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System-level understanding of gene expression and regulation for engineering secondary metabolite production in *Streptomyces*

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Abstract

The gram-positive bacterium, *Streptomyces*, is noticed for its ability to produce a wide array of pharmaceutically active compounds through secondary metabolism. To discover novel bioactive secondary metabolites and increase the production, *Streptomyces* species have been extensively studied for the past decades. Among the cellular components, RNA molecules play important roles as the messengers for gene expression and diverse regulations taking place at the RNA level. Thus, the analysis of RNA-level regulation is critical to understanding the regulation of *Streptomyces*' metabolism and secondary metabolite production. A dramatic advance in *Streptomyces* research was made recently, by exploiting high-throughput technology to systematically understand RNA levels. In this review, we describe the current status of the system-wide investigation of *Streptomyces* in terms of RNA, toward expansion of its genetic potential for secondary metabolite synthesis.

Keywords *Streptomyces* · Secondary metabolite · RNA · Transcription · Translation

Introduction

As a dominant soil bacterium, *Streptomyces* plays an important role in carbon recycling by decomposing the debris of other organisms, including lignocellulose [26, 99]. In addition to the ability to decompose saprophytic compounds, *Streptomyces* species are noticed in aspects of their (1) morphogenetic characteristics, (2) industrial potential for heterologous protein production and (3) ability to produce pharmaceutically active compounds. First, streptomycetes undergo multiple developmental stages during the life cycle like fungi, and thus are attractive models to study bacterial development processes [34]. Second, *Streptomyces* is considered as an important industrial microorganism as the heterologous protein production host [72]. As a member of gram-positive bacteria lacking outer membrane, the proteins secreted by *Streptomyces* are directly released to the culture medium, simplifying protein purification steps. In addition, their protein secretion systems are well-characterized [3, 38]. Finally, the most important feature of streptomycetes is the ability to produce pharmaceutically active secondary metabolites, including antibiotics, anthelmintics, and immunosuppressants [9, 30]. Especially, the antibiotics produced by *Streptomyces* account for more than two-thirds of clinically useful antibiotics. To cope with the emerging multidrug resistant bacteria and the discovery of other valuable compounds, the secondary metabolism of *Streptomyces* is of great interest.

With the aid of next-generation sequencing (NGS) techniques and in silico genome mining tools, the potential of *Streptomyces* for novel secondary metabolite discovery has been elevated. More than 200 complete genomes were obtained so far, and about 20–50 secondary metabolite biosynthetic gene clusters (smBGCs) are predicted from a single *Streptomyces*' genome, which is a number far outpacing the previously discovered secondary metabolites [12, 56]. It

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is noteworthy that the genus *Streptomyces* is one of the biggest genera and more than 1000 species are described in the NCBI taxonomy database. Thus, tens of thousands of smBGCs remain undiscovered and *Streptomyces* species still hold great importance as the dominant reservoir for novel secondary metabolite discovery. However, even if the genome of *Streptomyces* promises the existence of numerous novel bioactive compounds, the actual discovery of the compounds is not guaranteed [8]. Since the secondary metabolites are generally non-essential for survival, the smBGCs remain silent under routine conditions and are activated for their own purposes under specific conditions, such as osmotic pressure, nutrient limitations and interspecific competition [10, 14, 20, 55, 95]. Therefore, the smBGCs are subjected to complex regulation to produce secondary metabolites in accordance with their necessity. Thus, to fully harness the industrial and pharmaceutical potential of *Streptomyces*, understanding the complex regulation, especially underlying the secondary metabolism, is essential.

RNA is the central molecule subjected to significant portion of bacterial regulation [13]. According to the central dogma, genes are expressed to proteins through transcription and translation [27]. An RNA molecule takes part in the process as an intermediate, and thus RNA-level regulation, especially transcription initiation regulation, is the most economical steps to regulate gene expression with small expenses of resources [13]. In addition, RNA possesses regulatory functions by interacting with other transcripts or by modulating the expression of downstream genes. In bacteria, small non-coding RNAs (sRNAs) have diverse regulatory functions, including anti-transcription termination, translational inhibition and supply of substrate specificity for RNase [76, 85]. In addition, certain RNA elements, including riboswitches, mediate conditional gene expression regulation in cis via premature transcription termination or translational control [28].

The 7–10 Mbp GC-rich linear genome of *Streptomyces* typically encodes about 7000 genes, more than twice of the bacterial average [101]. As a result, the number of regulatory genes, including about 60 sigma factors, is also high, which gives rise to a complex transcriptional regulatory network [56, 84]. To understand the complex regulation of *Streptomyces* arising from the enormous coding potential, assessing the system-level transcriptome information is essential. In this review, we discuss about the system-level understanding of gene expression regulation in *Streptomyces* based on NGS techniques and its utilization for secondary metabolite research.

Tools for assessing genome-scale gene expression and regulation information

The secondary metabolism of *Streptomyces* undergoes complex regulation in response to specific environmental stimuli [14, 55]. Underlying the complex regulation, numerous regulatory genes are involved directly or indirectly to dictate the limited number of RNA polymerase molecules to transcribe an appropriate set of genes required for secondary metabolite synthesis [63, 81, 82]. For example, a γ-butyrolactone compound called A-factor antagonizes ArpA, the transcriptional repressor of AdpA, and promotes transcription of AdpA in *S. griseus* [69]. Then, AdpA directly controls the expression of more than 500 genes, including the StrR, a pathway-specific transcriptional activator of streptomycin BGC [39]. In addition, the synthesis of secondary metabolites consumes primary metabolites, including coenzyme A derivatives and amino acids, which are also utilized for self-propagation of host, and hence, a timely coordination of precursor accumulation and activation of secondary metabolism is crucial for the secondary metabolite synthesis [41, 97]. Thus, assessing the gene expression dynamics along with the involved regulators is important to understand the secondary metabolism of *Streptomyces* and further provide rationales to improve secondary metabolite production.

