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REVIEW PAPER

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AfsR as an integrator of signals that are sensed by multiple serine/threonine kinases in *Streptomyces coelicolor* A3(2)

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Abstract The genome sequence of *Streptomyces coelicolor* A3(2) has revealed the presence of about 40 protein serine/threonine or tyrosine kinases. AfsK, which is able to phosphorylate AfsR, a transcriptional activator with ATPase activity, represents the first instance in which a bacterial Hanks-type protein kinase phosphorylates a specific protein and exerts biologically important functions. The AfsK-AfsR system in *S. coelicolor* A3(2) globally controls secondary metabolism. The signal transduction pathway so far demonstrated or suggested is as follows: AfsK loosely attached to the membrane autophosphorylates threonine and serine residues, perhaps on sensing some external stimulus, and enhances its kinase activity. The kinase activity is modulated by KbpA, an AfsK-binding protein, by means of protein-protein interactions. The activated AfsK phosphorylates threonine and serine residues of AfsR in the cytoplasm, by which the DNA-binding activity of AfsR is greatly enhanced. In addition to AfsK, other kinases—including PkaG and AfsL—also phosphorylate AfsR, suggesting that AfsR serves as an integrator of multiple signals sensed by these kinases. The phosphorylated AfsR binds the promoter of *afsS*, which encodes a protein of 63 amino acids, and forms a closed complex with RNA polymerase. The closed complex is then converted to a transcriptionally active open complex by the energy available from ATP hydrolysis by AfsR. AfsS induced in this way activates transcription of pathway-specific transcriptional activators, such as *act*II-ORF4 for actinorhodin production and *redD* for undecylprodigiosin, in an as yet unknown manner.

Keywords Signal transduction · Serine/threonine kinase · Transcriptional factor · Secondary metabolism · *Streptomyces*

Dedicated to Professor Sir David A. Hopwood

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Introduction

Protein phosphorylation-dephosphorylation is a key element in cellular mechanisms for the detection, transmission, and integration of intra- and extra-cellular signals in both eukaryotes and prokaryotes. In bacteria, eukaryotic-type protein serine/threonine kinases, protein serine/threonine phosphatases, protein tyrosine phosphatases, and dual-specific protein phosphatases have been found [for reviews, see 21, 30, 37, 42], in addition to the two-component regulatory systems that target aspartate residues rather than the hydroxyl amino acid residues for modification, and to the phosphoenolpyruvate-dependent phosphotransferase systems [6]. Shi et al. [30] assume that all prokaryotic organisms at one time contained the genetic information necessary to construct protein phosphorylation-dephosphorylation networks that target serine, threonine, and/or tyrosine residues on proteins.

In the filamentous, soil-inhabiting, Gram-positive bacterial genus *Streptomyces*, a protein serine/threonine kinase AfsK that phosphorylates serine and threonine residues of AfsR—a regulatory protein involved in secondary metabolism in *Streptomyces coelicolor* A3(2) [24] and in morphological differentiation in *Streptomyces griseus* [36]—was first discovered, representing the first instance in the bacterial world in which the ability of a bacterial Hanks-type protein kinase to phosphorylate an exogenous protein has been demonstrated. Recently, the genome project of *S. coelicolor* A3(2) has predicted the presence of more than 40 such kinases [3]. Although little is known about eukaryotic-type protein phosphorylation-dephosphorylation in *Streptomyces*, it is conceivable that multicellular *Streptomyces* species showing complex morphological and physiological

differentiation have retained protein kinases and phosphatases that target hydroxyl amino acid residues for signal transduction to ensure healthy growth and survival in competition with other microorganisms in the soil and wider ecosystem.

Since we discovered the AfsK/AfsR regulatory system in *S. coelicolor* A3(2), we have studied the mechanism by which a phosphorylated form of AfsR enhances secondary metabolism in this strain and modulates aerial mycelium formation in response to glucose in *S. griseus* [36]. We have recently revealed that AfsR serves as a transcriptional activator for *afsS*, which encodes a small protein of 63 amino acids, and that phosphorylation of AfsR greatly enhances its DNA-binding activity. In eukaryotes, protein phosphorylation is frequently employed by cells to modulate the activities of transcriptional factors [40]. In this review, I summarize 20 years of work in this laboratory on signal transduction via AfsK and AfsR.

