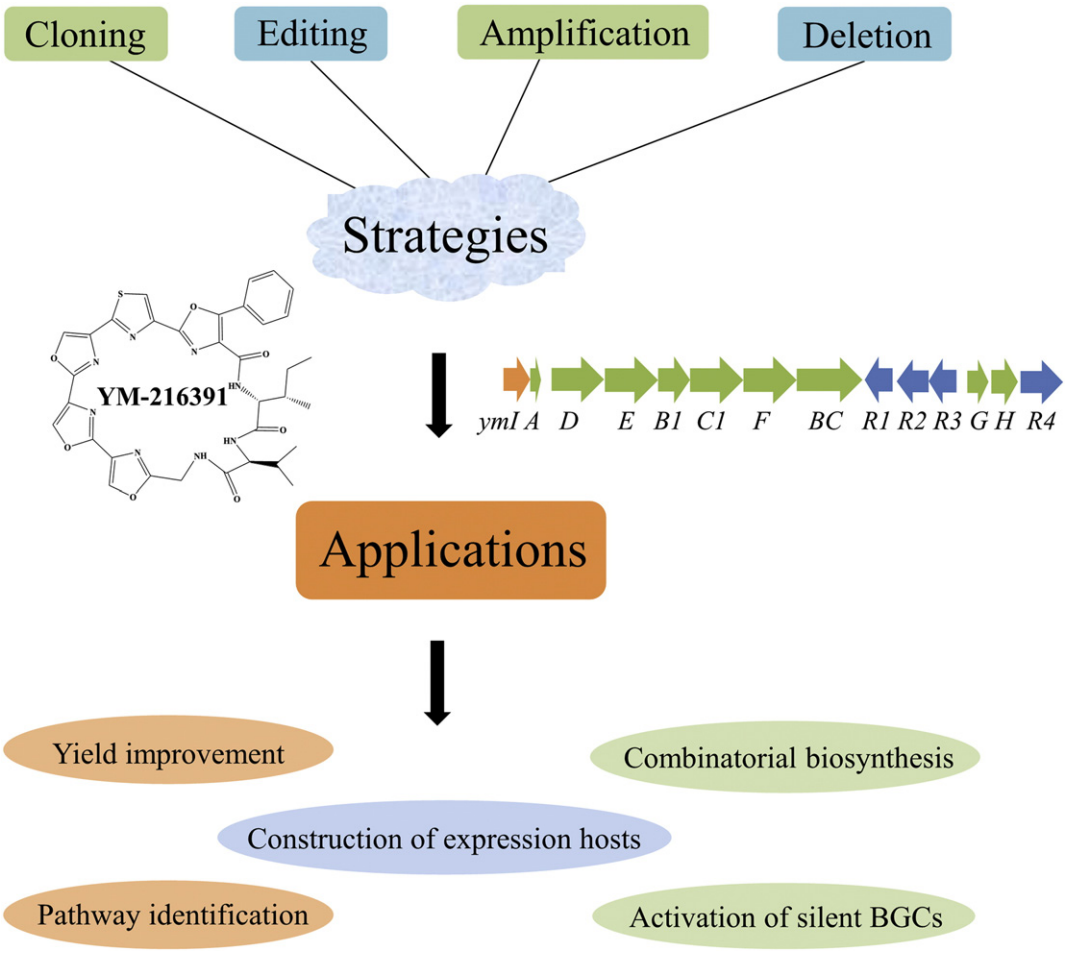

Fig. 2. Overview of the strategies for engineering biosynthetic gene clusters (BGCs) and their applications to NP discovery and development. As an example, an anti-tumor compound YM-216391 and its gene cluster are shown.

the TATA box, yeast transformants that contain pCAP03 with target BGCs can survive in the presence of 5-fluoroorotic acid (5-FOA). Using pCAP03, the Moore group successfully captured two thiotetronic acid BGCs ~30 kb in size with an efficiency of 20–75% (Tang et al., 2015). The second strategy is to use the RNA-guided Cas9 endonuclease to cleave chromosomal DNA instead of restriction enzymes. In the past, the Kouprina group discovered that homologous recombination is more efficient when the linearized capturing vector hooks (homology arms) are located closer to the ends of the target DNA sequences (Kouprina and Larionov, 2006). Although unique restriction enzymes can be theoretically obtained to cleave near the 5′- and 3′-ends of target DNA, it is often difficult to find suitable cutting sites. In 2015, the Kouprina group used the programmable CRISPR/Cas9 system to precisely cleave two sides of the target DNA, significantly improving TAR cloning efficiency up to 32% (Lee et al., 2015) (Fig. 3A). Currently, capturing target chromosomal regions only requires screening fewer than a dozen transformants. It is conceivable that TAR cloning, combined with a counter-selection marker and the CRISPR/Cas9 system, will further accelerate the direct cloning of microbial natural product BGCs.

2.1.3. SSR-mediated cloning

Derived from temperate *Streptomyces* bacteriophages, such as ΦC31 and ΦBT1, a variety of serine integrase-mediated site-specific recombination (SSR) systems has been developed to stably integrate exogenous DNA into the chromosomes of prokaryotes, eukaryotes and archaea (Baltz, 2012). Recently, the ΦBT1 integration system was extended for the cloning of target BGCs in native *Streptomyces* hosts (Du et al., 2015). First, the paired ΦBT1 integration sites *attB*/*attP* and the replicative plasmid pKC1139 are individually introduced on either side of the target BGC *via* two single cross-over recombination events. Then, the ΦBT1 recombinase is expressed, which mediates the cleavage of the two paired integration sites, resulting in circularization of target BGC in the plasmid pKC1139 (Fig. 3B). Using this strategy, three different BGCs, including the actinorhodin (ACT) gene cluster (23 kb) from *S. coelicolor* and the napsamycin (45 kb) and daptomycin (157 kb) gene clusters from *Streptomyces roseosporus*, were successfully acquired with high efficiency (>80%). Then, ACT and daptomycin production were enhanced by increasing the copy numbers of their corresponding BGCs in the native hosts. However, the SSR-mediated cloning method has two major limitations. One limitation is that the procedure is time-consuming and unsuitable for difficult-to-manipulate microorganisms. The second is that the multi-copy plasmid pKC1139 carrying the target BGC is unstable in *Streptomyces*.