As an efficient means to obtain gene expression and regulation information in genome-scale, diverse strategies based on NGS have been developed (Fig. 1) [23, 28, 44, 48, 98]. The most well-established tool for monitoring genome-scale gene expression is RNA-Seq. Basically, DNA molecules of proper size bearing specific sequencing adaptor sequences can be sequenced using the NGS [7]. The availability of synthesizing complementary DNA (cDNA) from RNA through reverse transcription enabled measuring gene expression in the transcriptional level by sequencing the cDNA of transcripts.

The first step in gene expression involves transcription initiation and thereby, regulation at transcription initiation is important to minimize resource consumption for unnecessary processes. The RNA polymerase molecules in an organism are distributed to each genomic locus by transcription factors and sigma factors [4]. The genome-scale in vivo DNA–protein interactions, especially the binding of transcription factors and sigma factors to genome, orchestrate the expression of individual genes adequate for proper operation of the host. To monitor the genome-scale DNA–protein interactions, chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq) was developed [48]. ChIP-Seq is performed by immunoprecipitating the DNA bound by the protein of interest. The immunoprecipitated DNA is then sequenced to identify the binding sites of the protein of interest.

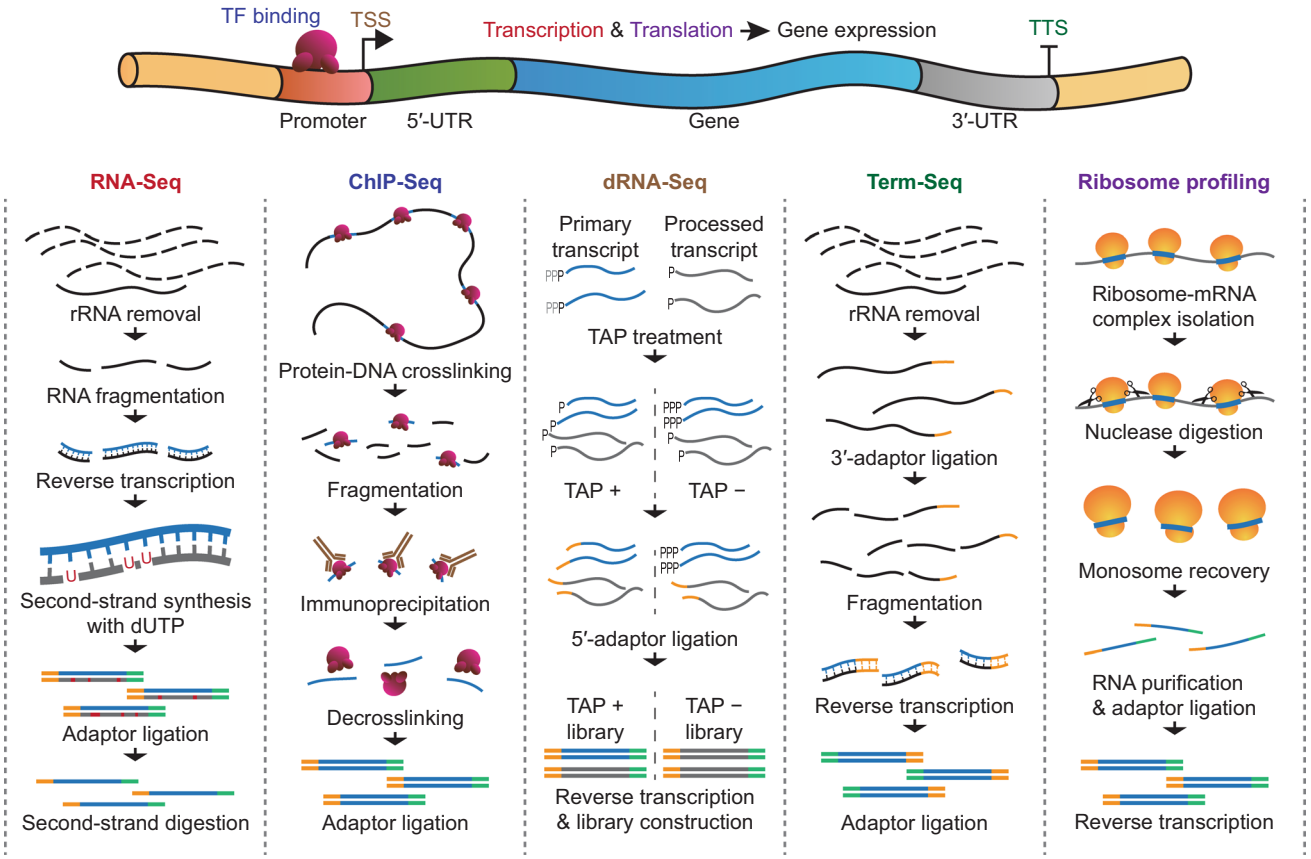

Fig. 1 NGS-based tools for assessing gene expression and identifying regulatory elements

From the ChIP-Seq approach, the *trans*-regulation of gene expression governed by transcriptional regulators can be monitored. In addition to the *trans*-regulation by transcriptional regulators, numerous *cis*-acting regulatory sequences, including promoters, 5′- and 3′-untranslated regions (UTRs), ribosome binding sites (RBSs) and transcription terminators, affect gene expression. Thus, identifying those *cis*-regulatory elements is also crucial to understand the gene expression regulatory mechanism. A significant portion of the *cis*-regulatory sequences can be identified by determining the 5′- and 3′-ends of RNA transcripts in genome scale by utilizing differential RNA-Seq (dRNA-Seq) and Term-Seq, respectively [23, 28, 57]. Unlike RNA-Seq where the random positions of a transcript are sequenced, only either 5′- or 3′-ends of RNA transcripts are sequenced in dRNA-Seq or Term-Seq, respectively. Since the 5′-end positions of RNA transcripts in vivo may indicate either the real transcription start sites (TSSs) or the processed 5′-ends, the presence of polyphosphate at the 5′-ends of unprocessed transcripts serves as a guide for the discrimination of a TSS from the processed 5′-ends in dRNA-Seq approach. On the other hand, since transcripts' termini lack such molecular features to distinguish real transcription termination sites from the processed 3′-ends, Term-Seq covers both types of transcripts' 3′-ends.