AfsR awakens "sleeping" antibiotic production genes in *Streptomyces lividans*

Historically, we discovered *afsR* earlier than *afsK*. During our shotgun cloning of DNA fragments from *S. coelicolor* A3(2) into *S. lividans*, we found a transformant that produced the blue pigment actinorhodin in a very large amount (Fig. 1) [16]. The transformant also overproduced the red pigment undecylprodigiosin [15]. These pigments are not usually produced on agar medium by *S. lividans*, and the cloned gene awakened "sleeping" multiple antibiotic biosynthetic genes in this strain. For overproduction of actinorhodin, this gene stimulated the transcription of almost all the *act* genes, including the pathway-specific activator *act*II-ORF4 [17]. The gene thus identified was mistakenly named *afsB* (A-factor biosynthesis), because it phenotypically complemented the *afsB* mutation of *S. coelicolor* A3(2) [16]. *afsB* of *S. coelicolor* A3(2) was thought to be a regulatory gene for antibiotic production because *afsB* mutants failed to produce these metabolites [10]. Later, Stein and Cohen [31] showed that this gene has a bypass function with regard to *afsB* complementation and named it *afsR*. Disruption of *afsR* on the *S. coelicolor* A3(2) chromosome results in significant, but not complete, loss of production of actinorhodin, undecylprodigiosin and calcium-dependent antibiotic. *afsR* is therefore called a global regulatory gene for secondary metabolite production. *afsR*-disruptants normally develop spores on any medium.

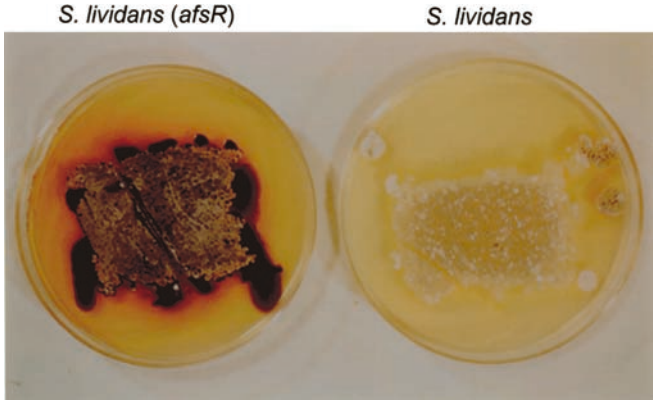

Fig. 1 Overproduction of pigments by *Streptomyces lividans* carrying *afsR* on a high-copy-number plasmid. *S. lividans* does not usually produce pigments on agar medium. Both strains were grown at 28°C for 7 days on Bennett medium [15]

afsR encodes a protein of 993 amino acids with a molecular mass of 106 kDa [18]. The NH_2-terminal portion of AfsR shows similarity to the pathway-specific transcriptional regulators of *Streptomyces*, also known SARP-family proteins (streptomycete antibiotic regulatory proteins), such as ActII-ORF4 for actinorhodin production [7] and RedD for undecylprodigiosin [26], neither of which contain the typical helix-turn-helix motifs found in many bacterial DNA-binding proteins. SARPs contain an OmpR-like DNA-binding motif at their NH_2-terminal ends and induce transcription of their respective gene clusters through DNA-binding to specific nucleotide sequences [41]. Despite partial similarity of AfsR to SARPs, AfsR could not substitute for ActII-ORF4 or RedD, but might function independently of these regulatory proteins to influence antibiotic production [8]. AfsR was therefore thought to bind specific DNA sequences and activate their transcription, as a result of which production of secondary metabolites in *S. coelicolor* A3(2) was enhanced. As described below, the true target(s) of AfsR as a DNA-binding protein is *afsS*.

A particular difference between AfsR and other SARPs is that AfsR contains A- and B-type ATP-binding consensus sequences in the middle of the protein. The importance of the ATP-binding sequences was confirmed by the finding that site-directed mutants at these sequences no longer enhanced antibiotic production in *S. lividans* [24]. A recent study demonstrated that the ATP-binding domain of AfsR serves as a core for its ATPase activity and is essential for transcriptional activation of *afsS* (see below).

Multiple kinases, including AfsK, that phosphorylate AfsR

We found that purified AfsR was phosphorylated when incubated with [γ-^{32}P]ATP in a cell-lysate of *S. coelicolor* A3(2) [13]. This phosphorylation was inhibited by K-252a and staurosporine [12, 14], known to be eukaryotic protein kinase inhibitors, which suggested that the phosphorylated amino acid residues might be serine/threonine or tyrosine. Later, phosphoamino acid analysis revealed that AfsR was phosphorylated on serine and threonine residues by AfsK (see below). While purifying a possible kinase able to phosphorylate AfsR from *S. coelicolor* A3(2), we identified a kinase activity that was loosely attached to the membrane.

