Original Research Article

Coordinated regulation for nature products discovery and overproduction in *Streptomyces*

Qun Zhou[a,1], Shuqing Ning[a,1], Yunzi Luo[a,b,*]

[a] *Frontier Science Center for Synthetic Biology and Key Laboratory of Systems Bioengineering (Ministry of Education), School of Chemical Engineering and Technology, Tianjin University, Tianjin, 300072, China*
[b] *Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin University, Tianjin, 300072, China*

A B S T R A C T

Streptomyces is an important treasure trove for natural products discovery. In recent years, many scientists focused on the genetic modification and metabolic regulation of *Streptomyces* to obtain diverse bioactive compounds with high yields. This review summarized the commonly used regulatory strategies for natural products discovery and overproduction in *Streptomyces* from three main aspects, including regulator-related strategies, promoter engineering, as well as other strategies employing transposons, signal factors, or feedback regulations. It is expected that the metabolic regulation network of *Streptomyces* will be elucidated more comprehensively to shed light on natural products research in the future.

1. Introduction

Natural products have served as important raw materials for the pharmaceutical industry for centuries [1]. Semi-synthetic natural products derivatives and synthetic analogs are widely used to treat various clinical diseases, including infectious diseases, cancer, high cholesterol, and transplant rejection [2]. *Streptomyces*, belonging to the *Actinomycetes* family, are gram-positive bacterium serving as a rich reservoir for natural products discovery [3,4], such as anthraquinones (e.g., adriamycin), lactones (e.g., rapamycin), and flavonoids (e.g., O-methylated phenylpropanoids) [5]. Since *Streptomyces* can produce a variety of bioactive natural products, the isolation of fermentation products from *Streptomyces* have attracted great attentions. However, the life cycle of *Streptomyces* is complicated, and their morphological differentiation is accompanied by complex physiological changes [6], indicating that the cell metabolism in *Streptomyces* is under sophisticated regulations.

Significant advances in next-generation sequencing technology reveal that *Streptomyces* is a rich resource for natural products discovery [7–9]. It also discloses that most secondary metabolites gene clusters in *Streptomyces* are unexpressed or low-expressed under standard laboratory fermentation conditions [2,10–12]. The expression of these gene clusters in *Streptomyces* is governed by complex and delicate regulatory networks [11,13]. At present, these complex regulatory networks have

not been clearly illustrated, which is an obstacle to the excavation of natural products in *Streptomyces*. After meticulous analysis via bioinformatics tools, researchers have attempted to coordinate the regulatory network by engineering regulatory elements, such as the regulators, promoters, ribosome binding sites, and terminators [14,15]. This review summarized several regulation strategies applied in natural products discovery and overproduction in *Streptomyces* (Fig. 1). First, strategies employing up-regulation and down-regulation of regulators in *Streptomyces* are summarized. Then, promoter engineering strategies applied in *Streptomyces* for natural products discovery were discussed. Finally, we probed into other regulatory engineering methods, such as the utilization of transposons, signal factors, and the feedback regulations. These strategies have efficiently promoted the overproduction of known natural products, as well as the discovery of novel natural products in *Streptomyces*.

2. Up and down regulation of the regulators

The transcription and translation processes guide the conversion of information from DNA molecules to functional proteins, which is precisely regulated *in vivo* to ensure the orderliness of cell metabolism. The coordination of important cellular process, such as osmotic pressure related transportation, catabolic process, differentiation, and the
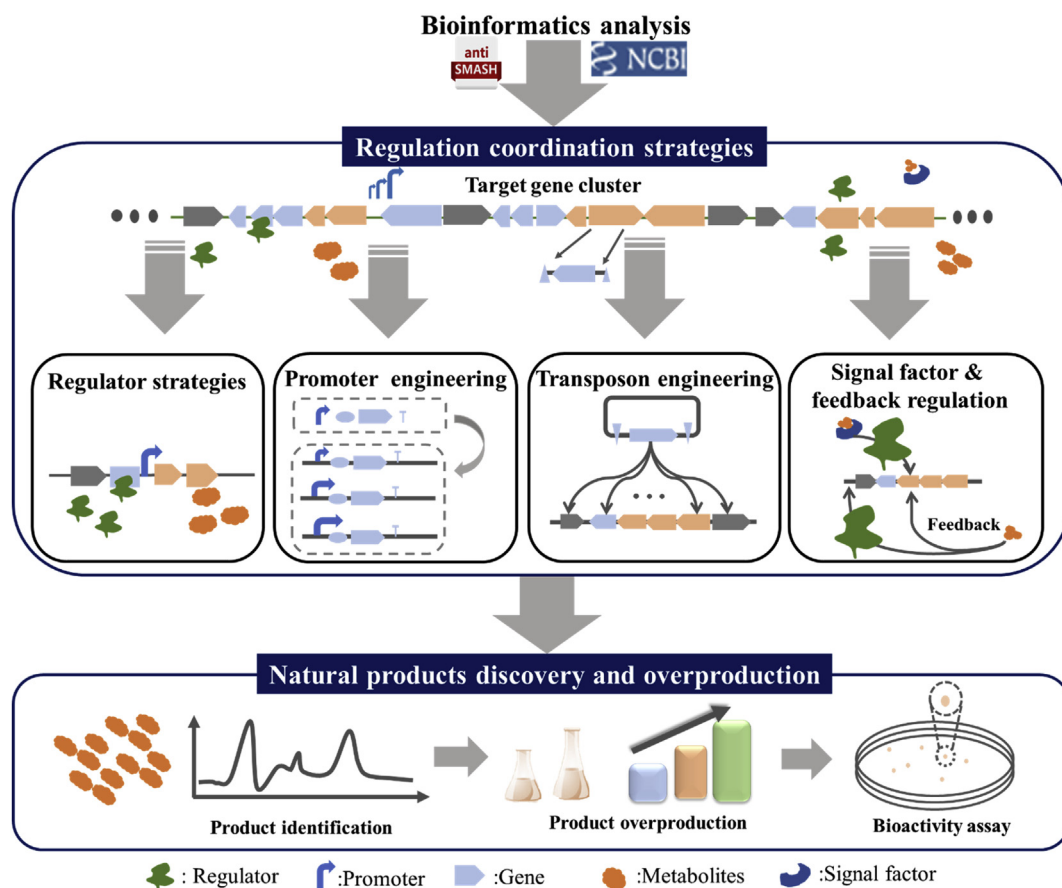
Fig. 1. Regulation coordination strategies for nature products discovery and overproduction in *Streptomyces*.