2.1.4. CATCH

As described above, *in vivo* cloning strategies have several limitations. LLHR in *E. coli* can only be used to clone BGCs smaller than 50 kb. The other two methods (TAR cloning in yeast and SSR-mediated cloning in *Streptomyces*) are time-consuming due to the slow growth rates of yeast and *Streptomyces*. Recently, a Cas9-associated targeting of chromosome segments (CATCH) method by combining the CRISPR/Cas9 system with Gibson assembly, was established for *in vitro* capture of bacterial genomic segments up to 100 kb in length (Jiang et al., 2015). After excision by the RNA-guided Cas9 endonuclease at two designated

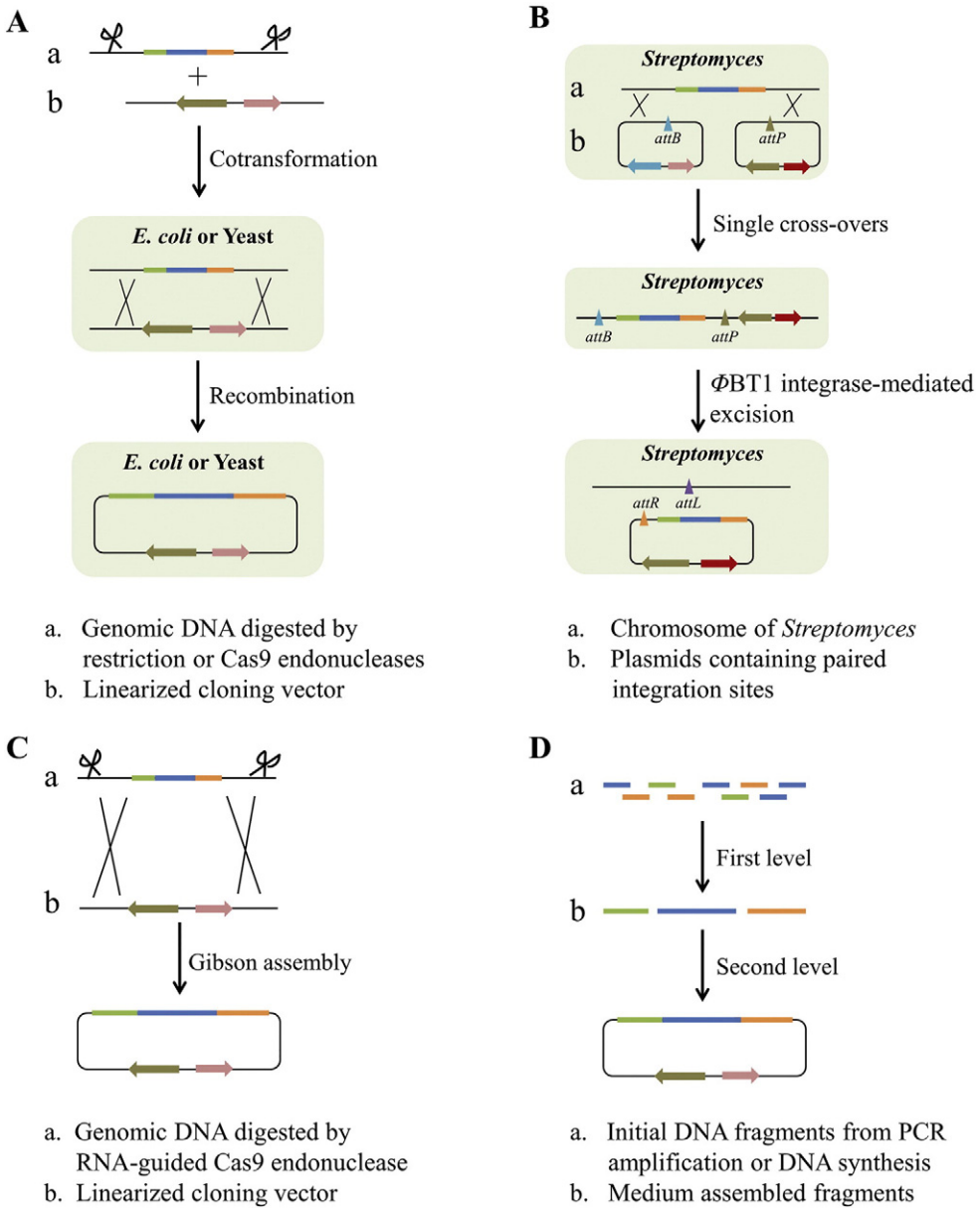

Fig. 3. Approaches for producing large DNA fragments. (A) Direct cloning of target BGCs by Cas9-facilitated transformation-associated recombination (TAR) in yeast or full-length RecET-mediated linear-linear homologous recombination (LLHR) in *E. coli*. (B) Direct cloning of antibiotic gene clusters *via* ΦBT1 integrase-mediated site-specific recombination in *Streptomyces*. (C) One-step targeted cloning of large gene clusters by combining the *in vitro* CRISPR/Cas9 system with Gibson assembly (CATCH). (D) Various DNA assembly methods for constructing large DNA fragments, including DNA assembler and Gibson assembly.

chromosomal loci, target DNA fragments can be one-step ligated to the capturing vector in a Gibson assembly mix (Fig. 3C). Using this approach, a 78-kb bacillaene-producing PKS gene cluster from *Bacillus subtilis* was successfully cloned into a BAC vector with a 12% positive rate. Two ~30 kb antibiotic gene clusters from *Streptomyces* were also captured into the p15A vector with an efficiency of approximately 90%. It is noteworthy that this approach requires very careful operation as preparing gel plugs to guarantee the integrity of the genomic DNA and high assembly efficiency (Jiang and Zhu, 2016).

2.2. Bottom-up assembly

With the exception of direct cloning of BGCs, DNA assembly provides an alternative strategy for acquiring target BGCs in a bottom-up manner (Fig. 3D and Table 1). With the rapid development of synthetic biology, numberous DNA assembly methods have been developed and are widely utilized to generate complex libraries, biosynthetic pathways and even microbial genomes (Ellis et al., 2011; Merryman and Gibson, 2012). Typically, these methods can be divided into three main types: (1) nontypical restriction enzyme-mediated methods, including Golden gate and MASTER ligation (Chen et al., 2013; Engler, 2009); (2) *in vitro* recombination-based assembly methods, including SLIC, CPEC and Gibson assembly (Gibson et al., 2009; Li and Elledge, 2007; Tian, 2009); (3) DNA assembler based on yeast homologous recombination (Shao and Zhao, 2009). Herein, four major DNA assembly methods for obtaining natural product BGCs are reviewed.

2.2.1. MASTER ligation

Type IIs restriction enzymes, which recognize asymmetric DNA sequences and cleave outside of their recognition sequences, hold great

Table 1
Strategies for cloning or assembling NP biosynthetic gene clusters (BGCs).

