

Strategies for Awakening Silent Gene Clusters in *Streptomyces* Based on Global Regulatory Systems such as AfsR-AfsS and Their Synthetic Biology Applications

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Abstract:

Streptomyces genomes harbor numerous biosynthetic gene clusters (BGCs), many of which remain transcriptionally silent under laboratory conditions. Among the regulatory systems involved in secondary metabolism, the AfsR–AfsS pathway has attracted considerable attention because of its role in controlling the expression of otherwise inactive BGCs. This system responds to environmental and metabolic cues through S-adenosylmethionine (SAM), influences the AfsK/KbpA phosphorylation cascade, and contributes to the regulation of AfsR–AfsS-dependent transcription. Evidence from different *Streptomyces* species suggests substantial variation in its regulatory functions, as well as interactions with other regulatory networks, including PhoR–PhoP and BldD. Recent studies have explored a range of synthetic biology approaches to activate silent BGCs, such as promoter engineering, regulatory circuit redesign, and chassis optimization. At the same time, advances in multi-omics analysis, machine learning-assisted genome mining, and standardized genetic part libraries are providing new opportunities for the discovery and development of previously inaccessible natural products and antibiotic candidates.

Keywords:

Streptomyces, Biosynthetic Gene Clusters, AfsR-AfsS Complex, Antibiotic Development

1. Introduction

Streptomyces has long been recognized as one of the most productive sources of bioactive natural products. Many clinically important secondary metabolites, including antitumor agents, immunosuppressants, and antibiotics, are derived from this genus. It has been estimated that nearly 70% of currently used antibiotics originate from *Streptomyces* species [1–3]. The rapid development of genome sequencing technologies has revealed that the biosynthetic capacity of these bacteria is substantially greater than previously appreciated. In many cases, dozens of biosynthetic gene clusters (BGCs) can be identified within a single genome, whereas only a small fraction of their corresponding metabolites are detected under conventional laboratory conditions [4,5]. This discrepancy between genomic potential

and observable metabolite production suggests that a large number of BGCs remain transcriptionally inactive or are expressed at levels too low to be readily detected. As a result, considerable attention has been directed toward strategies for accessing these cryptic biosynthetic resources and expanding the chemical diversity available from microbial natural products [6].

A variety of approaches have been explored to stimulate the expression of silent BGCs, including the One Strain Many Compounds (OSMAC) strategy, ribosome engineering, and heterologous expression systems [7,8]. More recently, efforts have increasingly focused on manipulating regulatory networks that coordinate secondary metabolism [9]. Unlike pathway-specific regulators (PSRs), which generally control individual biosynthetic pathways, global regulators influence multiple cellular processes and respond to a broad range of physiological and environmental cues [10]. Changes in nutrient availability, such as phosphate limitation, can be transmitted through these regulatory systems and subsequently affect the expression of numerous metabolic pathways [10]. Because of their extensive regulatory reach, global regulators have become attractive targets for activating otherwise silent biosynthetic pathways and reshaping metabolic flux distributions.

Among the global regulatory systems identified in *Streptomyces*, the AfsR–AfsS system has been studied extensively because of its involvement in secondary metabolite production [11]. However, its regulatory roles appear to vary considerably among species. In *Streptomyces coelicolor*, AfsR–AfsS promotes the production of secondary metabolites such as actinorhodin (ACT) through a positive regulatory mechanism [12]. By contrast, studies in *Streptomyces avermitilis* have shown that the same system negatively affects avermectin (AVE) biosynthesis [13]. These contrasting observations highlight the complexity of regulatory interactions in *Streptomyces* and suggest that the biological functions of AfsR–AfsS are shaped by species-specific regulatory contexts.