expression of natural product gene clusters, depends on complex interactions among various regulatory elements. Regulators are proteins that can directly or indirectly recognize or bind to the *cis*-acting elements and participate in regulating the transcription activity of target genes. The TetR family regulator is the most widely distributed family in *Streptomyces* [16]. In addition, there are regulators from other families such as the MarR family, LuxR family, ArgR family and so on. Here, we summarized the regulation strategies based on the regulators.

2.1. Positive regulation strategies

Production of secondary metabolites in *Streptomyces* is regulated by complex regulatory systems. Among them, the expression of some regulators is positively related to the yield of natural products, which are often called as positive regulators. Overexpression of positive regulators is a commonly used strategy to increase the production of secondary metabolites (Fig. 2A). In 2019, Xu et al. identified a regulator ToyA from the LuxR family, which directly activated the expression of the *toyB* and *toyE* operons [17]. They used promoters of different strengths to control the expression of ToyA in *S. disatotochromogenes* 1628. When promoters *SPL57*, *SPL21*, and *ermE*p* were used, the yield of toyocamycin was 2-fold, 1-fold, and 0.8-fold greater than that of the wild type strain, respectively. In order to understand the biosynthesis of lincomycin, Hou et al. identified a new regulator LmbU in *S. lincolnensis* NRRL 2936, which promotes the biosynthesis of lincomycin by regulating the transcription of key biosynthetic genes [18]. In another case, after the CtcS regulator knockout, the transcription of several biosynthetic genes was altered, leading to reduced production of tetracycline and chlorotetracycline, which indicates that CtcS is a positive regulator [19]. Chen et al. separately overexpressed genes *orf 22* and *orf 42* in *Streptomyces fungicidus* ATCC 31731, which increased
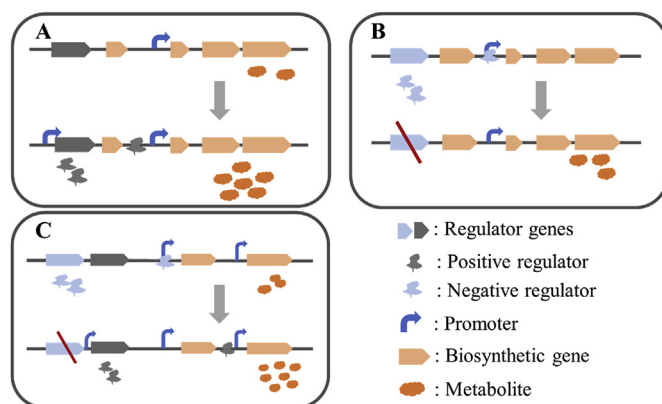

Fig. 2. Schematic diagram of positive and negative regulations. (A) positive regulation strategy: overexpression of positive regulators to promote the yield of metabolites; (B) negative regulation strategy: inhibition of the negative regulator expression to release the production of metabolites; (C) Combinatorial regulation strategy: coordination of positive and negative regulators to increase the yield of metabolites.

enduracidins titers by about 4-fold and 2.3-fold, respectively [20].

The positive regulators can not only regulate the expression of single gene, but can also coordinate the expression of multiple genes in some cases. A positive regulator HcdR2 belonging to the LuxR family can significantly increase the yield of herbicidin F by enhancing the transcription of several structural genes as well as transporters in the herbicidin biosynthetic gene cluster [21]. Under other circumstances, one regulator can take effects in different strains. For example, Liu et al. introduced the pluripotency regulator gene *adpa* into *Streptomyces* ZYJ-

6, leading to the highest reported sugar production of 9338 μg/mL [22]. The AdpA regulator was also used in the wild-type *S. griseus* strain to increase the yield of streptomycin [22], as well as in *S. diastatochromogenes* 1628 to increase the production of toyocamycin by 120.1% [23].

In addition to increasing the production of secondary metabolites, regulators also show considerable potential to activate silent gene clusters. Kang et al. found that the absence of AdpA$_{lin}$, a pleiotropic transcriptional regulator of *S. lincolnensis* NRRL 2936, interrupted the biosynthesis of lincomycin [24]. At the same year, Fu et al. confirmed that the CepR regulator served as a transcriptional activator of cephamycin C biosynthesis, which provided a theoretical basis for clavulanic acid production in *S. clavuligerus* F613-1 [25]. Generally, the activation of the putative operon is achieved by directing regulators to the corresponding promoter regions. For example, StaR is a regulator of the LuxR family, which activates staurosporine biosynthesis by binding to the promoter regions of *staO-staC* and *staG-staN* [26]. In another case, a TetR family regulator MilR2 was shown to be involved in the biosynthesis of 5-oxomilbemycin A3/A4 in *S. hygroscopicus*. Further studies revealed that MilR2 serves as an activator for 5-oxomilbemycin A3/A4 production, and its function is mediated by suppressing the transcription of its upstream hydrolase gene [27]. In another case, researchers overexpressed the PAS regulator from the LuxR family in *S. clavuligerus* ATCC 27064, which activated the biosynthesis of polyene macrolide antifungal drugs and other antibiotics. Moreover, the production of clavulanic acid, cephalosporin C, and tunicamycin complexes increased by 10, 7, and 5-fold, respectively [28]. Besides, different regulators can coordinate together to activate the expression of cryptic secondary metabolite gene clusters. Bu et al. identified three regulatory factors, Sxim22880, CVNABC$_{SX}$, and WblA$_{SX}$ in *Streptomyces* FR-008 and *S. albus* J1074, whose overexpression stimulated the production of new secondary metabolites, revealing the potential of these conserved regulators in activating the recessive secondary metabolite gene cluster in *Streptomyces* [29].