Methods		Principles	Sizes	Advantages	Disadvantages	Refs
Direct cloning strategy	Linear-linear homologous recombination (LLHR)	Red/ET recombination in *Escherichia coli*	52 kb (PKS-NRPS BGC)	High efficiency (17–50%); user friendly	Low efficiency for cloning large BGCs	(Fu et al., 2012)
	Transformation-associated recombination (TAR)	Homologous recombination in yeast	67 kb (taromycin BGC)	Cas9-facilitated high efficiency cloning (30%)	Time-consuming	(Lee et al., 2015)
	Site-specific recombination (SSR)-mediated cloning	SSR in native host *Streptomyces*	157 kb (daptomycin BGC)	Suitable for capturing large and complex BGCs	Time-consuming; instability of BGCs in the vector pKC1139	(Du et al., 2015)
	CATCH	CRISPR/Cas9 and Gibson assembly	78 kb (Psk BGC)	High efficiency (12–90%); suitable for cloning large genomic regions (>100 kb)	Fine operation is required to guarantee genomic DNA integrity	(Jiang et al., 2015)
Bottom-up assembly strategy	SSRTA	*In vitro* SSR	62 kb (epothilone BGC)	Useful for assembly of large DNA with high GC contents	Scars	(Zhang et al., 2011)
	MASTER ligation	Type IIs restriction enzyme (*Msp*JI)	29 kb (actinorhodin BGC)	Scarless cloning; user friendly	Low efficiency for assembling large DNA constructs	(Chen et al., 2013b)
	Modified Gibson assembly	Chew-back assembly	67 kb (pristinamycin II BGC)	High efficiency (20–40%) for assembling BGCs with high GC contents	Time-consuming	(Li et al., 2015)
	DNA assembler	Homologous recombination in yeast	45 kb (PKS BGC)	Scarless cloning; high efficiency (30%) for assembling fewer fragments	Time-consuming; instability of homologous regions in yeast	(Shao et al., 2011)

potential for a wide range of applications in the construction of gene libraries and biochemical pathways. For instance, the "golden gate cloning" method that commonly uses *Bsa* I, *Bsm*B I and *Bbs* I can assemble at least nine DNA fragments in a defined linear order in a single step with high efficiency (90%) (Werner et al., 2012). Recently, a similar strategy called MASTER (methylation-assisted tailorable ends rational) ligation based on *Msp*JI, a specific type IIs endonuclease, was developed for sequence-independent hierarchical DNA assembly (Chen et al., 2013). MspJ only recognizes methylation-specific 4-bp sites, (m)CNNR (R = A or G), and can produce four-base 5′-overhangs of any sequence. Using the *Msp*JI-mediated method, the ACT biosynthetic gene cluster (29 kb) from *S. coelicolor* was successfully assembled and heterologously expressed in the fast-growing strain *Streptomyces* sp. 4F. However, the assembly efficiency of the MASTER method is relatively low (~6.7%) when constructing large DNA fragments, which indicates that the ligation conditions still require further optimization (Chen et al., 2013).

2.2.2. SSRTA

In addition to the cloning of target BGCs in native hosts as described above, the site-specific ΦBT1 integration system has also been exploited for the *in vitro* assembly of multiple DNA parts, resulting in the establishment of a novel technology named 'site-specific recombination-based tandem assembly' (SSTRA) (Fogg et al., 2014; Zhang et al., 2011). Considering that DNA recombination cannot occur between *attB*/*attP* sites with different central di-nucleotides in the ΦBT1 integration system, the Ding group systematically identified 16 pairs of non-compatible *attB*/*attP* recombination sites (Zhang et al., 2008). Using these paired, non-compatible *attB*/*attP* sites, a 62.4 kb epothilone gene cluster was successfully assembled from seven DNA fragments in a single step. However, it is worth noting that this method will introduce multiple scar sequences (*attR*, 42 bp) between the assembly modules, which may be problematic for the efficient expression of target BGCs.

2.2.3. Modified Gibson assembly

The Gibson assembly method exhibits high simplicity and speed to produce large DNA constructs (up to 900 kb), and permit sequence-independent, one-pot assembly of multiple DNA fragments (Gibson et al., 2009). Three enzymes, T5 exonuclease, Phusion DNA polymerase and Taq DNA ligase, are included in the Gibson reaction mix to perform ligation reactions between DNA molecules. However, we found that this method could not be effectively used to assemble DNA fragments with high GC contents (>70%), mainly due to vector self-ligation (Li et al., 2015). It has been proposed that mismatched linker pairings are easily generated between overhangs with high GC contents due to the low reaction temperature (50 °C) (Casini et al., 2014). To address this limitation, a modified Gibson assembly method was developed in our group (Li et al., 2015). First, a pair of universal terminal single-stranded DNA overhangs with high AT contents (21 bp) was added to the ends of the BAC vector. Second, to allow for hierarchical and seamless assembly of large DNA molecules, two restriction enzyme sites were introduced to the respective sides of the designed overlaps. A 67 kb pristinamycin II (PII) gene cluster from *Streptomyces pristinaespiralis* was hierarchically assembled from 15 PCR-amplified fragments. Although the assembly efficiency is increased from 2.5% to 20–40%, the modified Gibson method requires further optimization, such as extending the length of overlaps between the DNA fragments and the vector.

2.2.4. DNA assembler

DNA assembler, an *in vivo* DNA assembly approach based on homologous recombination in *S. cerevisiae*, was recently developed to construct large biochemical pathways with high efficiency (70–100%) (Shao and Zhao, 2009). Afterwards, this robust method was used to