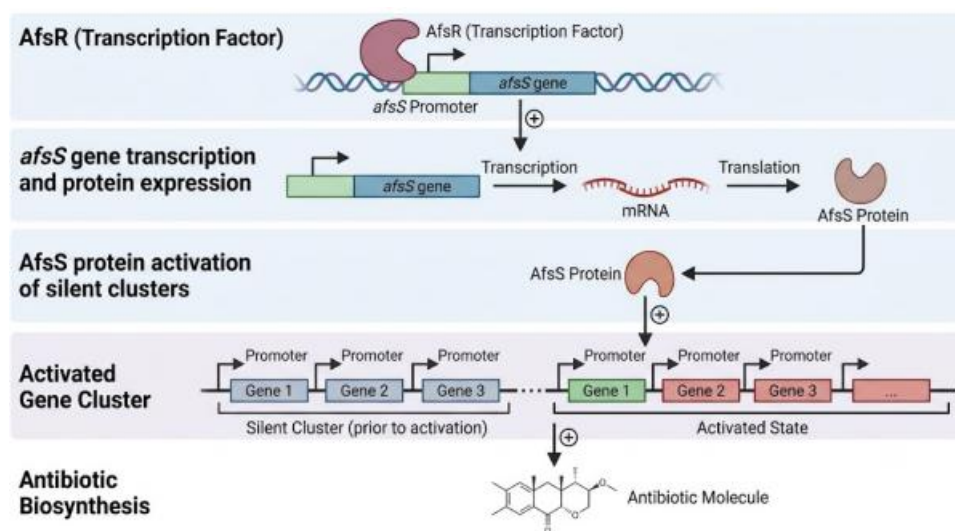


Figure 1. *AfsR–AfsS-Mediated Activation of Silent Biosynthetic Gene Clusters and Mechanisms of Antibiotic Biosynthesis.*

The AfsR–AfsS system has also become a target for synthetic biology-based strain engineering. Regulatory circuit redesign, promoter engineering, and the elimination of competing pathways have been employed to enhance the expression of previously silent BGCs in different *Streptomyces* hosts [14]. Nevertheless, manipulation of

global regulators is not without limitations. Excessive activation may disrupt cellular homeostasis and lead to undesirable outcomes, including impaired growth, cytotoxic effects, and widespread transcriptional perturbations [15]. Given these opportunities and challenges, a clearer understanding of the molecular functions and regulatory characteristics of the AfsR–AfsS system remains necessary. This review summarizes current knowledge of its regulatory mechanisms, examines its functional variation across different *Streptomyces* species, and discusses recent advances in its application to chassis engineering and metabolic reprogramming.

2. Structural Characteristics and Molecular Mechanisms of the AfsR-AfsS Two-Component System

2.1. Domain Architecture of AfsR and AfsS

AfsR is a large multidomain regulatory protein that belongs to the SARP (*Streptomyces* antibiotic regulatory protein) family of transcription factors [16]. Structural analyses indicate a modular organization composed of several conserved functional regions. At the N-terminus, AfsR contains a characteristic SARP domain that includes an OmpR-type DNA-binding domain (ODB) and a bacterial transcriptional activation domain (BTAD) [17]. The ODB region is involved in the recognition of specific promoter sequences, whereas the BTAD domain is thought to facilitate transcriptional activation through interactions with the transcriptional machinery [18].

The central portion of AfsR contains a nucleotide-binding oligomerization domain (NOD). Similar domains in other regulatory proteins are frequently associated with ATPase or NTPase activity and have been implicated in conformational regulation, signal transmission, and protein–protein interactions. Although the precise role of the NOD domain in AfsR has not been fully resolved, available evidence suggests that it contributes to the regulation of AfsR activity [16,17,19]. Multiple tetratricopeptide repeat (TPR) motifs are located in the C-terminal region of AfsR [16,19]. TPR-containing domains are widely recognized as platforms for protein–protein interactions and have been associated with signal perception and regulatory complex assembly in diverse biological systems. In AfsR, these repeats are proposed to participate in the integration of intracellular signals and the modulation of downstream regulatory responses, although the underlying molecular mechanisms remain incompletely understood [17,20].