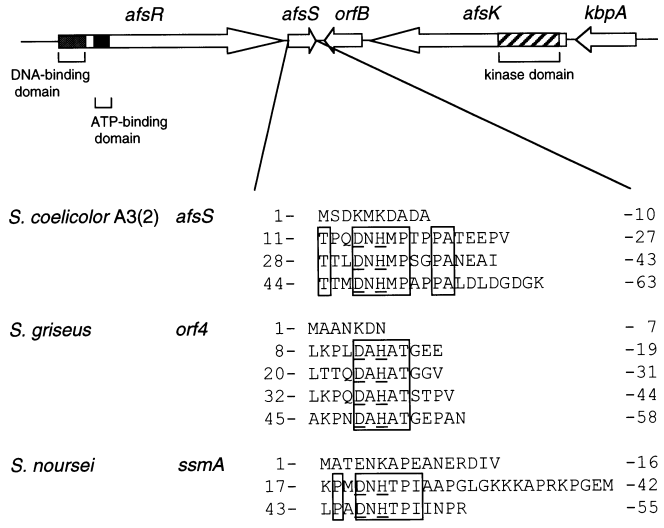

Fig. 2 Organization of *Streptomyces coelicolor* A3(2) *afs* genes and repeated sequences in AfsS homologues. AfsR contains a DNA-binding domain at its NH_2-terminal part and an ATP-binding domain in the middle. AfsK contains the kinase catalytic domain at its NH_2-terminal part. The repeated amino acid sequences in AfsS, Orf4 in *Streptomyces griseus*, and SsmA in *Streptomyces noursei* are shown

During this work, we noticed the presence of a gene just downstream of *afsR* (Fig. 2) that encodes a 799-amino-acid protein containing a kinase catalytic domain very similar to eukaryotic protein kinases. We then set out to produce the protein, named AfsK, in *Escherichia coli* and phosphorylate AfsR by the purified AfsK, on the assumption that functionally related genes are usually organized as an operon in bacteria. As expected, AfsK phosphorylated serine and threonine residues of AfsR [24]. In addition, the AfsK protein produced in *E. coli*, which appeared as a smeared band on SDS-polyacrylamide gel electrophoresis, was found to autophosphorylate on serine and threonine residues. Thus, AfsK and AfsR became the first example of eukaryotic-type protein phosphorylation in the bacterial world.

afsK-disruptants produced smaller amounts of actinorhodin and undecylprodigiosin, as was observed for *afsR*-disruptants. The degree of reduction in the *afsR*-disruptants was much greater than the *afsK*-disruptants. This is probably because AfsR is phosphorylated not only by AfsK but also by other multiple kinases. A cell-lysate prepared from the *afsK* null mutants still possessed the ability to phosphorylate AfsR, indicating that *S. coelicolor* A3(2) contains an additional kinase(s) able to phosphorylate AfsR [24].

Recent accomplishment of the genome sequence of this strain [3] has revealed the presence of more than 40 proteins that show significant similarity to the kinase catalytic domain of AfsK. We chose two such proteins and determined their potential ability to phosphorylate AfsR. PkaG (592 amino acids) and SCD10.09 (named AfsL; 580 amino acids), both produced in *E. coli*, were found to autophosphorylate threonine and serine residues and to phosphorylate threonine and serine residues of AfsR (our unpublished data). Furthermore, *pkaG*-disruptants produced less actinorhodin than the parental strain, suggesting the involvement of *pkaG* in secondary metabolism.

KbpA, an AfsK-binding protein that modulates the degree of AfsK autophosphorylation

Functionally related genes are often organized as an operon in bacteria. We focused on an open reading frame named *kbpA* that is located just upstream of *afsK* (Fig. 2). Overexpression of *kbpA* reduced actinorhodin production, and *kbpA*-disruption resulted in overproduction of actinorhodin, showing that *kbpA* serves as a repressor for actinorhodin biosynthesis [35].

Consistent with the in vivo observation that *kbpA* serves as a repressor for actinorhodin production, in vitro experiments showed that KbpA binds the catalytic domain of the unphosphorylated form of AfsK but does not bind the autophosphorylated form [35]. The binding of KbpA to AfsK inhibited autophosphorylation of AfsK. *kbpA* is transcribed throughout growth, and its transcription is enhanced when actinorhodin and undecylprodigiosin production has already begun. We therefore assume that KbpA puts a brake on the unlimited production of the pigments under the control of the AfsK-AfsR system, thus playing a role in a negative-feedback system.