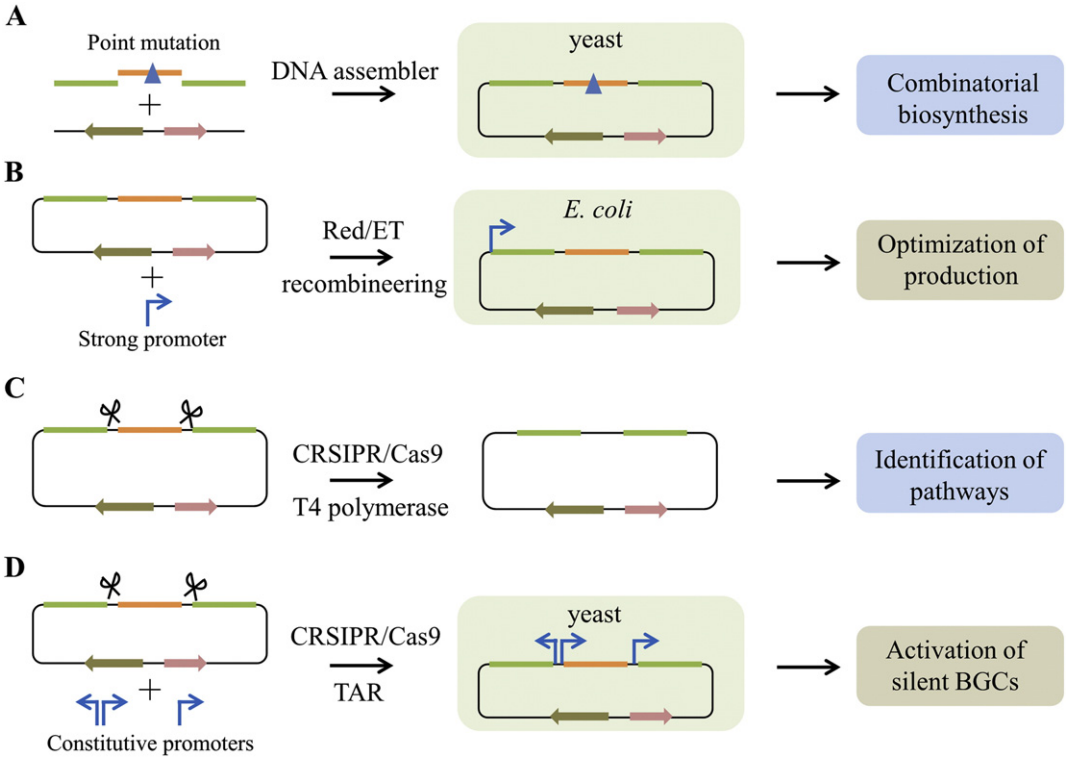

Fig. 4. Methods for refactoring BGCs with diverse applications. (A) Point mutations in core biosynthetic enzymes introduced by DNA assembler for combinatorial biosynthesis. (B) Red/ET recombineering applied to replace a native promoter with a strong promoter for overexpression of structural or regulatory genes. (C) *In vitro* CRISPR/Cas9 system combined with T4 DNA polymerase (ICE system) for in-frame deletion of single genes. (D) Multiplexed CRISPR/Cas9 and TAR-mediated promoter engineering (mCRISTAR) in yeast. The method is used to rapidly activate silent BGCs in a heterologous host.

assemble microbial natural product BGCs. Using DNA assembler, expression vectors containing both target BGCs and genetic elements needed for DNA maintenance in different hosts can be generated in a single-step manner (Shao et al., 2011). For example, seven PCR-amplified fragments (4–5 kb) from the aureothin gene cluster (29 kb) were co-transformed with three helper fragments into *S. cerevisiae*, generating a single circular DNA molecule with high efficiency (60%). Furthermore, because the assembled fragments are derived from PCR amplification, the DNA assembler method allows different genetic modifications, such as point mutations and gene deletions, to be easily introduced into target BGCs (Fig. 4A). Several new aureothin derivatives have been generated by introducing point mutation(s) into the DH domain of AurB (Shao et al., 2011). As such, DNA assembler exhibits unprecedented versatility and flexibility for assembling and refactoring natural product BGCs.

3. Strategies for editing BGCs—optimizing NP biosynthetic pattern

After acquiring target BGCs, the next key step is to edit or refactor BGCs to achieve the following three goals: (1) increasing the expression levels of target BGCs for strain improvement; (2) generating new derivatives by combinatorial biosynthesis; (3) activating silent BGCs for novel NP discovery. Currently, the available molecular genetic/synthetic biology approaches applicable to editing BGCs mainly include: (1) Red/ET recombineering in *E. coli* (Jiang et al., 2013); (2) multiplexed CRISPR/Cas9 and TAR-mediated promoter engineering in yeast (Kang et al., 2016); (3) *in vitro* CRISPR/Cas9-mediated editing systems (Liu et al., 2015); and (4) combinatorial design and assembly (Smanski et al., 2014; Temme et al., 2012) (Fig. 4). It should be noted that, as just mentioned, because initial fragments are obtained from PCR amplification or DNA synthesis, DNA assembler can be conveniently used to refactor BGCs (Shao et al., 2011) (Fig. 4A).

3.1. Red/ET recombineering

Red/ET recombineering technology has been exploited for a wide variety of applications (Wang et al., 2016). In addition to LLHR-mediated direct cloning of microbial natural product BGCs, Red/ET recombineering has also been used to edit BGCs to generate novel antibiotic derivatives *via* combinatorial biosynthesis as well as for strain improvement (Fig. 4B). For instance, Nguyen and coworkers used Red/ET recombineering to generate a library of novel lipopeptides *via* nonribosomal peptide synthetase (NRPS) subunit exchanges, inactivation of tailoring enzymes and variations of the lipid tail (Nguyen et al., 2006). Similarly, this technology has been used to replace multiple native promoters in the gougerotin gene cluster with the strong *hrdB* promoter, which led to significant improvements of gougerotin production in the native producer (Jiang et al., 2013).

3.2. In vitro CRISPR/Cas9 system

The programmable CRISPR/Cas9 system has been exploited for a wide variety of *in vivo* and *in vitro* applications (Choi and Lee, 2016; Luo et al., 2015a; Selle and Barrangou, 2015). Recently, Liu and coworkers developed a new *in vitro* CRISPR/Cas9-mediated editing (ICE) system by combining the RNA-guided Cas9 endonuclease with T4 DNA polymerase, providing a simple and highly efficient strategy for refactoring BGCs (Liu et al., 2015) (Fig. 4C). Using this approach, Liu et al. rapidly knocked out the *rkD* and *homE* genes in the RK-862 and holomycin gene clusters, respectively. However, the ICE system can only be used to delete or insert single genes; furthermore, T4 polymerase-mediated DNA repair of Cas9-generated sticky ends would generate frame-shift mutations. Therefore, this approach may not be suitable for precise combinatorial biosynthesis and requires further optimization (Kim et al., 2015).

3.3. mCRISTAR

Large-scale sequencing efforts of the microbial (meta)genome have revealed that most natural product BGCs are not expressed in laboratory fermentation conditions. Along with the new era of genomics-driven NP discovery, activation of these silent BGCs provides a simple and generic strategy to unearthing novel NPs. In recent years, a variety of pleiotropic and pathway-specific approaches has been developed to induce the expression of BGCs, including variations of growth conditions, transcriptional/translational machinery engineering, epigenetic perturbation and manipulation of global or pathway-specific regulators (Rutledge and Challis, 2015). Interested readers are referred to some excellent reviews on the awakening of silent BGCs using such approaches (Chiang et al., 2011; Rutledge and Challis, 2015). Here, we focus on the refactoring of BGCs of interest, especially *via* promoter exchange involving the replacement of natural promoters with constitutive promoters. This straightforward strategy has been widely used to trigger the expression of many NPs from *Streptomyces* and *Burkholderia* by Red/ET recombineering or DNA assembler (Biggins et al., 2011; Franke et al., 2012; Luo et al., 2013; Olano et al., 2014).