In contrast to AfsR, AfsS (also designated AfsR2 in earlier studies) is a small protein comprising only 63 amino acids. Sequence analyses and structural predictions indicate that AfsS lacks a canonical helix–turn–helix (HTH) DNA-binding motif and is therefore unlikely to function as an independent DNA-binding regulator [2,22]. Instead, AfsS appears to exert its regulatory effects through interactions with other proteins. Previous studies have suggested that AfsS associates with AfsR and participates in the regulation of secondary metabolite biosynthesis, although the precise nature of this interaction and its contribution to transcriptional control continue to be investigated [2,23].

2.2. Upstream Environmental Signal Perception and Kinase Phosphorylation Cascade

The production of secondary metabolites in *Streptomyces* is influenced by a complex network of environmental and intracellular signals, many of which are transmitted through protein phosphorylation pathways [24]. Among the molecules implicated in this process, *S-adenosylmethionine* (SAM) has attracted particular attention because of its close association with secondary metabolism and cellular physiological status [25,26,27]. As a central intermediate in one-carbon metabolism, SAM has been reported to affect antibiotic production and the expression of secondary metabolic genes in several *Streptomyces* species [28]. Experimental observations further suggest that changes in intracellular SAM levels may be linked to the activity of the AfsK–KbpA regulatory system, thereby connecting metabolic state to regulatory responses involved in secondary metabolite biosynthesis [20,28].

AfsK is a serine/threonine (Ser/Thr) protein kinase capable of autophosphorylation and subsequent phosphorylation of downstream regulatory proteins. Biochemical studies using γ -³²P-ATP labeling and phosphorylation-specific immunodetection have shown that AfsK phosphorylates AfsR at conserved serine residues, a modification that is closely associated with the activation of AfsR-dependent transcription. Through this phosphorylation event, metabolic and environmental signals can be transmitted to downstream regulatory networks involved in secondary metabolism.

An additional layer of regulation is provided by KbpA, a protein that interacts directly with AfsK [29]. Binding analyses, including surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC), indicate that KbpA associates with the ATP-binding region of AfsK and negatively affects its kinase activity. As a consequence, phosphorylation of downstream targets, including AfsR, is reduced. Current evidence suggests that elevated intracellular SAM concentrations weaken the interaction between KbpA and AfsK, thereby alleviating KbpA-mediated inhibition and enhancing signal transmission through the phosphorylation cascade [30]. This regulatory relationship has been proposed as a mechanism by which metabolic status is coupled to secondary metabolite production in *Streptomyces*. Although several aspects of the pathway remain to be clarified, the AfsK–KbpA module provides an important framework for understanding how intracellular metabolic cues are integrated into global regulatory networks controlling secondary metabolism.

2.3. Protein Interactions and Transcriptional Activation

The regulatory activity of the AfsR–AfsS system relies on a series of interactions involving both DNA and regulatory proteins. Following activation, AfsR binds to specific sequences within the promoter regions of target genes through its N-terminal SARP domain. These binding sites, often referred to as *afs* boxes, have been identified in the promoters of several genes associated with secondary metabolism, including *afsS* [17]. The transcriptional activation domain of AfsR is subsequently thought to facilitate interactions with the transcriptional machinery, thereby promoting expression of downstream target genes [20].

Recent co-immunoprecipitation (Co-IP) studies have further confirmed that AfsS can directly physically bind to AfsR, assembling into an AfsR–AfsS protein complex. Within this complex, AfsS acts as a co-activator, inducing a more favorable conformational change in AfsR for DNA binding through allosteric regulation [2]. In addition, genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) data analysis has revealed that the AfsR–AfsS complex can more efficiently enrich at the promoter regions of numerous secondary metabolism-related genes.

Taken together, current evidence supports a model in which AfsS functions as an accessory regulatory factor that modulates AfsR activity rather than acting as an independent transcription factor. Through its interaction with AfsR, AfsS may contribute to the regulation of downstream gene expression and influence the production of secondary metabolites. Further studies will be required to clarify the structural basis and mechanistic consequences of this regulatory partnership.