2.2. Negative regulation strategies

Apart from positive regulators, negative regulators also involve in the biosynthesis of numerous secondary metabolites in microorganisms (Fig. 2B). Negative effects of these regulators were often relieved by suppressing or removing these negative regulators. For instance, Planckaert et al. confirmed that the regulator CebR could inhibit the production of taxtomina. Additionally, they revealed that the CebR deletion mutants grew faster compared to the wild type strain [30]. The ArsR/SmtB family regulator BlmR was an inhibitor for bleomycin production. Chen et al. obtained a 34% increase in bleomycin B2 production by BlmR deletion in *S. verticillus* [31]. Negative regulators usually hinder product synthesis by binding to the promoters of the key biosynthetic genes. Mao et al. reported for the first time that the transcription regulator DepR2 from the ArsR family could directly interact with the *dptEp* promoter of the daptomycin gene cluster. DepR2 knockout led to increased daptomycin production by approximately 2.5-fold [32].

Negative regulators may show different regulatory mechanisms. Apart from down-regulating biosynthetic genes, regulators can also be self-regulated. In a previous research, transcriptomics study was combined with Chromatin immune precipitation to explore the *in vivo* interactions between the regulator AbsA2 and the *S. coelicolor* genome. They disclosed that AbsA2 performed two different inhibition mechanisms. Besides bounding to several sites in the calcium-dependent antibiotic gene cluster to inhibit the transcription of CdaR, AbsA2 also bound to its own gene to directly inhibits the transcription of itself [33]. Luo et al. introduced a Himar1-based random mutation screening strategy and identified a transcription regulator PhaR. The disruption of PhaR increased the expression of the gene cluster by approximately 2.68-fold, and therefore the daptomycin titer increased approximately

6.14-fold. They found that in addition to directly binding to the promoter of the daptomycin biosynthetic gene cluster, PhaR can also directly bind to its own promoter [34].

Negative regulator can regulate the expression of multiple genes. In 2019, Xu et al. identified a TetR family transcriptional regulator SLCG_2919, which had a negative effect on the biosynthesis of lincomycin in *S. lincolnensis* LCGL. SLCG_2919 specifically binds to the promoter region of the lincomycin biosynthetic genes (including 25 structural genes, three resistance genes, and one regulatory gene), inhibiting the transcription of these genes [35]. Gou et al. identified a new TetR family transcriptional regulator CalR3, whose disruption resulted in significant increase in calcimycin and cezomycin production, which was 30-fold and 171-fold that of the wild type strain, respectively. Also, they have identified two CalR3 binding sites within the promoter region of *calT* and *calR3* to illustrate the yield change in this research [36]. Zhao et al. introduced a TFD (transcription factor decoy) strategy to successfully activate eight silent BGCs in multiple *Streptomyces*. In this study, DNA molecules interfering with gene regulations are designed to bind to regulators, thus preventing the latter from binding to their cognate DNA targets. The targeted and high-throughput activation of silent BGCs in *Streptomyces* demonstrated the potential of TFD strategy for natural product discovery [37].

2.3. Combinatorial regulation strategies

To obtain a high yield of the target product, up-regulation of positive regulators and down regulation of negative regulators are often performed simultaneously (Fig. 2C). The chromomycins gene cluster in *S. reseiscleroticus* has two representative regulatory factors, the activator SrcmRI and the inhibitor SrcmRII. Overexpression of SrcmRI or disruption of SrcmRII starts the biosynthesis of tryptomycin. Therefore, by deleting SrcmRII and overexpressing SrcmRI, a high-titer tryptomycin production strain was constructed [38]. Similarly, Overexpression of the regulator AcyB2 greatly increased the production of carbomycins, while overexpression of the regulator CbmR hindered the production of carbomycin. Therefore, high-yield of carbomycins was achieved by overexpressing AcyB2 and knocking out CbmR simultaneously [39].

Coordination among regulators with the same effect can also achieve enhancement of natural product production. Simultaneous overexpression of positive regulatory genes *ccaR* and *claR* increased the production of clavulanic acid in *S. clavuligerus* OR by about 43% [40]. In another case, MonH, MonRI, and MonRII co-regulate the expression of the post-PKS genes, which helps to increase monensin production in *S. cinnamonensis*. A synergistic cascade process was identified, in which MonH up-regulates the transcription of MonRII while MonRII enhances the transcription of MonRI [41]. Bu et al. identified three transcriptional regulatory factors, namely Sxim22880, CVNABC$_{SX}$, and WblA$_{SX}$, which played positive regulation roles in polycyclic tetramate macrolactams biosynthesis in *S. mangrove Xiamen* 318. Simultaneous overexpression of these three regulators resulted in a 24.5% increase of polycyclic tetramate macrolactams production [29].

Coordinating the regulators with other functional proteins is also an effective approach to boost product yields. Atratumycin biosynthesis is regulated by a number of factors, including two LuxR-regulated genes, two ABC transporters, and one streptomycin antibiotic-regulated gene (*atr32*). Yang et al. identified a rare *Streptomyces* antibiotic regulatory protein Atr32 as a negative regulatory protein. Through rational engineering of these regulatory genes and transporters, the yield of atratumycin was 1.7-fold–2.3-fold greater than that of the wild-type [42].