Recently, a more powerful tool that combines the CRISPR/Cas9 system and TAR in yeast achieved marker-free, multiplexed replacement of up to four BGC promoters in a single round (Kang et al., 2016) (Fig. 4D). In this mCRISTAR (multiplexed-CRISPR-TAR) method, the CRISPR/Cas9 system is first used to induce DNA double-strand breaks (DSBs) in BGC promoters. The resulting linearized cluster fragments are then reassembled with synthetic promoters containing BGC-specific homology sequences. To avoid inter-promoter recombination, a set of orthogonal, active promoters and RBS sequences with different expression strengths was constructed. It is worth noting that natural CRISPR arrays rather than sgRNA (single guide RNA) were used to simplify the construction of CRISPR expression modules. In addition, a mCRISTAR webapp was developed to automatically generate all of the sequences required for the modified process. With this highly efficient DNA editing method, three silent BGCs, including the tetarimycin, lazarimide and aromatic polyketide AB1210 gene clusters, were rapidly refactored and successfully expressed in the genetically tractable heterologous *Streptomyces albus* host.

3.4. Functional optimization by combinatorial design and assembly

Because of the complex and redundant host regulation of natural product biosynthesis, it is sometimes difficult to achieve expected goals only *via* genetic engineering of BGCs of interest. Rebuilding target BGCs in a bottom-up manner using orthogonal, well-characterized parts provides an alternative approach to bypass all native regulation. As a proof-of-concept, the Voigt group developed a systematic platform to completely refactor the nitrogen fixation gene cluster from *Klebsiella oxytoca* (Smanski et al., 2014; Temme et al., 2012). At first, all seven operons in the native gene cluster were organized and expressed under the control of synthetic regulatory parts, including promoters, RBS and terminators (Temme et al., 2012). To further obtain radically different cluster architectures, Smanski et al. constructed 122 variants of the complete 16-gene pathway, in which 103 genetic parts were combinatorially designed and assembled by varying gene order, gene orientation and operon occupancy. A fully refactored nitrogen fixation gene cluster was finally produced, which recovered 57% of its wild-type activity (Smanski et al., 2014). It is conceivable that this rebuilding approach will provide a useful synthetic biology tool for the functional optimization of BGCs of interest in the future (Smanski et al., 2016).

4. Strategies for amplifying BGCs—improving NP production

Today, microbial NPs remain an important source for novel drug discovery and development; however, their production titers are often very low and must be improved (Baltz, 2016b; Katz and Baltz, 2016;

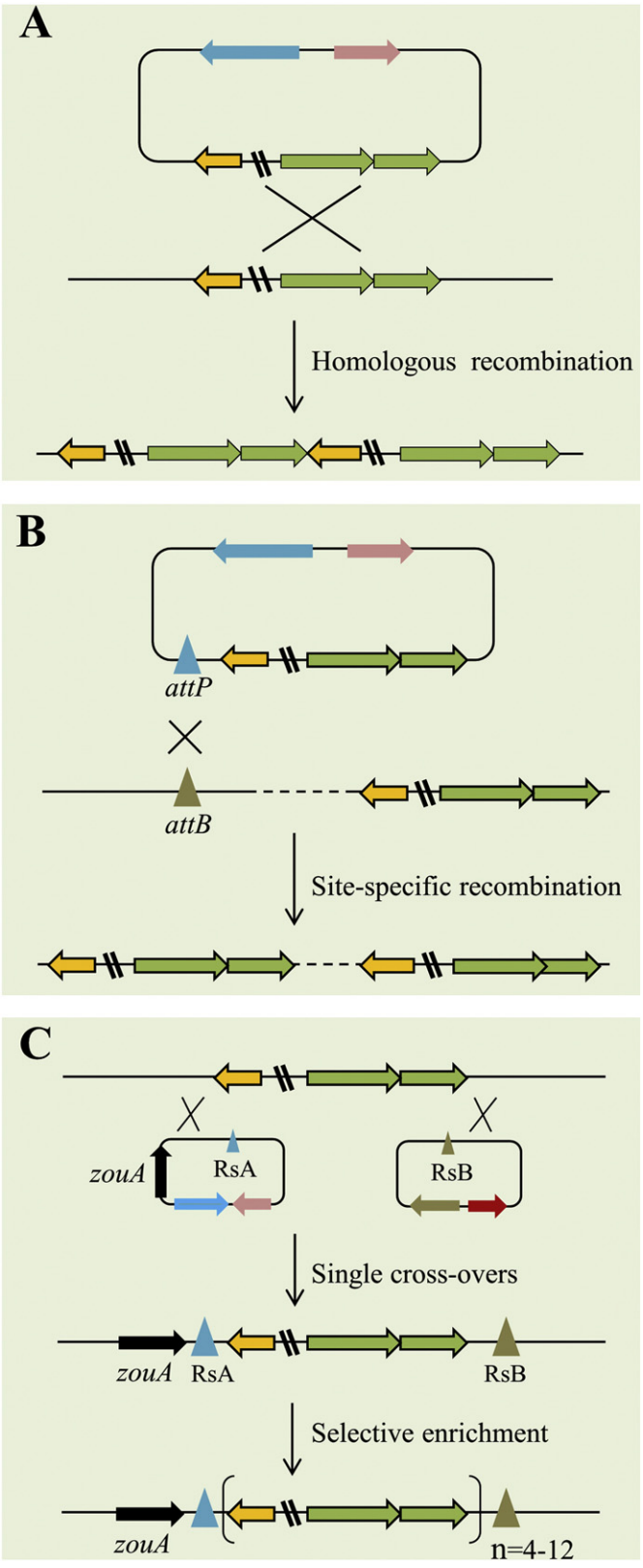

Fig. 5. Tools for the amplification of natural product BGCs in actinomycetes. (A) Homologous recombination-mediated tandem amplification of BGCs in native hosts. (B) Bacteriophage site-specific recombination-mediated chromosomal integration of BGCs in native or heterologous *Streptomyces*. (C) ZouA relaxase-mediated site-specific recombination. This method facilitates the tandem amplification of genomic regions between the RsA and RsB sites.

Lok, 2015). Over the last two decades, rational metabolic engineering strategies, including increasing precursor supply, manipulating regulatory genes and amplification of BGCs, have been successfully employed to improve the biosynthesis of important NPs in industrial strains or to activate the expression of silent BGCs in heterologous hosts (Baltz, 2016b; Olano et al., 2008; Zhang et al., 2016). As a biotechnological tool, the duplication or amplification of special regions of genomic DNA plays important roles in industrial applications, including NP biosynthesis, bioconversions and degradation of toxic compounds. Intriguingly, some overproducing strains generated *via* traditional mutagenesis and screening, such as *Penicillium chrysogenum*, *Streptomyces kanamyceticus* and *Streptomyces linolnensis*, contain tandem amplifications of their antibiotic gene clusters (Fierro et al., 1995; Peschke et al., 1995; Yanai et al., 2006).