3. Complexity of the Regulatory Network of the AfsR-AfsS Two-Component System

3.1. Interspecies Regulatory Diversity of the AfsR-AfsS System

The regulatory effects of the AfsR–AfsS system differ considerably among *Streptomyces* species, suggesting that its function is strongly influenced by strain-specific regulatory networks and genomic contexts. Evidence from different model organisms indicates that AfsR–AfsS can exert positive, negative, or pathway-dependent effects on secondary metabolite biosynthesis.

In *Streptomyces coelicolor*, AfsR–AfsS is generally associated with enhanced production of secondary metabolites. Genetic and transcriptional studies have shown that activation of this regulatory system is accompanied by increased expression of the pathway-specific regulators *actII-ORF4* and *redD*, which control actinorhodin (ACT) and undecylprodigiosin (RED) biosynthesis, respectively [31,32]. RNA-seq analyses further indicate that elevated AfsS expression is associated with widespread changes in gene transcription, affecting both primary and secondary metabolic pathways [33]. Similar observations have been reported in *Streptomyces lomondensis*, where heterologous expression of the *afsR-afsS* locus resulted in an approximately 2.5-fold increase in lomofungin production [34]. Together, these findings support a positive regulatory role for AfsR–AfsS in several antibiotic-producing species.

A different pattern has been observed in *Streptomyces avermitilis*. In this organism, disruption of *afsR* was reported to increase rather than decrease avermectin production [2]. Consistent with this observation, transcription factor decoy experiments designed to reduce functional AfsR activity led to higher avermectin yields [35]. Available evidence suggests that AfsR interacts with regulatory elements associated with *aveR*, a pathway-specific activator of avermectin biosynthesis, although the precise molecular basis of this regulation remains under investigation [2,35,36]. These findings indicate that the regulatory consequences of AfsR activity can vary substantially depending on the genomic and regulatory background of the host strain [35].

Species-specific regulatory behavior has also been reported in *Streptomyces pristinaespiralis*, the producer of pristinamycin. Pristinamycin biosynthesis involves two chemically distinct components, PI and PII, which are encoded by separate biosynthetic loci and regulated by different pathway-specific factors [37]. Overexpression of *afsR* has been shown to affect the two pathways unequally, with a stronger stimulatory effect on PII production than on PI production [38]. ChIP-seq analyses further suggest that AfsR displays distinct binding patterns across promoters associated with the two biosynthetic branches [39]. Such differences may contribute to the differential regulation of metabolite production within complex biosynthetic systems.

3.2. Crosstalk with Other Global Regulatory Networks

The AfsR–AfsS system functions within a broader regulatory framework that coordinates nutrient sensing, morphological development, and secondary metabolism in *Streptomyces*. A growing body of evidence indicates that its activity is influenced by interactions with other global regulatory systems, allowing environmental and physiological signals to be integrated at multiple levels.

One of the best-characterized examples involves the phosphate-responsive PhoR–PhoP two-component system. Phosphate availability is a major determinant of cellular metabolism, and under phosphate-replete conditions, phosphorylated PhoP has been associated with the activation of primary metabolic processes and the repression of secondary metabolism [9]. Analysis of the afsS promoter region revealed substantial overlap between the PhoP recognition sequence (Pho box) and the AfsR-binding site (Afs box) [40]. Competitive electrophoretic mobility shift assays (EMSA) further showed that PhoP can compete with AfsR for promoter occupancy, resulting in reduced afsS transcription under certain conditions [24]. These observations suggest that PhoP and AfsR participate in interconnected regulatory pathways through which phosphate availability can influence secondary metabolite production [41].