The measurement of transcriptome and the identification of transcriptional regulatory information are good means of understanding gene expression. Meanwhile, inconsistencies between transcript's abundance and actual amount of proteins are sometimes observed, suggesting that gene expression is also substantially regulated in post-transcriptional level, including the translation step [86]. Hence, acquiring genome-scale translation information is also crucial to understand bacterial gene expression regulation. By capturing the mRNA bound by translating ribosomes, the in vivo translation process can be monitored in genome-scale by harnessing NGS, through so-called ribosome profiling [44]. Treatment of translational inhibitors and maintaining high salt condition arrest translation while maintaining the interaction between ribosome and mRNA. Nuclease digestion leaves the footprints of ribosomes, since ribosome bound to mRNA physically blocks the nuclease. By sequencing those ribosomal footprints, the allocation of ribosomes to individual transcripts can be determined in genome-scale.

Transcriptome-based understanding of *Streptomycetes* and their secondary metabolism

The representative RNA-Seq application for studying the secondary metabolism of *Streptomyces* is the comparative transcriptomics approach. RNA-Seq can be performed in genetically heterogeneous conditions to analyze the effect of genetic variance on transcriptome, or can be performed in phenotypically heterogeneous conditions to elucidate the key genetic or environmental factors for the phenotypic variance. The former strategy is especially effective for studying regulatory genes, including transcription factors and sigma factors, about their functions on the transcriptional regulatory network. For example, SCO4117, the ECF52 sigma factor of *Streptomyces coelicolor*, was knocked out and the perturbation of transcriptome was monitored, identifying that SCO4117 is an activator for secondary metabolic genes (i.e., deoxysugar synthases and actinorhodin biosynthetic genes) and developmental genes related to aerial mycelium formation and sporulation [63].

Furthermore, the latter comparative transcriptomics approach is also extensively exploited to study the secondary metabolism of *Streptomyces*. As a representative example, RNA-Seq was utilized to address the actinorhodin production by *S. coelicolor* during co-culture with *Myxococcus xanthus* [55]. RNA-Seq was applied to both organisms to monitor transcriptional responses of the two organisms during co-cultivation. While actinorhodin production related genes were up-regulated in *S. coelicolor* during the co-culture, iron uptake related genes were up-regulated in *M. xanthus*. From the transcriptional landscape, depletion of iron in the culture medium by *M. xanthus* was pointed out as the trigger for actinorhodin production by *S. coelicolor*. Also, further RNA-Seq analysis revealed that the iron depletion promotes the synthesis of certain secondary metabolites, mainly siderophores, by *Streptomyces* species. In addition, another mechanism of actinorhodin production and transport was proposed from the RNA-Seq result. SCO6666, a gene distal from the actinorhodin BGC, is involved in actinorhodin production under co-culture condition by replacing the function of cluster-situated transporter genes. Furthermore, RNA-Seq was carried out to determine the rate-limiting step of enduracidin production by *Streptomyces fungicidicus* [105]. During enduracidin production, the transcription level of EndC gene which encodes a non-ribosomal peptide synthase in the enduracidin biosynthetic gene cluster was lower than the transcription levels of other biosynthetic genes in the cluster. It was pointed out as the rate-limiting gene for enduracidin production due to its lower transcription level, and improvement of its transcription led to an increased production of enduracidin. As described above, RNA-Seq can be utilized to comprehend the regulatory functions of a gene toward global transcriptome dynamics and elucidate the driving force for a specific phenotype. And the accumulating knowledge based on the genome-scale transcriptome information will serve as a fundamental resource for understanding the secondary metabolism of *Streptomyces*.

Defining the genome-wide binding sites of transcriptional regulators

While comparative transcriptomics approaches can be utilized to reveal the set of genes affected by a specific transcriptional regulator under a specific condition, it does not necessarily reveal the presence of a direct link between genes and their regulators. Specifically, multiple transcriptional regulators may form a complex hierarchical regulatory network and change in gene expression alone is not sufficient to indicate direct regulation by regulators [77]. To date, ChIP-Seq serves as the most efficient tool for collecting in vivo binding sites of transcription factors and sigma factors in genome-scale [48]. Several ChIP-Seq experiments were applied to *Streptomyces*, including MtrA (a member of two-component regulatory system related to cell division), ScbR (γ-butyrolactone binding protein with TetR family transcriptional regulator domain situated in coelimycin BGC), ScbR2 (homolog of ScbR also situated in coelimycin BGC) and NdgR (IclR type transcriptional regulator) of *S. coelicolor*, and WhiA (a regulator related to development) and WhiB (a regulator related to development) of *S. venezuelae* (Table 1) [15, 17, 50, 59, 82]. The number of regulons directly regulated by a transcription factor may not be correlated to the actual number of genes affected by the regulator. For example, BldO only targets WhiB for repression in *S. venezuelae*, and overall development is repressed due to the repression of WhiB [18]. Also, ChIP-Seq has been applied to the housekeeping sigma factor, HrdB, of *S. coelicolor* [81]. From the ChIP-Seq result, 1245 HrdB binding sites were determined and about 2300 genes, including essential genes for primary metabolism and vegetative growth, were assigned as potential HrdB regulon.