afsS, a target of the transcriptional activator AfsR

We next focused on the two genes located between *afsK* and *afsR* (Fig. 2). Disruption or overexpression of *orfB* caused no detectable effects on actinorhodin or undecylprodigiosin production. However, we found that overexpression of the other gene, *afsS*, led to overproduction of actinorhodin and undecylprodigiosin [25]. Vögtli et al. [38] also reported that *afsR2*, the *afsS* counterpart in *S. lividans*, showed the same phenotype. Furthermore, transcription of *afsS* was found to depend on *afsR*; no transcription of *afsS* was detected in *afsR*-disruptants [22]. Consistent with the dependence of *afsS* transcription on *afsR*, AfsR bound the promoter region of *afsS*, as determined by gel mobility shift assays. DNase I footprinting showed that AfsR covered a sequence on the sense strand from positions −15 to −40 with respect to the transcriptional start point of *afsS*, and a sequence from −20 to −42 on the antisense strand. The −35 and −10 promoter sequences are in many cases the sites to which repressors bind. However, several transcriptional activators bind the promoter of target genes; MerR (mediating mercury resistance [2]) and SoxR (mediating a global response to superoxide-generating agents [11]) bind the promoter of their target genes and are believed to optimize the spacing between the −35 and −10 elements by introducing a bend and untwisting the DNA. The spacing of the −35 and −10 elements of *afsS* is

20 bp, which is larger than those (17–18 bp) of standard *Streptomyces* promoters [32]. We thus speculate that AfsR activates transcription of *afsS* by inducing a DNA bend to optimize the spacing for RNA polymerase to recognize and bind the promoter elements.

What is the role of the phosphorylation of AfsR in transcriptional activation of *afsS*? We found that phosphorylation of AfsR by AfsK greatly enhanced its ability to bind the *afsS* promoter [22]. The ATPase activity of AfsR did not affect its DNA-binding ability, although it is essential for transcriptional activation (see below). We therefore speculate that AfsR is modular in that it is composed of physically separable DNA-binding and ATPase domains that can function independently of one another, as found in many eukaryotic enhancer-binding proteins and some prokaryotic transcriptional factors, including NtrC for nitrogen regulation and NifA for nitrogen fixation [9, 27]. NtrC, a response regulator of a two-component regulatory system, acquires DNA-binding activity on acceptance of a phosphate at Asp-54 from its sensor kinase NtrB. Although the enhancement of DNA-binding activity of AfsR by phosphorylation is analogous to that of NtrC, the phosphorylation of AfsR is of the eukaryotic type, whereas that of NtrC is a typical prokaryotic type.

Site-directed mutants at the ATP-binding motifs of AfsR did not cause *S. lividans* to overproduce actinorhodin, indicating the importance of the ATP-binding motifs [24]. We found that AfsR can hydrolyze ATP and GTP but AfsR proteins with mutations at the ATP-binding motifs lack ATPase activity. The ATP-binding motifs of AfsR therefore serve as a catalytic domain for ATPase activity. What is then the role of the ATPase activity of AfsR? As described above, *afsS* is not transcribed in *afsR*-deficient backgrounds. Introduction of *afsR* into the *afsR*-deficient mutants restores *afsS* transcription, but AfsR proteins lacking ATPase activity cannot. Since the AfsR mutant proteins still retain the ability to bind the *afsS* promoter, we assume that the ATPase activity of AfsR is essential for a step after AfsR has bound the *afsS* promoter, perhaps for forming an open complex ready for transcription initiation. As was found for NtrC [39], the energy available from ATP hydrolysis may be required for the isomerization of a closed complex consisting of RNA polymerase and AfsR into a transcriptionally competent open complex.

AfsS is a 63-amino-acid protein containing three repeats of the sequence Thr-X_2-Asp-Asn-His-Met-Pro-X_2-Pro-Ala (X: non-conserved amino acids) (Fig. 2). A similar repeat is conserved in *orf4* in *S. griseus* [36] and in *ssmA* in *S. noursei* [29], both of which are encoded just downstream of an *afsR* homologue in their respective strains. These observations suggest the importance of the repeat sequence and the close functional correlation between the *afsR* and *afsS* homologues. In fact, our preliminary experiments have shown that the Asp-X-His sequence in the repeated sequences within AfsS is essential for stimulation of antibiotic production. This sequence is conserved in all the repeated sequences in AfsS and in the two AfsS counterparts. Although introduction of multiple copies of *afsS* has been found to enhance production of actinorhodin and undecylprodigiosin by enhancing transcription of *act*II-ORF4 and *redD*, respectively, how AfsS stimulates transcription of these pathway-specific transcriptional regulator genes remains unknown.