3. Promoters engineering strategies

Although bioinformatics analysis of the sequenced microbial genome revealed a large number of uncharacterized biosynthetic gene clusters [43,44], it is still difficult to obtain the potential natural products of these gene clusters as most of them are silent under normal
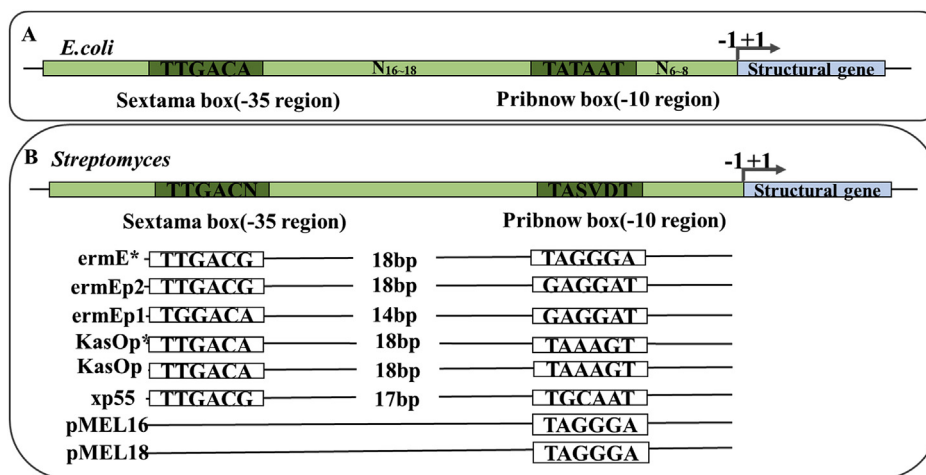
Fig. 3. The structure of promoters in prokaryotes. (A) Promoter structure of *E. coli,* including Pribnow box and Sextama box with conserved sequences, as well as interval region and transcription initiation sequence. (B) Putative *Streptomyces* promoter structures.

laboratory culture conditions. Therefore, tools that can systematically activate silent biosynthetic gene clusters are in urgent need. Promoter engineering is a widely used strategy to activate cryptic biosynthetic gene clusters. Through promoter engineering, native regulatory sequences that are strictly regulated by pathway-specific or pleiotropic regulators are replaced by regulatory sequences with known or controlled characteristics [45–47]. Therefore, promoter engineering owns the potential to serve as a universal tool for natural products discovery and overproduction.

3.1. Promoter structures

Promoter is a DNA sequence located upstream of the 5′ end of a structural gene, allowing the recognition and binding of RNA polymerase. It is a typical *cis*-acting element that coordinates with transcription factors (*trans*-acting factors) to regulate the manner, location and level of gene expression. The length of the promoter varies according to the type of the organisms, and the length of a prokaryote promoter is generally 20–200bp.

Prokaryotic promoters usually consist of 4 parts (Fig. 3): (1) Transcription initiation sequence: locates in the transcription initiation position and encodes a base complementary to the first nucleotide of a new RNA strand, usually a purine; (2) Pribnow box (−10 region): locates 10 bp upstream of the transcription start site with a conserved consensus sequence TATAAT [48]. It is predicted that the initial TA and the final T plays an important role in RNA polymerase binding in this conserved sequence. A Pribnow-like region, named Hogness region, also exists in eukaryotes [49]; (3) Sextama box (−35 region): a sequence upstream of the −10 region, whose center is about −35bp with a conserved sequence TTGACA [50]. This sequence is the recognition site for RNA polymerase, which largely determines the strength of the promoter; (4) Interval region: the area between the Pribnow box and the Sextama box [50]. When the center position of the two is 16–18 bp, the promoter has a strong transcription function, but if the distance between the two central positions is closer or farther, the initiation of transcription will be weakened.

Some *Streptomyces* promoters are different from the above-mentioned typical prokaryotic promoters. There are several different types of *Streptomyces* promoters (Fig. 3). (1) The −10 region and −35 region are similar to promoters of *E. coli* [51]. For example, the erythromycin related promoter *ermEp*2 [52], and xp55 promoter are similar to the *E. coli* promoters [53]. (2) The −10 region is similar, while the −35 region is different, some even do not have the −35 region. For example, Manome et al. identified two strong promoter pMEL16 and pMEL18, with similarity to the sequence in −10 region of PI promoter which

drives the expression of the thiostrepton-resistance gene, but different in sequences of the upstream region [54]. (3) Another type of *Streptomyces* promoter does not show any similarity with the known promoter sequences. In one study, 139 promoters were compared and many of them do not display the typical −10 and −35 regions [51].

The nucleotide sequences of the −10 and −35 regions are much less conserved in Gram-positive bacteria [55,56]. Earlier, the consensus sequences of 28 *Streptomyces* promoters were analyzed, and a consensus sequences TTGAC-(Pu) (where Pu is A or G) for the −35 region and TAG-(Pu)-(Pu)-T for the −10 region were identified [51]. In 2011, researchers constructed a synthetic promoter library. The hexamers TTGACN (where N is A, T, C, or G) and TASVDT (where S is G or C, V is G, A, or C and D is A, T, or G) corresponding to the −35 and −10 consensus sequences were preserved, while a 17 bp spacer region between the −10 and −35 sequences were totally randomized [57,58]. The distance between the −10 region and the −35 region of the *Streptomyces* promoter varies from 7 bp to 24 bp [59]. In short, the *Streptomyces* promoters show more diversity compared with the general prokaryotic promoter.

3.2. Methods for promoter identification and characterization

Promoters are important elements involved in gene transcription regulations. Currently, more than 20 databases can be used for prediction as well as analysis of promoter structures and functions. The commonly used databases include BDGP (promoter prediction), BIMAS (Prokaryotic promoter prediction), CONSITE (transcription factor binding site prediction), TRES (transcriptional regulatory factor analysis), TESS (transcription factor binding site prediction), Gene-Regulation (prediction of eukaryotic transcription factor binding sites), TRANSFAC (prediction of transcription factor binding sites) and so on [60]. The commonly used software for promoter identification includes: Core-Promoter Prediction Program, Finding Promoter (NCBI), Neural Network Promoter Prediction, Promoter 2.0, Promoter Scan, and The Markov Chain Promoter Prediction Server [61–63] (Fig. 4A).