Meanwhile, ChIP-Seq cannot solely reveal transcriptional effects of the transcriptional regulators on gene expression in their regulon. By integrating RNA-Seq and ChIP-Seq, the effect of regulators on transcription of genes in their regulons can be identified. For example, the effects of CagR, a member of two-component regulatory system located next to the clavulanic acid BGC of *S. clavuligerus*, were identified on the expression changes of genes in its regulon [35]. The genes in CagR regulon were identified using the ChIP-Seq approach, and the transcriptional effects of CagR on expression changes

Table 1 ChIP-Seq applications for determining regulons of transcriptional regulators

Target gene	Organism	Note	References
c-di-GMP tetramer bound BldD	*S. venezuelae*	Binding of c-di-GMP is required for proper function of BldD	[75, 91]
MtrA	*S. coelicolor* *S. venezuelae*	MtrA activates antibiotics production, including chloramphenicol of *S. venezuelae* and actinorhodin and undecylprodigiosin of *S. coelicolor*. MtrA also regulates developmental genes	[82, 83]
σ^E	*S. coelicolor*	σ^E regulates cell envelope related genes along with some regulatory genes including HrdD	[89]
CagR	*S. clavuligerus*	CagR regulates genes involved in fatty acid degradation, glyceraldehyde 3-phosphate metabolism, arginine biosynthesis and clavulanic acid production	[35]
BldO	*S. venezuelae*	BldO represses WhiB as the only target	[18]
AdpA	*S. griseus*	The regulons of AdpA were identified by combining ChIP-Seq and ChAP (chromatin affinity precipitation)-Seq	[39]
BldC	*S. venezuelae*	BldC regulates genes involved in chromosomal condensation and segregation and cell division during sporulation as well as regulatory genes related to development	[16]
RsrR	*S. venezuelae*	The regulons of RsrR, including genes related to glutamine synthesis, NADH/NAD(P)H metabolism and DNA/RNA and amino acid/protein turn over, were identified	[67]
ScbR and ScbR2	*S. coelicolor*	The regulons of ScbR and ScbR2 were identified and the majority of the regulons were related to secondary metabolism and stress response	[59]
NsrR	*S. coelicolor*	Only three targets, HmpA1, HmpA2 and NsrR, were determined as regulons of NsrR	[25]
WhiA and WhiB	*S. venezuelae*	WhiA and WhiB cooperatively regulate genes related to cell division and sporulation	[15, 17]
NdgR	*S. coelicolor*	NdgR regulates genes involved in branched-chain amino acid and cysteine biosynthesis pathways	[50]
BldM and WhiI	*S. venezuelae*	BldM function as homodimer or heterodimer with WhiI to regulate different groups of genes	[1]
HrdB	*S. coelicolor*	Regulons of HrdB were identified and dependence of the regulons on RbpA was evaluated	[81]
Lsr2	*S. venezuelae*	Lsr2 represses genes in multiple smBGCs	[36]
MacR	*S. coelicolor*	Three targets were identified as in vivo targets of MacR, including sco2101 (putative carotenoid dehydrogenase), sco6728 (small membrane protein) and sco7460 (putative lipoprotein)	[62]
WblC	*S. coelicolor*	The regulation by WblC under the presence of ribosome-targeting antibiotics was elucidated	[53]

of genes in its regulon were determined by exploiting the RNA-Seq. The expression changes of the genes in CagR regulon between the wild-type and CagR deletion mutant were analyzed to determine whether CagR functions as an activator or a repressor for its regulon. Thus, ChIP-Seq approach is crucial to understand the complex genetic regulation of *Streptomyces* and clarify their transcriptional regulatory networks.

Also, some transcriptional regulators are dedicated to a specific metabolic pathway, and engineering those regulators is an effective strategy to modulate the entire pathway at a lower cost compared to engineering each component [16]. For instance, the smBGCs of *Streptomyces* often possess *Streptomyces* antibiotic regulatory protein (SARP) family transcriptional regulators for transcriptional activation of secondary metabolite biosynthetic genes, and the overexpression of those regulators generally resulted in an increased production of secondary metabolites [22, 60, 100].

Understanding genetic regulations from transcription unit architecture

Since the transcriptional regulators control genes in a unit of transcripts, obtaining transcription unit (TU) information and determining the transcriptional regulatory elements for the regulatory unit are crucial to understand the gene expression regulation of *Streptomyces*. Conventionally, operons can be predicted by analyzing features such as intergenic distance, transcriptional homogeneity and the presence of terminator-like sequence between adjacent genes [21]. However, the operons and TUs can be determined from the transcripts' 5′-ends and 3′-ends information which can be obtained by dRNA-Seq and Term-Seq, respectively [57].

The TU information is important in three aspects. First, the TU information determines the minimal unit of

transcriptional regulation and clarifies the transcriptional regulatory network. By combining the genome-scale binding site information of a transcriptional regulator with the TU information, the exact regulons of the transcriptional regulator can be identified [81]. Especially, multiple secondary metabolite synthase genes are often co-transcribed in one TU and modifying the promoter for the first gene can modulate the expression of entire genes in the TU [106].

Second, regulatory RNAs, such as sRNAs and premature transcripts resulting from premature transcription termination, can be determined from the TU information. In bacteria, the sRNAs may bind to mRNA to modulate the stability of RNA by offering substrate specificity to RNases, or translationally regulate gene expression by modulating the accessibility of ribosomes to mRNA [85]. In addition, sRNAs can bind to proteins to modulate the functions [70]. *Streptomyces* also possesses sRNAs with regulatory functions as other bacteria. For example, the sRNA scr5239 of *S. coelicolor* represses the expression of DagA, the agarose gene, MetE, the cobalamin independent methionine synthase, and phosphoenolpyruvate carboxykinase by binding near the start codons of their mRNAs [31, 93, 94]. In addition, premature transcription termination plays an important role for gene expression regulation [28]. For example, in *Streptomyces griseus*, glycine responsive riboswitches are present in the 5′-UTRs of glycine cleavage related genes [87]. The transcription termination activities of the riboswitches are reduced when glycine binds to the riboswitches and the glycine cleavage system can be activated in the presence of glycine. The on/off switching by ligand binding to RNA element is an efficient means of engineering bacteria for inducible gene expression [37]. Among the TSSs determined in the genomes of *S. coelicolor* and *S. clavuligerus*, more than 200 TSSs are present in reverse strand of genes, suggesting the presence of antisense transcripts which may regulate gene expression by directly binding to their complementary sequences [43, 47]. In addition, from the TU determined from dRNA-Seq and Term-Seq, more than a hundred of short transcripts without open reading frames are newly detected as the potential sRNAs and *cis*-regulatory elements such as riboswitches, suggesting the presence of diverse RNA species with regulatory functions in *Streptomyces lividans* [57].