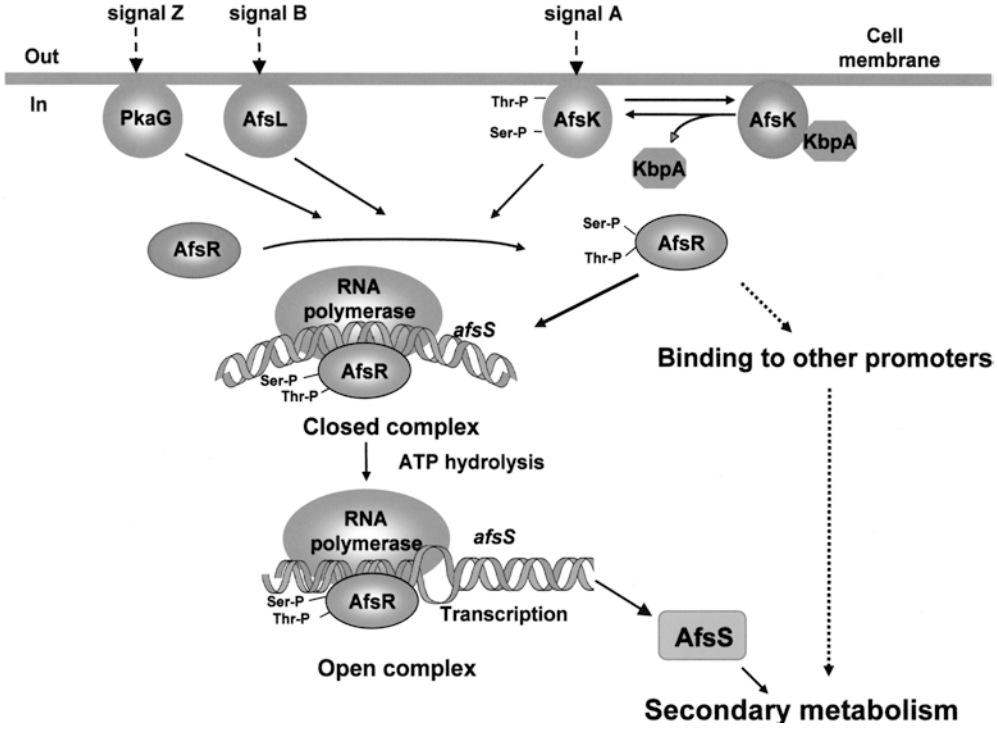

Fig. 3 Model of a signal transduction pathway involving AfsK, KbpA, PkaG, AfsL, AfsR, and AfsS. Reproduced in a modified form from Lee et al. [22]. See text for details

Signal transduction pathway involving AfsK and AfsR

Signal transduction pathways involving eukaryotic protein phosphorylation systems have been revealed (Fig. 3). By analogy with eukaryotic systems, we assume that AfsK attached to the inner side of the membrane autophosphorylates upon sensing some environmental condition and transmits the signal to AfsR in the cytoplasm by phosphorylating its threonine and serine residues. Under some conditions and at a later stage of growth, KbpA binds a newly translated, nonphosphorylated form of AfsK or dephosphorylated AfsK and inhibits the conversion from the inactive to active form of AfsK. Additional kinases, PkaG and AfsL, also sense some environmental condition, autophosphorylate, and transfer the signal to AfsR. Phosphorylation of AfsR enhances its DNA-binding activity and allows AfsR to bind the *afsS* promoter. The ATPase activity of AfsR supplies energy to convert an RNA polymerase-AfsR closed complex into an open complex for transcriptional initiation. AfsS induced in this way activates *act*II-ORF4 and *redD* in an as yet unknown manner and induces production of the pigments actinorhodin and undecylprodigiosin. Because *afsR* enhances the yields of these pigments in the absence of *afsS*, AfsR appears to activate not only *afsS* but also some other genes.

Secondary metabolism in *S. coelicolor* A3(2) is influenced by apparently independent regulatory systems, such as *bld* genes [5], ppGpp [4, 23], two-component regulatory systems [1, 20], γ-butyrolactone-type regulators [28, 34], cAMP [19, 33], and the AfsK-AfsR system. These systems supposedly sense nutritional conditions such as carbon, nitrogen, and phosphate, and environmental conditions such as temperature, osmolality, and a variety of stresses. Some of the stimuli sensed by these systems are transferred to pathway-specific activators, such as *act*II-ORF4 and *redD*, via the respective signal transduction pathways and lead to antibiotic production. Other stimuli are transferred to the many sets of genes required for healthy growth in response to such stimuli and morphological differentiation in the soil environment of the ecosystem.

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