Besides bioinformatics prediction, other experimental methods for promoter identification and characterization are also available, including the classic DNase I foot printing method and the Chromatin immune precipitation (ChIP). DNase I foot printing can be used to accurately predict the binding sites of transcription factors and promoters (Fig. 4B) [64]. The basic principle is that DNA will not be degraded by DNase I while binding with a protein. At present, ChIP [65] is the only method to study the interactions between DNA and proteins *in vivo* (Fig. 4C). Its basic principle is to fix the protein-DNA complex in a living cell and randomly cut it into small chromatin fragments within a
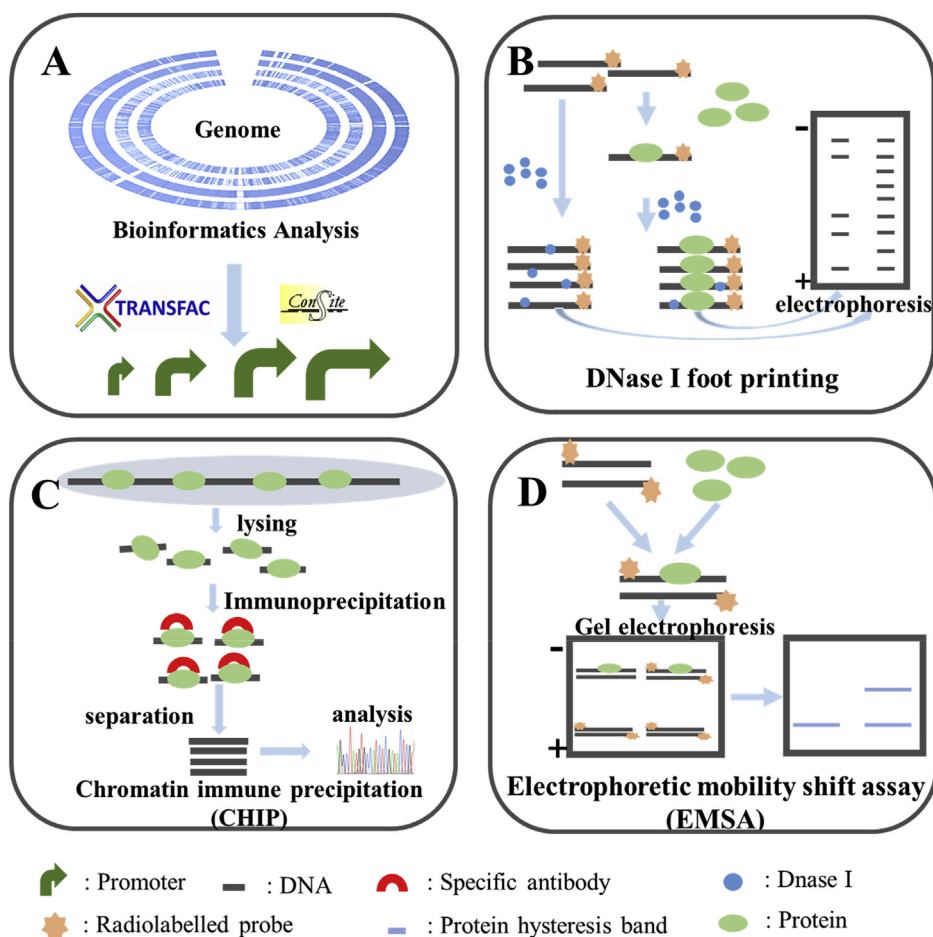
: Promoter **— : DNA** **: Specific antibody** **: Dnase I**

: Radiolabelled probe **— : Protein hysteresis band** **: Protein**

Fig. 4. Methods for promoter identification and characterization. (A) Bioinformatics analysis. (B) DNase I foot printing. DNA will not be degraded by DNase I once binding with a protein. This can be used to predict the binding sites of transcription factors and promoters. (C) Chromatin immune precipitation. DNA fragment bounds to the target protein will be specifically enriched, which can be used to analyze the promoter sequences. (D) Electrophoretic mobility shift assay. Protein-DNA complexes migrate slower than DNA alone. This can be used to identify promoter binding proteins.

certain length range. DNA fragments bound to the target protein will be specifically enriched after precipitated via immunological methods. Finally, DNA sequences of promoters can be obtained through purification and sequencing of the target fragments.

Other methods for promoter function prediction and verification are also based on the principle of protein-DNA interactions, such as the electrophoretic mobility shift assay (EMSA). Electrophoretic mobility shift assay (EMSA) [66] is a typical method to study the interaction between promoters and proteins (Fig. 4D). The basic principle is that the electrophoretic migration speed of the protein-DNA/RNA complex is slower than DNAs/RNAs without protein binding. This method can detect the interaction of nucleic acids (DNA or RNA) with binding proteins, therefore it has been widely used to identify promoter functions via known proteins [67,68].

It is of practical significance in optimization of the above-mentioned methods. For example, combining with gene chip and high-throughput sequencing, ChIP has been developed to ChIP-on chip and ChIP-seq [69] technology. Lewis et al. used ChIP-on chip technology to uncover the binding of absA2 to the promoter regions of redZ and actII-orfIV, which affects the production of calcium-dependent antibiotic [33]. Pepe et al. applied ChIP-sequencing in combination with RNA-sequencing to study the binding sites of HspR and identified the special conservation sequence of the promoter that HspR bound [70]. Through the combination of EMSA and DNase I foot printing assays, the PhoP binding sequence consisting of 11 nucleotide direct repeat units was identified [68]. In another research, FkbR1 was found to bind to the intergenic region of fkbR1-fkbE via EMSA and ChIP-qPCR assays

together, providing a foundation for subsequent engineering of the biosynthesis of ascomycin [71]. The advances in the development of effective DNA-protein interaction technologies shed lights on the identification and characterization of promoters, laying a foundation for future promoter engineering.

3.3. Promoter engineering in natural products discovery and overproduction

Promoter engineering is an effective regulatory strategy to increase the yield of natural products or activate the expression of silent gene clusters (Table 1). The commonly used "plug-and-play" method in synthetic biology often employs different promoters, which have been widely applied to activate silent biosynthetic gene clusters and increase the production of microbial secondary metabolites [72].