Interactions between AfsR–AfsS and developmental regulatory pathways have also been reported. Morphological differentiation in *Streptomyces*, including aerial hypha formation and sporulation, is closely associated with changes in secondary metabolism [10]. BldD, a developmental regulator whose activity is controlled by the second messenger c-di-GMP, has been shown to bind directly to the promoter region of afsR [42,43]. Genome-wide ChIP-seq analyses further identified afsR as part of the BldD regulon [44]. These findings support a regulatory connection between developmental processes and AfsR-dependent control of secondary metabolism, although the precise temporal relationships between these pathways remain to be fully clarified.

In addition to interactions with nutrient- and development-related regulators, evidence also points to links between the AfsR–AfsS system and the basal transcriptional machinery. Previous studies reported that AfsS can interact with the C-terminal domain (α -CTD) of the RNA polymerase (RNAP) α subunit [45]. Taken together, current evidence suggests that AfsR–AfsS participates in a broader regulatory network involving phosphate-responsive signaling, developmental regulation, and transcriptional control [2]. Rather than functioning as an isolated regulatory module, the system appears to integrate multiple physiological inputs that collectively influence secondary metabolite biosynthesis.

4. Synthetic Biology “Awakening” Strategies Based on the AfsR–AfsS System

4.1. Promoter Engineering and Expression System Reconstruction

Promoter engineering has been widely applied to modulate gene expression and enhance the production of secondary metabolites in *Streptomyces*. Because the native promoters controlling afsR and afsS are often subject to complex regulatory constraints, promoter replacement or promoter optimization has been explored as a means of altering the expression levels of these global regulators and influencing downstream biosynthetic pathways.

The use of constitutive promoters represents one of the most common approaches for activating silent biosynthetic gene clusters (BGCs). In many studies, heterologous or synthetic promoters have been employed to drive the expression of *afsR*, *afsS*, or other regulatory genes associated with secondary metabolism. Among these, *ermEp** has been extensively used because of its robust transcriptional activity across multiple *Streptomyces* species [46]. In *Streptomyces albus*, for example, expression driven by *ermEp** was associated with the activation of two previously cryptic polyketide biosynthetic gene clusters [49]. Another widely used promoter, *kasOp*, was developed through promoter optimization and has been reported to exhibit stronger transcriptional activity than *ermEp* under certain conditions [47]. Expression of the pathway-specific regulator *actII-ORF4* under the control of *kasOp** resulted in a 2.5–3-fold increase in actinorhodin production in *S. coelicolor* [48]. More recently, promoter libraries containing variants with different strengths have been constructed to facilitate the fine-tuning of gene expression and to reduce the risk of metabolic imbalance associated with excessive regulatory activity [50].

Inducible expression systems provide an alternative strategy for regulating secondary metabolism. Constitutive overexpression of global regulators may impose a metabolic burden on the host cell, potentially affecting growth and precursor availability [2]. Consequently, inducible systems have been investigated as a means of separating biomass accumulation from secondary metabolite production. Chemically inducible promoters, including the thiostrepton-responsive *tipA* system [51,52] and tetracycline-regulated Tet-ON/Tet-OFF systems [53], have been incorporated into a variety of *Streptomyces* expression platforms. However, the requirement for chemical inducers may introduce additional costs and practical limitations, particularly in large-scale fermentation processes. Recent advances in optogenetics have expanded the range of available regulatory tools. Light-responsive systems based on the LitR/LitS photoreceptor module have been developed to control gene expression in response to blue–green light. These systems have been applied to the regulation of global regulators, including AfsR, and have enabled external control of secondary metabolite production without the addition of chemical inducers. Such approaches provide an additional framework for investigating dynamic control of secondary metabolism and may offer new opportunities for the engineering of complex biosynthetic pathways [54].

4.2. Regulatory Factor Cascades and Chassis Microenvironment Optimization

Because the expression of silent biosynthetic gene clusters (BGCs) is often controlled by multiple regulatory layers, modification of a single promoter is not always sufficient to achieve sustained activation. As a result, recent studies have increasingly combined transcriptional regulation with chassis engineering and metabolic network optimization to improve the expression of target biosynthetic pathways.