Third, the *cis*-regulatory sequences for gene expression can be determined along with their effects on gene expression. At current, dRNA-Seq has been applied to five *Streptomyces* species, including *S. clavuligerus*, *S. coelicolor*, *S. lividans*, *S. tsukubaensis* and *S. venezuelae*, offering more than 1900 TSSs per each species [5, 43, 47, 57, 67]. From the promoter sequence analysis of four *Streptomyces* species (except *S. venezuelae*) based on the TSS information, a highly conserved sequence motif 'ANNNT' was found as the − 10 element of the promoter for most TSSs. While the − 10 elements are highly conserved, the − 35 elements are rather ambiguous in terms of the sequence and position relative to the TSSs, suggesting that the − 35 elements can serve as the key recognition sequences for specific sigma factor binding [43, 47, 57]. From the TSS information, 5′-UTRs, which contain the ribosome binding sites (RBSs) for translation, also can be determined. From the 5′-UTRs, the conserved RBS sequence for *Streptomyces* was found as RRGGAG (R: A or G), typically 6nt apart from start codon. On the other hand, Term-Seq has been applied only to *S. lividans*, offering more than 1600 transcripts' 3′-end positions (TEPs) resulting from transcription termination or processing by RNases [57]. In general, GC-rich sequence, which may form RNA stem structure, is found upstream of the TEPs. However, TEPs ending with poly U sequence, which is a key characteristic for bacterial rho-independent termination, are rare. Yet, a distinct sequence motif, composed of GC-rich sequence followed by U rich sequence (considering the GC-rich genome of *Streptomyces*) and ending with CGT, is found for TEPs exhibiting strong transcription termination signal.

Interestingly, the determination of 5′-UTRs revealed that about 20% of the total transcriptome of *Streptomyces* consists of leaderless mRNAs, whose 5′-UTRs are absent [43, 47, 57]. For leaderless mRNAs, RBSs are also absent, which means that those genes transcribed as leaderless are subjected to distinct translational regulation. In contrast to the canonical translation initiation that the ribosome 30S subunit binds to mRNA prior to formation of ribosome 70S complex, fMet-tRNA charged ribosome 70S complex initiates translation of bacterial leaderless mRNA [66]. The leaderless mRNAs are considered to be important for maintaining cell viability during stressful conditions. In *Mycobacterium tuberculosis*, about 26% of genes are transcribed as leaderless, and substantial portion of those genes are up-regulated during starvation [24]. In addition, about 60% of genes are leaderless in *Deinococcus deserti*, a desiccation and radiation tolerant bacterium, and about 85% of defense mechanism related genes are leaderless [29]. Furthermore, some portion of leaderless mRNAs in *Streptomyces*' transcriptome may originate from the self-resistance to their own secondary metabolites. For example, the kasugamycin, produced by *Streptomyces kasugaensis*, selectively inhibits translation initiation of leadered mRNA and induces formation of 61S ribosome particles, which lack more than six proteins (S1, S2, S6, S12, S18 and S21) and are capable of translation initiation of leaderless mRNA [6, 49]. Therefore, transcribing genes in leaderless form would be favored for the producers of kasugamycin or its functional homologs for efficient gene expression under the producing condition.

The identification of regulatory elements with their effects on gene expression is essential for current bioengineering

theme, the synthetic biology, by providing bioparts for fine-tuning the expression of individual genes [104]. The available regulatory elements for tuning the gene expression in *Streptomyces*, such as promoters and terminators, are limited in number, and acquiring a wide array of bioparts is essential for engineering *Streptomyces* to enhance secondary metabolite production [54]. Especially, the development of inducible gene expression system is crucial since the secondary metabolite production by *Streptomyces* involves metabolic shift from primary metabolism to secondary metabolism, and timely coordination of precursor supply to secondary metabolite production is crucial to reduce the consumption of cellular resources for undesired products [2]. As an example, dynamic degradation of triacylglycerols (ddTAG) was applied to various *Streptomyces* species to enhance polyketide production [97]. The strategy involves controlled degradation of triacylglycerols using inducible promoters for timely supply of precursors during polyketide synthesis. However, the inducible promoters currently in use are substantially leaky, leading to gene expression in un-induced states [42]. In addition, the limited number of inducing agents could hamper multiplexed gene expression control [54].

To expand the availability of bioparts for a more precise gene expression control, transcriptome information is a good resource. Thousands of regulatory elements can be determined from transcriptome and TU information, and their strength on transcriptional regulation under various conditions can be measured quantitatively. There are some examples describing identification of multiple bioparts from transcriptome information for controllable as well as constitutive gene expression. From the RNA-Seq experiments done in multiple growth conditions, 32 and 35 promoter candidates were screened for constitutive transcription in *S. albus* and *S. coelicolor*, respectively [64, 104]. In addition, an inducible promoter activated at stationary growth phase was discovered from time-series RNA-Seq data of *S. coelicolor* [51]. The exploitation of the inducible promoter for expression of ActAB, the transporter genes of actinorhodin, was superior for actinorhodin production compared to utilizing a strong constitutive promoter for ActAB expression with regards to cell fitness. Collectively, the transcription unit and *cis*-regulatory element information could serve as a fundamental resource for understanding the regulation of *Streptomyces* and offer a wide array of genetic tools to engineer *Streptomyces* for an enhanced secondary metabolite production.