Directly replacement of native promoter with a well-characterized one is one of the most popular promoter engineering strategies. Xu et al. replaced the promoter of toyF gene in S. diastatochromo 1628 with the SPL-21 promoter and the yield of toyocamycin was 2-fold greater than that of the wild-type, reaching 489.7 mg/L [73]. Promoter engineering can also be combined with regulator related strategies to achieve increased yield. Wang et al. identified the promoter thlM4p based on microarray analysis, and found that it was 7-fold more active than the commonly used promoter ermE*p. The application of the thlM4p promoter to drive the expression of the regulator gene scnRII leading to 30% higher production of natamycin [74]. At the same year, they identified another strong promoter, groESp, through proteomic analysis of the natamycin-producing strain, Chattanogenesis L10. Under the

Table 1
Applications of promoter engineering.

Strain	effect	promoter	Reference
S.avermitilis	Lycopene yield was increased to 82 mg/g dry cell weight	Sp44	[79]
S. hygroscopicus **XM201**	Geldanamycin yield was increased by 88%	5063p	[80]
S. lividans **TK24**	P*tac**$_{RBS3}$ activity is 17.6-fold than that of P*tac* and 3.6-fold than that of P*kasO**$_{R15}$	P*tac**$_{RBS3}$	[81]
S. albus **J1074**	Production of the blue pigment indigoidine and activation of the biosynthesis of 6-*epi*-alteramides A/B	*ermE**p	[82]
S. lividans **TK24**	Gene expression control at the posttranslational level in Actinobacteria	An RBS selector *in vivo*	[83]
S. lividans	The production of transglutaminases reached 5.73 U/mL	TGase promoter	[84]
S. avermitilis **MA-4680**	Chitobiase activity was 24-fold higher	*xylA*	[85]
S. fradiae **CGMCC 4.576**	Neomycin production was increased by 36%	P*kasO**	[86]
S. natalensis	Activation of pimaricin biosynthesis	*pimM*	[87]
*S. coelicolor*A3(2)	Actinorhodin production tripled	SPL-20	[88]
S. rimosus **M527**	β-glucuronidase activity increased by 2.2-fold	SPL-21	[89]
S. lividans	Enzymatic activity of Phospholipase D reached 69.12 U/mL	P*tip*	[90]

control of *groESp*, the yield of natamycin was approximately 20% higher [75]. This study also revealed that proteomics is an effective method for promoter identification, which can be widely applied to other *Streptomyces* species.

In addition to increasing secondary metabolites production, promoter engineering can also be used to activate silent gene clusters. Zhou et al. transferred the constitutive promoter *ermE**p to marine-derived S. *chattanoogensis* L10, thereby specifically activating the production of chattamycin B, which showed significant antitumor and antibacterial activities [76]. Horbal et al. described a cluster reconstruction method that replaced the native promoter with a randomly generated constitutive synthetic promoter. Depending on this method, they optimized the titer of botulinycin and characterized new derivatives that had not been described previously [77]. Saha et al. used promoter engineering and heterologous expression to activate silent gene clusters in marine *Streptomyces* SCSIO 02999, they successfully identified six new antitumor polycyclic tetraamino macrolactam antibiotics pactamide A–F [78].

Since promoter engineering is an efficient strategy for natural products discovery and overproduction, the development of these methods grows fast. In 2017, researchers combined the CRISPR-Cas9 technology with promoter engineering by using CRISPR technology to efficiently and accurately introduce foreign promoters into the *Streptomyces* genome to activate silent biosynthetic gene clusters [91]. Ji et al. constructed a library of *Streptomyces* regulatory sequences and used the blue pigment indigo gene cluster to rapidly screen the library. In subsequent applications, they inserted four regulatory sequences to the silent actinomycin gene cluster and successfully activated it in *S. albus* J1074 [92]. Other than constitutive promoters, inducible promoters can also be applied in dynamic regulation of gene expression. Li et al. identified several natural inducible promoters and applied them to increase the production of actinomycin and oxytetracycline in *S. coelicolor M1*45 by 1.3-fold and 9.1-fold, respectively [93]. In summary, the continuous development of synthetic biology technologies will further increase the applications of promoter engineering.

4. Other gene expression regulation strategies

4.1. Engineering the transposon related elements

Transposable Elements (TEs), also known as mobile genes or jumping genes, are DNA sequences that can be copied and displaced on the chromosomal DNA [94]. There are three main types of transposons: Insertion Sequence (IS), Composite Transposon (Tn), and Transposable Phage [95]. TEs have *cis*-regulatory elements that can be replicated throughout the genome, therefore the occurrence of the transposition process can lead to the spread of such regulatory elements throughout the genome, which could increase the potential for simultaneous regulation of different genes and promote the spatiotemporal innovation of genes [96]. The general transposition processes are as follows [97,98]

(Fig. 5A): (i) Transposase expression: TEs are regulated by their corresponding transposases, whose expression marks the beginning of the transposition process. (ii) Formation of a transposase-inverted repeat (transposase-IR) complex: The transposase recognizes and binds the inverted repeats at opposite ends of the transposon and combines to form a transposase-IR complex. (iii) Donor DNA excision: the transposase-IR complex has the ability to recognize and excis the donor DNA, making the transposable gene fall off. (iv) Target sequences recognition: the target is identified and attacked by transposable gene, generating staggered cuts at both ends of the attacked sites. (v) Completion of transposition: polymerase and ligase are recruited to fill the DNA gap and form an approximately 4–9 bp repeat sequence, completing the "gene jumping" process [95].