One approach involves the construction of regulatory cascades that couple global and pathway-specific regulators. In this design, the AfsR–AfsS system is combined with pathway-specific activators, including members of the SARP family, to strengthen transcriptional output from target gene clusters. For example, co-expression of *actII-ORF4* together with *afsR*–*afsS* has been reported to enhance expression of the actinorhodin biosynthetic pathway beyond that achieved by either regulatory component alone [13]. These observations suggest that interactions

between global and pathway-specific regulators may provide an effective means of increasing BGC expression.

Optimization of the host strain represents another important component of secondary metabolite engineering. In addition to transcriptional activation, removal of competing pathways can alter the distribution of intracellular resources and improve precursor availability for the target biosynthetic process. The development of CRISPR-Cas-based genome editing technologies has greatly expanded the ability to modify *Streptomyces* chassis strains in a targeted manner. For example, CRISPR-Cas9 and CRISPR-Cas12a systems have been used to delete endogenous biosynthetic gene clusters in *S. avermitilis*, thereby redirecting metabolic flux toward heterologous or otherwise prioritized pathways [55,56].

The concept of streamlined chassis strains has also received considerable attention. Representative examples include *Streptomyces coelicolor* M1146 and members of the *S. avermitilis* SUKA series. In these strains, multiple endogenous secondary metabolite gene clusters, including those responsible for actinorhodin, undecylprodigiosin, and calcium-dependent antibiotic (CDA) production, have been removed through large-scale genome engineering [14,57]. Such modifications reduce background metabolite production and simplify downstream analysis of heterologously expressed pathways. One study combined a streamlined SUKA chassis with AfsR–AfsS-mediated regulation and heterologous expression approaches, leading to the activation of three previously silent BGCs derived from different *Streptomyces* species [58]. These findings illustrate the potential benefits of combining regulatory engineering with chassis optimization.

Enhancing precursor supply represents an additional strategy for improving secondary metabolite production. Activation of transcription alone may not be sufficient when biosynthetic pathways are constrained by precursor availability. Studies have shown that co-expression of afsR–afsS together with genes involved in malonyl-CoA generation, including malonyl-CoA carboxylase, can increase the availability of key precursors required for polyketide biosynthesis and further improve product yields [13,59].

Recent work has increasingly emphasized the integration of these different engineering approaches into coordinated optimization strategies [60]. In general, such strategies combine transcriptional activation, genome editing, and metabolic pathway engineering. A typical workflow may involve enhancing expression of afsR and afsS, removing competing biosynthetic pathways through CRISPR-mediated genome editing, and subsequently identifying metabolic bottlenecks through metabolomic analyses for further engineering intervention. Rather than relying on a single modification, these approaches seek to coordinate regulatory and metabolic processes at multiple levels, providing a framework for the activation and expression of otherwise silent BGCs.

5. Multi-Omics Analysis of the Regulatory Network and Effect Evaluation

5.1. Transcriptomic Analysis of Global Gene Expression Profile Remodeling

The activation of secondary metabolism in *Streptomyces* is closely associated with growth phase and physiological status. Time-series transcriptomic analyses have therefore become an important approach for examining changes in gene expression

during different stages of development and secondary metabolite production. Genome-wide expression profiling has indicated that AfsS activity is particularly relevant during the transition to and maintenance of the stationary phase. Deletion of *afsS* was reported to affect the expression of more than 117 genes and to abolish actinomycin production, suggesting a role for AfsS in coordinating secondary metabolism under specific physiological conditions [22]. Additional studies combining RT-qPCR and ChIP-qPCR analyses at multiple fermentation stages have provided further insight into AfsR–AfsS-dependent regulation. In *Streptomyces avermitilis*, the AfsR–AfsS system was associated with reduced transcription of genes within the avermectin biosynthetic cluster during intermediate stages of fermentation. Deletion of either *afsR* or *afsS* relieved this regulatory effect and was accompanied by sustained expression of both structural genes and pathway-specific regulatory genes involved in avermectin biosynthesis during the exponential and stationary phases. These transcriptional changes correlated with increases in avermectin production ranging from 4.2- to 4.8-fold [2].