Till now, three different strategies have been reported for BGC amplification. In general, entire BGCs can be quickly duplicated in native hosts based on homologous recombination (Nah et al., 2015) (Fig. 5A). However, this simple approach only increases one copy of target BGCs, and the resultant strains are typically genetically unstable due to the presence of a tandem amplification. Based on site-specific recombination, two different approaches have been developed for multi-copy amplification of BGCs. The serine integrase-mediated recombination system has been exploited to produce stable recombinants by discrete insertion of target BGCs into actinomycete chromosomes (Baltz, 2012) (Fig. 5B). The relaxase ZouA-mediated DNA recombination system can facilitate the tandem amplification of more than ten BGC copies (Murakami et al., 2011) (Fig. 5C).

4.1. Bacteriophage Att/Int system

Phage-encoded large serine integrases have been widely used in the genetic engineering of actinobacteria, particularly *Streptomyces* (Baltz, 2012). The integration process involves a single DNA cross-over between the bacterial *attB* site and the phage *attP* site, generating two hybrid sites, *attL* and *attR*. Five compatible integration systems (Att/Int systems) using the ΦC31, ΦBT1, R4, TG1 and SV1 integrases have been identified in streptomycetes and successfully used for strain improvement *via* target BGC amplification (Baltz, 2012; Fayed et al., 2014). For instance, an additional copy of the nikkomycin gene cluster was integrated into the native host by a ΦC31-based vector, leading to significantly enhanced production of nikkomycins (4-fold nikkomycin X and 1.8-fold nikkomycin Z) (Liao et al., 2010) (Fig. 5B). In addition, we demonstrated that starting from a pristinamycin II (PII)-overproducing strain with systematic engineering of cluster-situated regulatory genes, duplication of the PII gene cluster led to a further 1.5-fold improvement of PII titers (Li et al., 2015). However, in most cases, only one extra copy of target BGCs can be added to host genomes. To achieve a higher-order amplification of target BGCs, a new amplification strategy based on the ΦC31 Att/Int system was developed by inserting an artificial ΦC31 *attB* site into the genome of *S. albus* J1074, which harbors two native ΦC31 *attB* sites (Manderscheid et al., 2016). Three copies of antibiotic gene clusters were integrated into the engineered host, leading to significantly enhanced production. However, because the artificial *attB* site was introduced by transposon mutagenesis, the resultant strains exhibited great fluctuations in antibiotic production (Manderscheid et al., 2016). Finally, different Att/Int systems can be used in conjunction for BGC amplification (Baltz, 2012). For example, ΦC31 and TG1 integration systems were sequentially used to duplicate and triplicate the goadsporin gene cluster, leading to 1.5- and 2.3-fold increases in goadsporin production (Haginaka et al., 2014).

4.2. Relaxase ZouA-RsA/B system

As mentioned, the amplification of antibiotic gene clusters has been observed in several overproducing strains, including *S. kanamyceticus* and *S. linolnensis* (Peschke et al., 1995; Yanai et al., 2006). An in-depth investigation by Murakami and coworkers demonstrated that the ZouA relaxase and two *oriT*-like recombination sites RsA and RsB are required for amplification of the kanamycin gene cluster in the native producer *S. kanamyceticus* (Murakami et al., 2011). To demonstrate the generality as well as efficiency of the ZouA-mediated recombination system, an amplified unit DNA (AUD, 35 kb) harboring the 23 kb actinorhodin (ACT) gene cluster in *S. coelicolor* was constructed. After selective enrichment, 4–12 tandem copies of the cluster were obtained, resulting in a 20-fold increase in ACT production (Fig. 5C). Recently, the entire validamycin A (VAL-A) gene cluster (40 kb) was also amplified to 3–5 tandem copies using the ZouA-mediated system in the industrial strain *Streptomyces hygroscopicus* 5008, leading to a 34% enhancement of VAL-A production (from 15 to 21 g/L) (Zhou et al., 2014). However, although the ZouA-mediated system enables multi-copy amplification of target BGCs, the engineered strains may be genetically unstable in the absence of antibiotic selection due to the presence of tandem amplification.

5. Strategies for deleting unnecessary BGCs—constructing genome-minimized hosts

When BGCs of interest are cloned from difficult-to-culture microorganisms or eDNA, heterologous expression in a genetically amenable host is a good choice for product identification (Baltz, 2010, 2016b). In recent years, various excellent heterologous hosts, including actinomycetes, fungi and myxobacteria, have been developed for the expression of diverse natural product BGCs (Baltz, 2010; Luo et al., 2016; Ongley et al., 2013). Interested readers are referred to other reviews regarding fungal hosts for the expression of eukaryotic NPs (Lazarus et al., 2014; Luo et al., 2015b; Unkles et al., 2014). Here, we focus on the gram-positive bacteria *Streptomyces*, which are the best hosts for expressing BGCs with actinomycete origins. While *S. albus* J1074 and *Streptomyces lividans* are widely used due to their weak restriction/modification barriers and low endogenous secondary metabolite profiles, *S. coelicolor* and *Streptomyces avermitilis* have also been engineered for efficient heterologous expression of actinomycete-derived natural product BGCs (Gomez-Escribano and Bibb, 2011; Ikeda et al., 2014; Komatsua et al., 2010). Usually, three metabolic engineering approaches are used in the development of heterologous hosts: deletion of unnecessary IS elements and BGCs, optimization of primary metabolism and engineering of global transcriptional or translational machinery (Baltz, 2016b; Gomez-Escribano and Bibb, 2014). Of these metabolic engineering strategies, the deletion of unnecessary BGCs has been demonstrated as an efficient strategy to simplify product characterization or to increase target BGC expression, possibly by enhancing precursor supply. For instance, the *S. coelicolor* M145-derived host M1152, which has four active BGC deletions and an *rpoB* point mutation, produced 20–40 times more antibiotics than the parental strain (Gomez-Escribano and Bibb, 2011). In fact, the deletion of actively transcribed non-target BGCs can also improve titers of desired known metabolites in industrial strains. Recently, Lu and coworkers showed that individual deletion of seven PKS/PKS-NRPS gene clusters in *S. albus* DSM 41398 resulted in 91–850% higher salinomycin production compared to the parental strain (Lu et al., 2016). Herein, we present different genome editing tools for deleting unnecessary BGCs, which have greatly facilitated the construction of genome-minimized actinomycete hosts (Fig. 6).