Deciphering translational regulation by monitoring ribosomal allocation on the mRNA

Translation is another key step for gene expression and diverse regulatory mechanisms take place for gene expression regulation. The translation process also can be monitored by exploiting one variant of NGS technique called ribosome profiling [44]. While RNA-Seq is the most efficient and common strategy to quantify gene expression in genome-scale, the actual gene expression may be inconsistent from the RNA-Seq result, due to the varying translation levels across the individual genes. The existence of ribosome on mRNA, which can be inferred from ribosome profiling studies, indicates that the mRNA is being translated. By combining the ribosome profiling data with RNA-Seq data, the translational efficiency (TE), which stands for the translation level of a single mRNA molecule, can be evaluated. The analysis of TE and transcript abundance along the growth phase revealed another layer of gene expression regulation in *Streptomyces* called translational buffering, a phenomenon in which the translation is maintained or repressed while transcription increases [47]. The translational buffering is observed for regulatory genes in the smBGCs of *S. coelicolor*, including CdaR of calcium-dependent antibiotic BGC, ActII-ORF4 of actinorhodin BGC, and RedD and RedZ of prodiginine BGC. The finding indicates that secondary metabolism is also subjected to a significant regulation in translational level, and dampening the translational buffering may serve as a novel engineering strategy for improving secondary metabolite production. Interestingly, the TE values of leaderless mRNA increase relative to the growth phase. In *E. coli*, the activity of mRNA interferase toxin, MazF, generates a distinct 70S ribosome complex with a 3′-43 nt removed 16S rRNA which is capable of translation of leaderless mRNA [92]. Since the stringent response, which prevails in late growth phase, involves activation of mRNA interferase toxins, the exploitation of leaderless transcription for the late-growth-phase-activated secondary metabolic genes may increase the secondary metabolite production [74].

In addition, TE information can be utilized for synthetic biology applications. As the key regulatory sequence for ribosome recruitment, the RBS can be associated to the TE index to evaluate its strength for ribosome recruitment. The gene expression datasets obtained from RNA-Seq and ribosome profiling integrated with regulatory element information, including promoters, 5′-UTRs and RBSs, leads to the evaluation of the effects of the regulatory elements on gene expression, which is an important resource for engineering *Streptomyces*. For instance, in *S.*

coelicolor, a total of eight promoter and 5′-UTR pairs were chosen from the multi-omics dataset and their performance was verified for tunable gene expression [104].

While translational buffering can be regarded as regulation at translational initiation, translational elongation is another important point to understand secondary metabolism. The guanosine tetraphoshpate (ppGpp) is produced by ribosome-associated ppGpp synthetase, RelA, when uncharged tRNA binds to ribosomal A site, and accumulation of ppGpp is closely related to the stringent response and the onset of secondary metabolism, indicating that regulation at translational elongation affects secondary metabolism [19, 68]. In particular, conferring resistance to translation inhibitors, such as streptomycin, paromomycin and gentamicin, resulted in an increased secondary metabolite production in *Streptomyces* [96]. Monitoring the translation elongation with ribosome profiling will provide insights for secondary metabolism. In addition, ribosome profiling suggests another novel engineering points for secondary metabolite production enhancement. Along a single gene, the ribosomes are not evenly distributed in the ribosome profiling data and the position where higher number of reads are mapped may indicate the position where ribosomes dwell longer during translation and thus indicates the presence of translational pause or arrest at the position [65, 102]. Then the codons can be replaced to alleviate translational pause or arrest for enhancing protein production. The ribosomal pausing during translation plays important role for gene expression, not only directly affecting translation but also affecting transcription as well. Since bacteria lack nucleus, transcription and translation are taking place in the same environment. As a result, both procedures are highly coupled and even, the RNA polymerase directly interacts with the ribosome [32]. Particularly, the leading ribosome, the first ribosome behind the transcribing RNA polymerase, serves as a barrier for Rho factor accessing to RNA polymerase and pausing of the leading ribosome may lead to premature transcription termination by Rho [108]. Therefore, the alleviation of ribosome pausing in secondary metabolic genes can result in higher gene expression level, leading to improved secondary metabolite synthesis (Fig. 2). Taken together, monitoring translation elongation is important to understand secondary metabolism and improve secondary metabolite production.

Other approaches for system level investigation of *Streptomyces*

Prior to the application of NGS techniques on *Streptomyces* research, proteomics and metabolomics approaches were utilized for system level analysis by mass spectrometry or nuclear magnetic resonance (for metabolomics) following the separation of molecules [33, 90, 103]. Those tools are as effective as NGS-based tools for system level analysis. For example, based on the finding that polyphosphate kinase (Ppk) disruption mutant of *S. lividans* TK24 produced higher level of antibiotics than wild type, the proteome of the mutant were compared to the proteome of wild type to find differentially expressed proteins that may affect antibiotics production [52]. By linking the proteome to metabolic pathways, degradation of triacylglycerol was identified as the cause for antibiotics overproduction by the PpK disruption mutant. In addition to the direct measurement of protein abundances, which may differ from gene expression level measured in NGS tools due to the post-translational regulations and varying half-life, proteomics can further give insights on regulatory mechanisms by capturing post-translational modification states [40, 46, 61]. For example, StrM, a gene involved in streptomycin biosynthesis, was identified as a highly acetylated protein from proteomics analysis, and acetylation of the Lys70 of StrM was found to inhibit the enzymatic function of StrM [45].