The combined use of RNA-seq and ChIP-seq has further expanded the ability to investigate regulatory networks at the genome-wide level. By integrating transcriptional profiles with transcription factor binding data, these approaches can facilitate the identification of regulatory targets, operon structures, and potential interactions between global regulators and silent biosynthetic gene clusters (BGCs) [61]. Such analyses have contributed to a broader understanding of the regulatory architecture underlying secondary metabolism. At the same time, transcriptomic studies have highlighted potential limitations associated with the overexpression of global regulators. Elevated AfsR–AfsS activity has been linked to changes in the expression of developmental regulators, including members of the *bld* and *whi* gene families, as well as genes involved in central carbon metabolism such as glycolysis and the tricarboxylic acid (TCA) cycle. These transcriptional changes have been associated with altered developmental phenotypes, including delayed aerial hypha formation and impaired sporulation [62]. Consequently, transcriptomic analyses not only provide insight into the activation of silent BGCs but also offer a means of evaluating unintended regulatory effects and guiding the optimization of engineering strategies [63,64].

5.2. Metabolomics Reveals Metabolic Flux and Precursor Supply

Metabolomic approaches have become increasingly important for evaluating the consequences of silent biosynthetic gene cluster (BGC) activation in *Streptomyces*. Non-targeted metabolomics based on LC-MS/MS, particularly when combined with Global Natural Products Social Molecular Networking (GNPS), enables the comparison and classification of large numbers of metabolic features and facilitates the detection of metabolites associated with newly activated pathways. Such approaches provide a complementary perspective to transcriptomic analyses by linking changes in gene expression to metabolite production.

One example was reported by Yanagisawa and colleagues, who employed a co-culture strategy to induce the expression of silent biosynthetic pathways in *Streptomyces*. Analysis of LC-MS/MS data using GNPS led to the identification of a previously uncharacterized pyranonaphthoquinone, actinoquinonal A. Comparative metabolomic analyses of intracellular and extracellular samples further supported the association of this compound with the activated biosynthetic pathway [65]. In addition, targeted LC-MS/MS-based studies of protein modifications suggested that the

upstream kinase AfsK participates in global regulation through phosphorylation of DivIVA, providing further insight into regulatory processes associated with AfsR–AfsS-dependent metabolic remodeling [66].

Comparative metabolomic investigations have also highlighted the diversity of metabolites associated with Afs-mediated regulation. In studies of clavulanic acid-producing strains, GNPS-based molecular networking revealed numerous unannotated metabolic features, suggesting that many biosynthetic products remain uncharacterized [67]. Similarly, the combination of the OSMAC (One Strain Many Compounds) strategy with non-targeted metabolomics has contributed to the discovery of additional secondary metabolites with potential agricultural applications [69].

Targeted metabolomics provides complementary information by quantifying intracellular metabolites and metabolic intermediates. Such analyses have been used to investigate cases in which transcriptional activation does not translate into proportional increases in product formation, a phenomenon frequently observed during BGC activation [68,70]. Furthermore, combining ^{13}C -MFA isotope tracing technology to track carbon metabolic flux allows precise localization of metabolic competition branches and rate-limiting enzyme deficiencies [71]. Taken together, transcriptomic and metabolomic datasets provide complementary information on the relationship between gene expression, metabolic flux, and secondary metabolite production. The integration of these datasets has become an important approach for evaluating the functional consequences of silent BGC activation and for guiding subsequent strain engineering and pathway optimization efforts [72].