5.1. PCR-targeting system

The PCR-targeting method was first applied to the one-step inactivation of chromosomal genes in *Escherichia coli* with PCR products containing an antibiotic resistance cassette flanked by 36–50 nt homology extensions (Datsenko and Wanner, 2000). To accelerate the functional analysis of *Streptomyces* genomes, the PCR-targeting method was then extended to single gene deletions in the model strain *S. coelicolor*

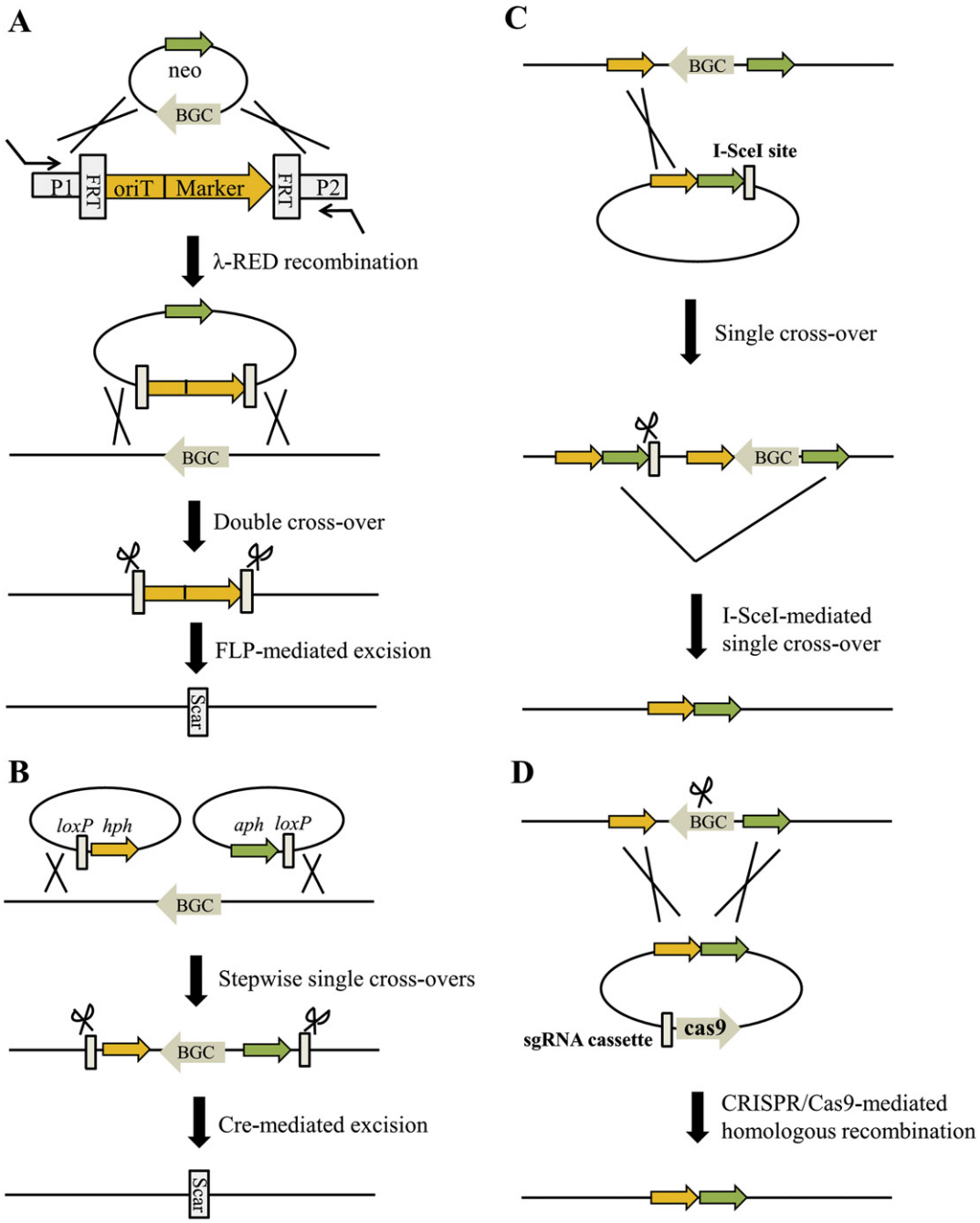

Fig. 6. Four major strategies for deleting large genomic regions in actinomycetes. (A) PCR-targeting method achieved by combining homologous recombination and site-specific recombination. (B) Cre-*loxP* recombination-mediated deletion of large chromosomal regions. (C) I-*Sce*I meganuclease applied to the markerless deletion of BGCs. (D) Cas9-facilitated one-step deletion of large-size BGCs.

(Bentley et al., 2002; Gust et al., 2003). Three steps are usually required in the PCR-targeting method: (1) the target gene within the cosmid in *E. coli* is replaced with the disruption cassette (containing a selectable marker flanked by *FRT* or *loxP* sites); (2) after the mutant cosmid is transferred into *S. coelicolor*, the single gene can be efficiently deleted by a double cross-over event; (3) the disruption cassette, flanked by *FRT* or *loxP* sites, is finally excised by tyrosine recombinase FLP or Cre to generate the unmarked, nonpolar mutation (Fig. 6A). Recently, this approach was successfully used to remove all three endogenous type III PKS genes in the engineered *S. coelicolor* strain M1152, generating an excellent expression host for the discovery and identification of actinobacterial type III polyketide metabolites (Thanapipatsiri et al., 2015). Although PCR-targeting is one of the most frequently method for single or multiple gene disruptions in streptomycetes, two major bottlenecks, including time-consuming cosmid library construction and limited size of cosmid for carrying DNA fragments (<50 kb), have hampered its application for deleting target BGCs.

5.2. Cre-loxP recombination system

In the PCR-targeting method, the Cre-*loxP* recombination system is used as an assistant tool to remove resistance markers. In fact, the site-specific recombination system is also alone used to delete unnecessary BGCs. First, two *loxP* sites in the same orientation are directly

inserted into flanking genomic regions by stepwise single cross-overs. Then, the Cre recombinase is expressed to excise the unnecessary genomic DNA (Komatsua et al., 2010) (Fig. 6B). In general, this strategy is faster than the tedious PCR-targeting method and can knock out large chromosomal regions. Using this approach, Komatsu and coworkers successfully deleted a region of >1.4 Mb stepwise from the 9 Mb *S. avermitilis* genome, which resulted in no longer production of most endogenous secondary metabolites. Three exogenous NPs, including streptomycin, cephamycin C and pladienolide, were then effectively produced in the genome-minimized engineered host (Komatsua et al., 2010).

5.3. I-SceI meganuclease-promoted recombination system

Traditional methods for in-frame deletion of genes or BGCs usually use suicide plasmids or temperature-sensitive replicative plasmids in combination with resistance markers to select for single and double cross-over events. However, when target microorganisms exhibit very low intrinsic frequencies of homologous recombination, the second single cross-over recombination for resistance marker removal is difficult. Yeast I-*Sce*I meganuclease, which can recognize an 18-bp unique sequence and induce a DNA double-strand break (DSB), has been widely used to promote homologous recombination in plant cells, mammalian cells and bacteria (Monteilhet et al., 1990; Plessis et al., 1992). To facilitate genetic manipulation of actinomycetes, a codon-optimized I-*Sce*I gene was synthesized and introduced into actinomycetes for gene or gene cluster deletions (Fig. 6C). Using the I-*Sce*I-mediated method, two colored secondary metabolites BGCs, ACT and RED gene clusters, were successfully deleted in the model *S. coelicolor* (Fernandez-Martinez and Bibb, 2014; Lu et al., 2010).