In recent years, the association between the Tn7-like transposon and the CRISPR/Cas system has been reported successively [99,100], proving that the evolution of the CRISPR/Cas system was closely related with the mobile elements [101]. Tn7 transposon that exists in *E. coli* is regulated by five transposases, TnsA-E [102–104]. Different from the Tn7 transposon, Tn7-like transposon lacks TnsE and TnsD, but possesses a homolog of TnsE named TniQ [100]. Klompe et al. discovered that the TniQ-Cascade complex exists in the mini-IF CRISPR/Cas system of *Vibrio cholerae*, where TniQ bound to the Cas6 subunit. This study represented the first example of a type I crRNA-guided effector complex that directly interacts with non-Cas proteins [105]. They subsequently structurally explained that the CRISPR system is mainly responsible for the recognition of target DNA, with the transposase TniQ for the transposition insertion [106]. However, how the TniQ-Cascade complex achieves "transposition" after identifying the target sequence is still inconclusive. Later, Zhang et al. characterized the CRISPR-related transposase of the cyanobacteria *Scytonema hofmanni* (ShCAST), which consists of a Tn7-like transposase subunit and a V–K CRISPR effector (Cas12k). Transposase genes *tnsB*, *tnsC*, and *tniQ* are on one side while *cas12k* and CRISPR arrays are on the other side (Fig. 5B). In this system, the Cas protein and the transposase were fused together. After Cas12k binding to the PAM region, the transposon inserts a fragment 60–66 bp downstream of the PAM region (Fig. 5B). ShCAST integrated DNA into unique locations in the *E. coli* genome with a frequency of up to 80% [107], establishing a new paradigm for precise DNA insertion.

Due to the randomness and autonomy of transposition process, increasing the transposition efficiency and frequency leads to a wide range of mutations in the genome. The transposons widely used in *Streptomyces* are IS204 and Tn5, which are derived from *Nocardia asteroides* and *E. coli*, respectively. Zhang et al. developed an efficient transposable elements delivery vehicle derived from IS204. A large mutation library has been established to screen important regulatory genes for natural product biosynthetic pathways. Twenty-five *S. coelicolor* mutants were obtained, one of those revealed an unknown gene in the undecylprodigiosyn (red) biosynthesis pathway, *rrdA*. This method achieves efficient gene delivery and becomes an attractive method for
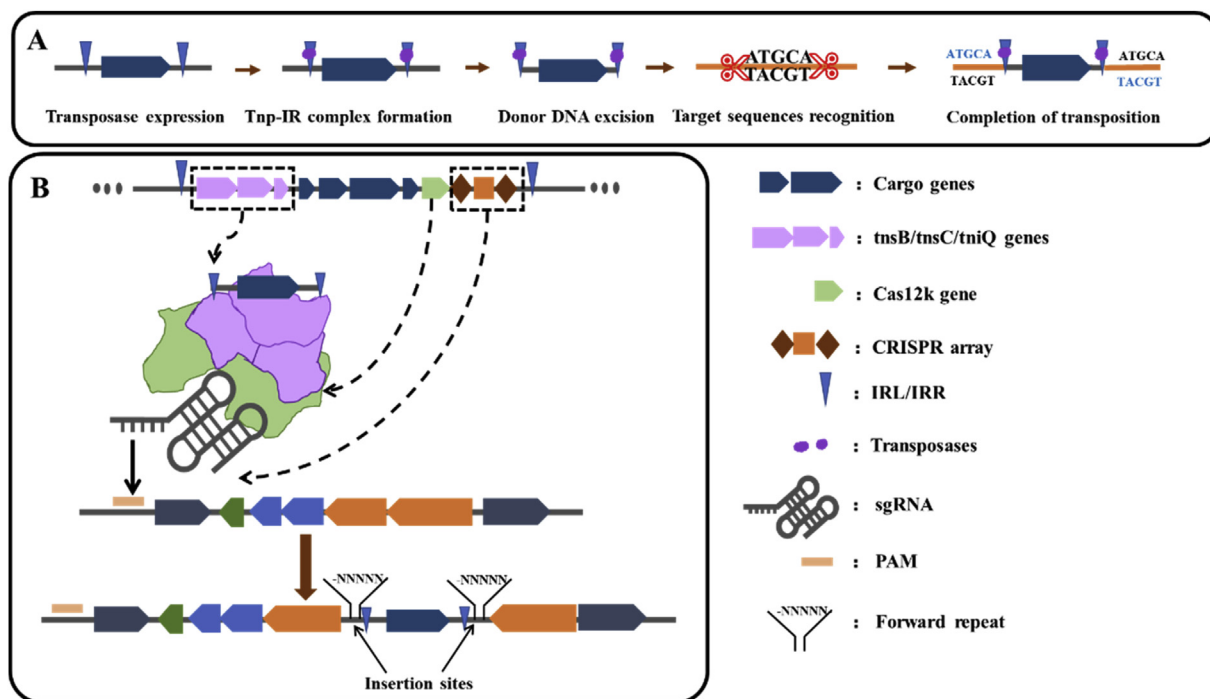
Fig. 5. Transposon transposition process and the RNA-guided DNA transposition. (A) Five steps of transposon "gene jumping". (B) The process of RNA-guided DNA transposition. The ShCAST complex consisting of Cas12 k, TnsB, TnsC, and TniQ mediates the insertion of DNA 60–66 bp downstream of the PAM. The IRL and IRR sequences of the transposon and other cargo genes were inserted into the DNA with a 5 bp forward repeat.

identifying remote positive and negative regulatory genes for natural products biosynthetic gene clusters [97].

Genome-wide mutagenesis using transposons can identify new genes and pathways that affect antibiotic production. Scientists have modified the Tn5 transposon in *S. coelicolor*. They confirmed that the transposable insertion was randomly happened in the *Streptomyces* genome [108–110]. Xu et al. mutated 5 positions of the Tn5 transposase (*E54K*, *M56A*, *P242A*, *E345K*, and *L372P*) to construct a highly-active mini-Tn5 transposase system [110]. A library of 50,000 independent mutants based on the highly-active Tn5 transposase system was constructed and 551 genes altering the production of actinorhodin were identified, more than half of which were new effectors [109]. In summary, highly active and efficient transposons can be applied to genome-wide mutagenesis study in *Streptomyces*, with the potential to identify important genes and pathways that may affect the metabolic regulation network of *Streptomyces* from an overall perspective.