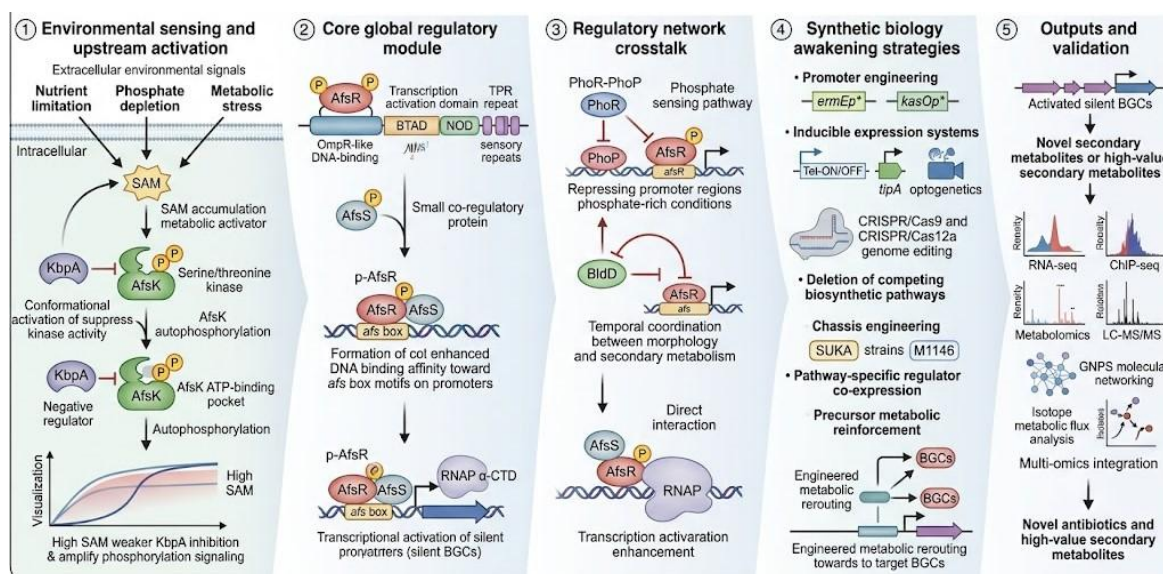


Figure 2. Global Regulatory Network of the AfsR–AfsS System in Awakening Silent Biosynthetic Gene Clusters.

6. Challenges and Future Prospects

Although manipulation of the AfsR–AfsS system has been applied in the activation of silent biosynthetic gene clusters, several limitations remain. In particular, elevated expression of global regulators may affect cellular fitness, while their broad regulatory scope can lead to unintended changes in metabolic flux and by-product formation. These issues continue to complicate the practical application of AfsR–

AfsS-based engineering strategies. Further progress will likely depend on a better understanding of the regulatory mechanisms underlying AfsR–AfsS activity. Approaches such as proximity-labeling and cross-linking mass spectrometry may help identify additional interaction partners involved in transcriptional regulation. At the same time, the growing availability of genomic and multi-omics datasets may facilitate the discovery of alternative AfsR/AfsS homologs and regulatory elements with distinct functional properties. Dynamic control systems, including optogenetic tools, may also provide greater flexibility for coordinating regulator expression with cellular physiological status. Together, these developments could contribute to more controlled activation of silent gene clusters while reducing unwanted effects on host metabolism.

7. Conclusions

The AfsR–AfsS system represents one of the major regulatory pathways associated with secondary metabolism in *Streptomyces*. Studies conducted in different species have shown that its regulatory effects vary according to genetic background and interactions with other regulatory networks. Current evidence indicates that AfsR–AfsS participates in the integration of environmental and intracellular signals and influences the expression of biosynthetic pathways through multiple regulatory mechanisms. Recent advances in promoter engineering, chassis development, genome editing, and multi-omics analyses have expanded the range of approaches available for investigating and manipulating AfsR–AfsS-dependent regulation. At the same time, transcriptomic and metabolomic studies have highlighted both the opportunities and the limitations associated with global regulatory engineering. Continued efforts to clarify the molecular basis of AfsR–AfsS function are likely to support future exploration of silent biosynthetic gene clusters and the discovery of previously inaccessible natural products.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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