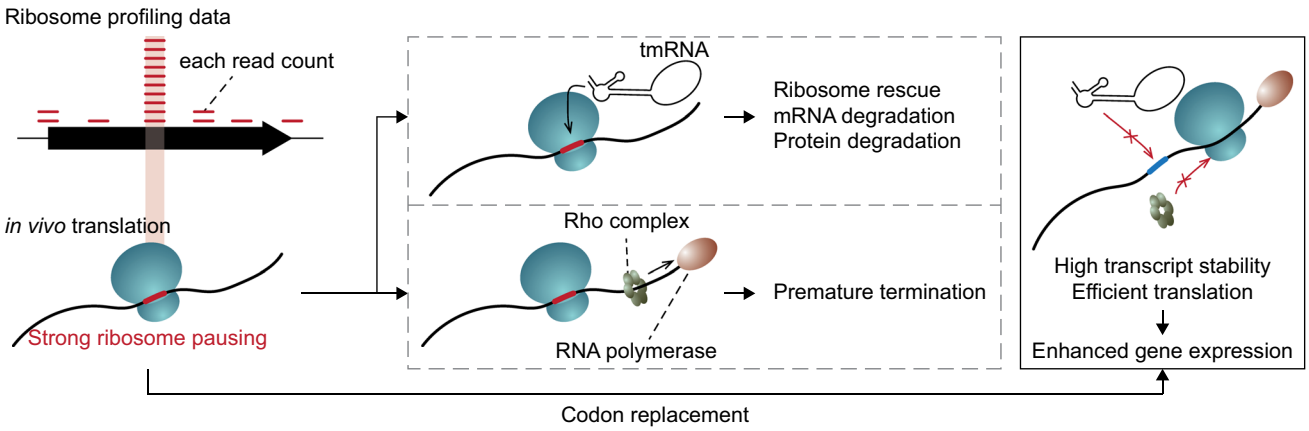

Fig. 2 Gene expression enhancement by alleviating ribosomal pausing

Compared to other omics tools, metabolomics has another advantage that it can be utilized for direct detection of the secondary metabolites [71, 78]. For example, novel siderophore type secondary metabolites were discovered from *S. coelicolor* under high temperature condition by exploiting metabolomics approach [79]. In addition to directly identifying the secondary metabolites, metabolomics is as effective as NGS or proteomics tools for system level understanding of secondary metabolism. Interestingly, metabolomics analysis also led to the similar conclusion for the system level secondary metabolism analysis as the example of proteomics. By comparing the metabolome of wild-type *S. coelicolor* to the metabolome of actinorhodin overproducing strain, decrease of intracellular triacylglycerol was observed to coincide with the increase of secondary metabolites, resulting in development of ddTAG strategy for timely coordination of precursor supply to polyketide synthesis [97]. As shown above, system level analysis of *Streptomyces* harnessing omics tools enables identification of key processes that are distal but highly related to secondary metabolite production. For coordination of those unlinked processes to secondary metabolism, understanding of genome-scale transcriptional network and responsible regulatory elements using NGS tools is crucial, and those omics approaches should give a great synergy when overlapped.

Linking omics-driven findings to secondary metabolite production enhancement by harnessing CRISPR-Cas system

While the omics-driven conclusions often suggest novel strategy for improving secondary metabolite production, only few exploitations are reported, perhaps due to the scarcity of highly efficient engineering tools compared to the rapid discovery of engineering targets from omics tools. As the breakthrough, CRISPR-Cas system is noticed for high efficient and multiplexed genome manipulation. At current, both Cas9 and Cpf1 based CRISPR-Cas system can be utilized for editing *Streptomyces*' genome [42, 58, 88]. Especially, the most solid and simple application for improving secondary metabolite production would be the reconstitution of promoters in smBGC with strong promoters [106]. As novel engineering targets for secondary metabolite production, ranging from a single codon to a single entire metabolic pathway, are identified and a palette of bioparts for tuned gene expression are collected from omics approaches, the CRISPR-Cas system will serve as the basis for linking the findings to practical applications.

In addition to directly manipulating the genome, CRISPR-Cas can be utilized for modulation of gene expression. The CRISPR interference (CRISPRi), which is mediated by targeted binding of nuclease deactivated Cas (dCas) protein to desired genomic position to block transcription by RNA polymerase, enables gene repression with less effort compared to refactoring regulatory sequences [58, 88, 107]. For CRISPRi application, the dCas binding position relative to the promoter or TSS of the target gene is critical for efficiency of gene repression [73]. In addition, the CRISPR activation (CRISPRa) for gene activation, which is not applied to *Streptomyces* yet, also requires TSS or promoter information for efficient genetic modulation [11]. Therefore genome-wide TSS information obtained from dRNA-Seq will assure the broad applicability of CRISPRi and CRISPRa for *Streptomyces*. As shown from the metabolomics analysis, the timely coordination of precursor supply to secondary metabolism activation is crucial for enhancing secondary metabolite production [97]. In that sense, CRISPRi and CRISPRa are attractive tools since they do not change the gene expression permanently and the activity can be controlled using inducible promoters for guide RNA expression (Fig. 3a, b). In addition, the recently developed toehold-gated guide RNA strategy which can modulate guide RNA activity of CRISPR-Cas system with the endogenous RNA molecule can be employed to CRISPRi or CRISPRa tools to temporally regulate gene expression [80]. In toehold-gated guide RNA strategy, the spacer region is hindered by the partial-complementary 5′-overhang sequence of the guide RNA (Fig. 3c). When trigger strand, complementary to the 5′-overhang sequence, is present, the spacer region is exposed and CRISPR-Cas system is activated. Endogenous transcripts can be utilized as the trigger strand to tightly control the Cas activity based on the endogenous cellular information. By employing toehold-gated guide RNA for CRISPRa based smBGC activation and utilizing the transcript of precursor supply gene as the trigger strand, the activation of precursor supply genes can be directly linked to activation of secondary metabolism, or vice versa (Fig. 3d).