5.4. CRISPR/Cas9 genome editing technology

In the past three years, the CRISPR/Cas9 system has become the most popular and efficient tool for genome engineering in many organisms, including bacteria, plants and mammals (Barrangou and Doudna, 2016; Hsu et al., 2014). Compared to the genome editing methods mediated by the meganuclease I-*Sce*I and tyrosine recombinase Cre, which require advance introduction of unique recognition sites into genomes, the Cas9 endonuclease can directly target to any genomic locus with the aid of a transcribed synthetic sgRNA, thereby significantly reducing the time and labor required for genome modification. Recently, three studies describing the development of the CRISPR/Cas9 system in multiple *Streptomyces* strains were reported, which provided a simple and highly efficient tool for in-frame deletion of individual or multiple genes as well as large-size BGCs (Cobb et al., 2015; Huang et al., 2015; Tong et al., 2015) (Fig. 6D). However, to perform repeated rounds of gene or gene cluster deletions, the Cas9 editing plasmid that harbors the temperature-sensitive replicon pSG5 needs to be removed by two or three passages at 37 °C. To address this limitation, Zeng and coworkers combined the counter-selection marker CodA (sm) with the CRISPR/Cas9 technology, significantly accelerating the screening process of target deletion mutants (Zeng et al., 2015).

Using the CRISPR/Cas9 genome editing tool, our group systematically investigated the efficiency of removing BGCs of different lengths from *S. coelicolor* (Huang et al., 2015). We discovered that three antibiotic gene clusters with sizes ranging from 21.3 to 82.8 kb, including the ACT, RED and CDA gene clusters, could be knocked out with high efficiency (70–100%) in a single step. Additionally, the ACT and RED gene clusters were also simultaneously deleted with an efficiency of 45%. The CRISPR/Cas9 system provides an effective tool for deletion of BGCs in actinomycetes, which is a very useful supplement to the above mentioned methods. It is worth noting that when the CRISPR/Cas9 system is used as a genome editing tool, target bacteria must harbor high intrinsic rates of transformation and homologous recombination (Luo et al., 2015b). In addition, so far off-target mutations were not checked in the CRISPR/Cas9 experiments in actinomycetes, which may be a problem for the wide application of the CRISPR/Cas9 genome editing tool. To reduce the risk of off-targets, optimized Cas9 endonucleases with improved specificity could be used (Kleinstiver et al., 2016; Slaymaker et al., 2016).

6. Concluding remarks

Over the past decade, a large number of cryptic or silent BGCs (called "biosynthetic dark matter") has been uncovered from the huge amount of microbial (meta)genome sequencing data, which hold great potential to produce a wide range of novel bioactive small molecules (Baltz, 2016a; Cimermancic et al., 2014; Lok, 2015). Recently, a survey conducted by Baltz showed that many gifted actinomycetes with large genomes have the capacity to encode 40–50 BGCs. Several Proteobacteria and Firmicutes also devote 9–17% of their genomes to encode NPs, ranging from 19 to 32 BGCs (Baltz, 2016a). Such extensive sequencing and bioinformatic data provide valuable starting materials for the discovery of novel NPs from microorganisms and will certainly facilitate a resurgence in new drug development. In the post-genomic era, the genome mining approach, consisting of whole genome sequencing, heterologous expression of silent BGCs and characterization of target metabolites, plays an increasing significant role in reinvigorating drug discovery pipelines (Rutledge and Challis, 2015). To accelerate the genomics-driven NPs discovery, rapidly unlocking these cryptic BGCs through simple and high-throughput strategies is nowadays a major priority. Over the past two decades, great advances in technologies based on DNA recombination systems and synthetic biology have provided renewed motivation for the exploration of microbial NPs (Kim et al., 2015; Smanski et al., 2016; Unkles et al., 2014). In particular, many dazzling and versatile synthetic biology tools, including DNA assembly, genome editing and standard genetic parts for precise control of gene expression, have opened opportunities for rapidly understanding NP biosynthetic pathways and discovering new therapeutic drugs. For instance, Luo and coworkers applied the "bottom-up" concept to reconstruct an uncharacterized BGCs by adding strong promoters into the upstream region of each gene, and discovered three new polycyclic tetramate macrolactams from *Streptomyces griseus* (Luo et al., 2013). On the other hand, refactoring and amplification of BGCs are efficient and straightforward strategies for strain improvement *via* promoter engineering or increased gene dosage. Currently, a series of host engineering tools has been developed to overproduce desired metabolites, including engineering of transcriptional/translational machineries and overexpression of phosphopantetheinyl transferase (PPTase) genes (Baltz, 2016b; Olano et al., 2008; Zhang et al., 2016). Given the need for high titers in commercial production, an integrated approach involving traditional mutagenesis screening, rational host engineering and optimization of biosynthetic pathways is likely required to optimize the biosynthesis of target metabolites.

Some major challenges for engineering microbial natural product BGCs remain to be addressed: (1) when target BGCs for heterologous expression are acquired from distantly related bacteria, they will usually be poorly expressed and require reconstitution from codon-optimized fragments. However, the high cost of DNA synthesis makes it infeasible for bottom-up assembly or editing of BGCs. To address this problem, current technologies for *de novo* gene synthesis, such as microarray-based DNA synthesis, require further improvement to reduce the cost of DNA synthesis (Kosuri and Church, 2014; Ma et al., 2012); (2) a generally applicable method for stable, multi-copy amplification of target BGCs remains to be developed in actinomycetes and filamentous fungi, which would be very useful for the robust genetic engineering of industrial strains. The concept involving discrete, multi-copy chromosomal integration of target BGCs may provide a solution to this problem, as similar solutions have been established in *S. cerevisiae* and *E. coli* based on the CRISPR/Cas9 system (Shi et al., 2016) and site-specific

recombination (Yin et al., 2015), respectively; (3) considering that a large number of BGCs are not expressed under standard laboratory conditions, it is still necessary and urgent to develop powerful heterologous hosts and universal refactoring strategies to rapidly activate silent BGCs (Iqbal et al., 2016; Katz et al., 2016). Nonetheless, the highlighted strategies for engineering microbial natural products BGCs presented in this review and future developments in this area are sure to play increasingly important roles in genome mining for NP discovery and development, as well as yield improvement for large-scale manufacturing.

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