4.2. Regulation strategies using signal factors

In *Streptomyces*, morphological development and secondary metabolism are simultaneously affected by multiple nutritional factors and also controlled by extracellular signal molecules. It is reported that most *Actinomycetes* may use γ-butyrolactone (GBL) to control the production of antibiotics, which is known as the "streptomycin hormone" [111]. The most well characterized signal factor of GBL is "Factor A", discovered in the early 1960s [112], which was identified to be associated with the production of streptomycin in *S. griseus* in 1967 [113]. ArpA is the receptor for Factor A, which recognizes and binds a 22 bp palindrome of DNA. ArpA usually binds with the promoter *adpA* to form an ArpA-DNA complex, thus inhibits the transcription of *adpA* and decreases the production of streptomycin. When Factor A is added, Factor A will bind to ArpA, releasing the transcription of *adpA* [111].

Recio et al. showed that limited endogenous PI [2,3-diamino-2,3-bis (hydroxymethyl) −1,4-butanediol] factors restricted the biosynthesis of pimaricin in wild-type strains. They restored the biosynthesis of pimaricin in mutant *S. natalensis* strain by supplementing Factor A or PI, which stimulated pimaricin production by 33% more [114]. In another case, two regulatory genes (*jadR2* and *jadR3*) encoding homologues of the γ-butyrolactone receptor were identified. Zou et al. purified a JadR3 interacting molecule SVB1, which has the same structure as γ-butyrolactone SCB3 in *S. coelicolor* [115]. The authors stated that the addition of SVB1 or extraction from *S. coelicolor* to the mutant strain could restore jadomycin production. This study indicated that the binding of JadR3 and SVB1 plays an important role in controlling jadomycin biosynthesis; on the other hand, it provided new insights into the γ-butyrolactone/receptor system. Zhang et al. found that GBL can be used as an interspecies signal in *Streptomyces*. They expressed the GBL biosynthetic gene deriving from *S.coelicolor* M145 in *S. albidoflavus* J1074 to synthesize *Streptomyces coelicolor* butanolides (SCBs) [116]. This showed that GBL has great potential in natural products biosynthesis regulations.

4.3. Regulation strategies involving feedback regulations

In natural products biosynthesis, some intermediates may cause feedback regulation to coordinate the expression of biosynthetic genes. For example, the polyketide gene cluster *aur1* is responsible for the production of auricin in *S. aureofaciens* CCM3239 [117]. Auricin and its intermediates could bind to the pathway-specific activator Aur1P, thus inhibited the expression of *aur1*. This process is obviously related to the acidity of the fermentation environment, which can be intervened by stabilizing the acidic conditions [118]. SsaA is a key activator for sansanmycin biosynthesis. SsaA strictly controls the production of sansanmycins via sensing the accumulation of the final products [119]. The regulator AtrA in *S. globisporus* serves as a transcriptional activator for actinorhodin biosynthesis in *S. coelicolor* [120]. The pleiotropic regulator AtrA could coordinate the production of lidamycin, which is inhibited by a biosynthetic intermediate. The activity of AtrA is also regulated by actinorhodin concentrations [121].

In addition to the aforementioned products feedback regulation, some biosynthetic processes require the participation of multiple inhibitors and activators, forming a complex regulatory network. For

example, pristinamycin production in *S. pristinaespiralis* Pr11 is tightly co-regulated by a γ-butyrolactone receptor gene (*spbR*), two TetR repressor genes (*papR3* and *papR5*), three *Streptomyces* antibiotic regulatory genes (*papR1*, *papR2*, and *papR4*), and a response regulator gene (*papR6*) [122]. In the absence of γ-butyrolactone, the auto-regulator SbpR inhibits the expression of all SARPs (*Streptomyces* Antibiotic Regulatory Protein) in this pathway, including the pristinamycin biosynthetic pathway. In the presence of a critical concentration of γ-butyrolactone, the inhibition is alleviated. PapR1 and PapR2, as the main activators of the pristinamycin biosynthetic gene cluster, activate the transcription of the pristinamycin structural genes. Repressor PapR3 inhibits the transcription of *papR4* and *papR5*, while PapR5 suppresses the transcription of *papR1* and *papR4* [122]. Deletion of *papR5* in combination with overexpression of *papR4* and *papR6* increased pristinamycin-II titers approximately 1.5-fold than that of the parental strain. At the same time, pristinamycin-II titer increased more than 5-fold by adding macroreticular resin to lessen end-product feedback inhibition and toxic effects [123]. Similarly, the resistance of *S. aureus* to oxytetracycline is controlled by genes encoding ribosome protection protein (OtrA) and efflux proteins (OtrB and OtrC), which in turn affects the production of oxytetracycline. Yin et al. adopted a three-protein co-expression strategy to improve the synergistic effect of drug resistance and increased the production of oxytetracycline by approximately 2-fold [124].

5. Summary and perspectives

With the rapid development in bioinformatics studies, more and more biosynthetic gene clusters (BGCs) and their related regulatory elements are predicted. Accordingly, to obtain diverse natural products, scientists have developed multiple strategies to coordinate the complex metabolic network in *Streptomyces*. This review mainly summarized the regulation strategies for *Streptomyces* natural products biosynthesis from three aspects: regulatory factors, promoters, and others (transposons, signal factors, and feedback regulations). The applications of these strategies should also improve the efficiency of genetic manipulation in *Streptomyces*. With the development of the CRISPR technology, there will be predictable improvements in this area.

Generally, synergistic effects among several regulatory elements exist to regulate certain metabolic pathways [14]. Therefore, understanding the relationship between different regulatory networks may open up the opportunities for new strategies. Some regulators involved in processes such as bacterial growth and spore production may also indirectly affect the regulation of secondary metabolites biosynthetic pathways. In addition, some competing pathways may lead to the loss of carbon sources, thereby reducing the production of target products. Based on current situations, a better understanding of the regulation in *Streptomyces* is in urgent need to help us to develop comprehensive regulation strategies. In short, rational application of regulatory strategies will help the discovery and overproduction of valuable natural products, and is expected to open a new century for natural product research.

CRediT authorship contribution statement

Qun Zhou: Writing - original draft. **Shuqing Ning:** Writing - original draft. **Yunzi Luo:** Writing - review & editing, Project administration.

Declaration of competing interest

None.

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