Conclusion

The NGS technologies have revolutionized our understanding on the secondary metabolism of *Streptomyces* by expanding the genetic potential for secondary metabolite production and offering genome-scale gene expression and regulatory information. Currently, limitations and strategies for secondary metabolite production are routinely screened out from the system-level information obtained by high-throughput technologies, and synthetic biology is of great interest in linking the observations to practical applications. Toward the synthetic biology-based engineering of *Streptomyces*, diverse regulatory elements for controlling gene expression are essential and NGS-derived genome-scale gene expression information could serve as a fundamental resource (Fig. 4). In addition, the

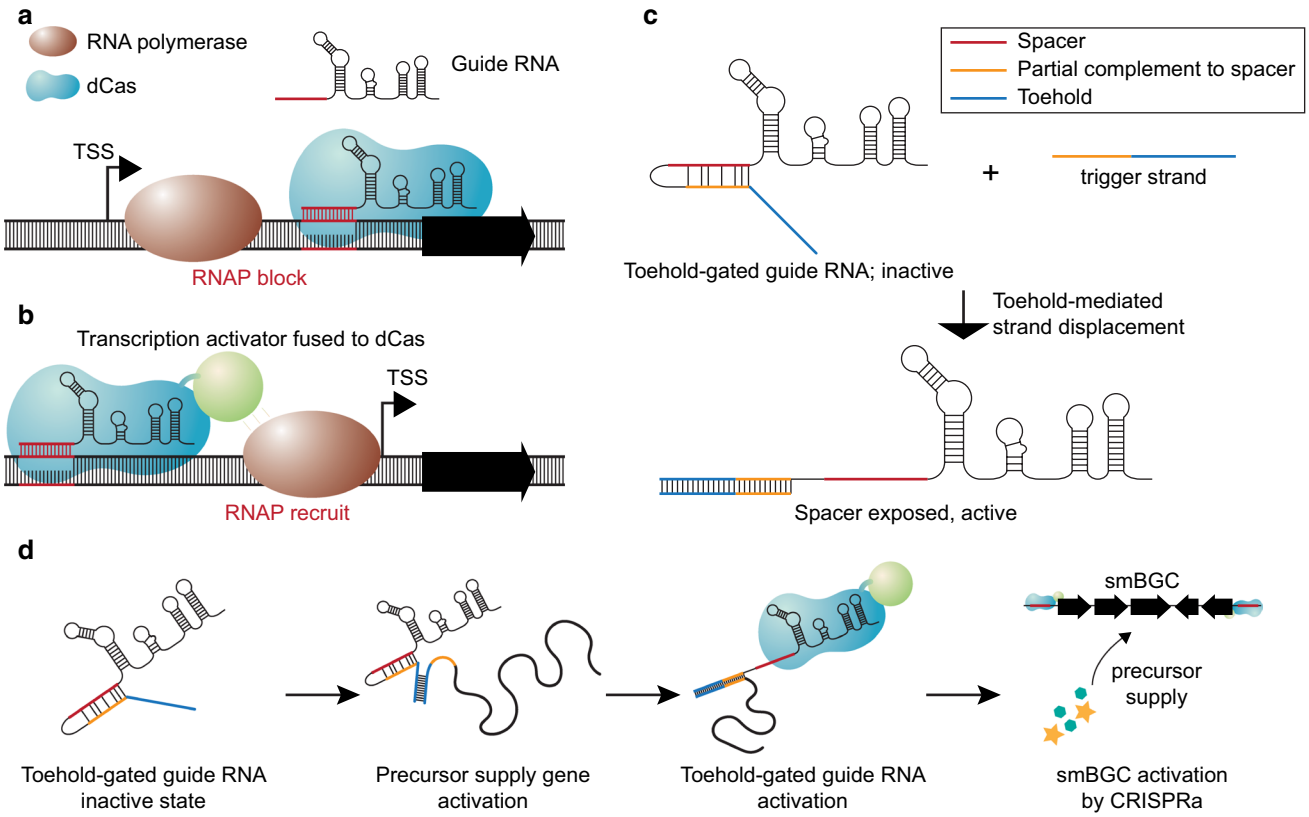

Fig. 3 CRISPR-Cas based gene expression modulation tools. **a** Basic principle of CRISPRi. **b** Basic principle of CRISPRa. **c** Activation of toehold-gated guide RNA through toehold-mediated strand displacement. **d** Utilization of toehold-gated guide RNA and CRISPRa to link activation of precursor supply gene to activation of the corresponding smBGC

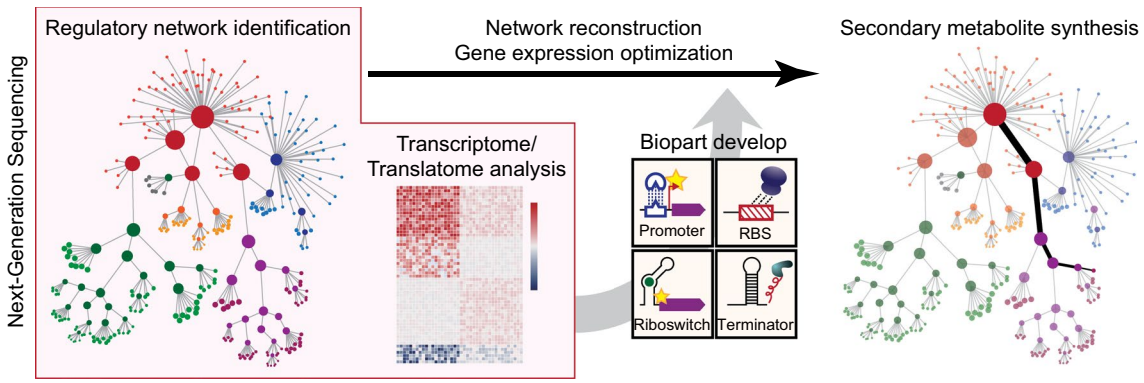

Fig. 4 Utilization of NGS-based knowledge for secondary metabolite production

transcriptional regulatory network information is crucial to rewire the endogenous interconnected regulation and construct a robust platform for novel secondary metabolite discovery. Thus, the accumulation of system-level regulatory information on gene expression will serve as a key driver for secondary metabolite research.

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