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The A-factor regulatory cascade and cAMP in the regulation of physiological and morphological development in *Streptomyces griseus*[†]

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In the A-factor regulatory cascade leading to the onset of streptomycin biosynthesis and aerial mycelium formation in *Streptomyces griseus*, the A-factor receptor protein (ArpA) serves as a DNA-binding repressor and A-factor releases the repression by binding to ArpA and dissociating it from the DNA. Mutants defective in *arpA* therefore produce streptomycin and aerial hyphae in the absence of A-factor. A gene that inhibits streptomycin production and aerial hyphae formation in an *arpA* mutant was cloned on a high-copy-number plasmid and found to encode a eukaryotic-type adenylate cyclase (CyaA). Consistent with this, an exogenous supply of cAMP at high concentration almost abolished streptomycin production and aerial hyphae formation. On the other hand, cAMP at lower concentrations stimulated or accelerated these developmental processes. The effects of cAMP were detectable only in *arpA* mutants, and not in the wild-type strain; an exogenous supply of cAMP or *cyaA* disruption in the wild-type strain caused almost no effect on these phenotypes. Thus the effects of cAMP became apparent only in the *arpA*-defective background. cAMP at high concentrations inhibited stringent response factor ppGpp production, which is important for the onset of antibiotic biosynthesis. cAMP also influenced the timing of tyrosine phosphorylation of more than nine proteins. These findings show that a cAMP regulatory relay for physiological and morphological development functions in a concerted and interdependent way with other signal transduction pathways. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 177–182.

Keywords: A-factor receptor protein; signal transduction; streptomycin biosynthesis; aerial hyphae formation; stringent response factor ppGpp; protein tyrosine phosphorylation

Introduction

The Gram-positive bacterial genus *Streptomyces* is characterized by its ability to produce a wide variety of secondary metabolites including antibiotics and biologically active substances and by its complex morphological differentiation culminating in the formation of chains of spores. Secondary metabolite formation is sometimes termed "physiological" differentiation because it occurs during the idiophase after the main period of rapid vegetative growth and assimilative metabolism. In *Streptomyces griseus*, an autoregulatory factor called A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) at a concentration as low as 10^{-9} M acts as a switch for physiological and morphological differentiation [10,12,13]. Accumulating evidence has shown that similar γ-butyrolactone-type autoregulators exert "hormonal" regulatory functions in these developmental processes in a variety of *Streptomyces* species [11]. It is also apparent that γ-butyrolactones act as ligands of their specific receptor proteins which, in the absence of the respective ligands, serve as DNA-binding repressors [16,23].

More than 20 years ago, Ragan and Vining [25] reported a possible, but indirect, relationship between cAMP and streptomycin biosynthesis by determining the timing of cAMP production in relation to that of streptomycin in *S. griseus*. Since then there have been no further studies, although the roles of cAMP as a second messenger in signal transduction in eukaryotes, as well as in catabolite repression in Gram-negative bacteria, have been elucidated. During our study on A-factor, we happened to find effects of cAMP on physiological and morphological differentiation. The effects of cAMP are detectable only in *arpA* mutants and not in the wild-type strain; we readily miss the effects of cAMP as long as we use the *S. griseus* strains containing intact *arpA*. We here show that cAMP exerts general pleiotropic effects on physiological and morphological development in *Streptomyces* species. Süsstrunk *et al.* [27] also reported pleiotropic effects of cAMP on germination, secondary metabolism and morphogenesis in *Streptomyces coelicolor* A3(2).

The A-factor regulatory cascade leading to onset of streptomycin biosynthesis

A-factor-deficient mutants of *S. griseus* are readily obtained by so-called plasmid-curing treatments, such as acridine orange treatment, incubation at high temperatures, and protoplast regen-

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eration. *afsA*, encoding a protein of 301 amino acids, was cloned as a gene that conferred streptomycin production and sporulation on an A-factor-deficient mutant [14]. *afsA* caused *Escherichia coli* to produce A-factor when introduced on a plasmid [2], suggesting that AfsA is a key enzyme for synthesis of A-factor, probably from a glycerol derivative and a β-keto acid derived from the fatty acid biosynthetic pathway. The presumptive β-keto acid may contain either acetyl-coenzyme A or acyl carrier protein at the end (Figure 1). In *S. griseus*, A-factor is produced in a growth-dependent manner until the end of exponential growth phase. A-factor at a critical concentration binds A-factor receptor protein (ArpA), which has repressed the *adpA* promoter by binding its -10 and -35 regions, dissociates it from the DNA, and allows RNA polymerase to transcribe *adpA*. The *adpA* promoter overlaps with the ArpA-binding consensus sequence, 5′-GG(T/C)CGGT(A/T)(T/C)G(T/G)-3′, as one half of the 22-bp palindromic sequence [22,23]. AdpA encodes a protein of 405 amino acids that contains an α-helix–turn–α-helix DNA-binding motif at the central portion and shows sequence similarity to members in the AraC/XylS family [21]. AdpA thus induced acts as a transcrip-

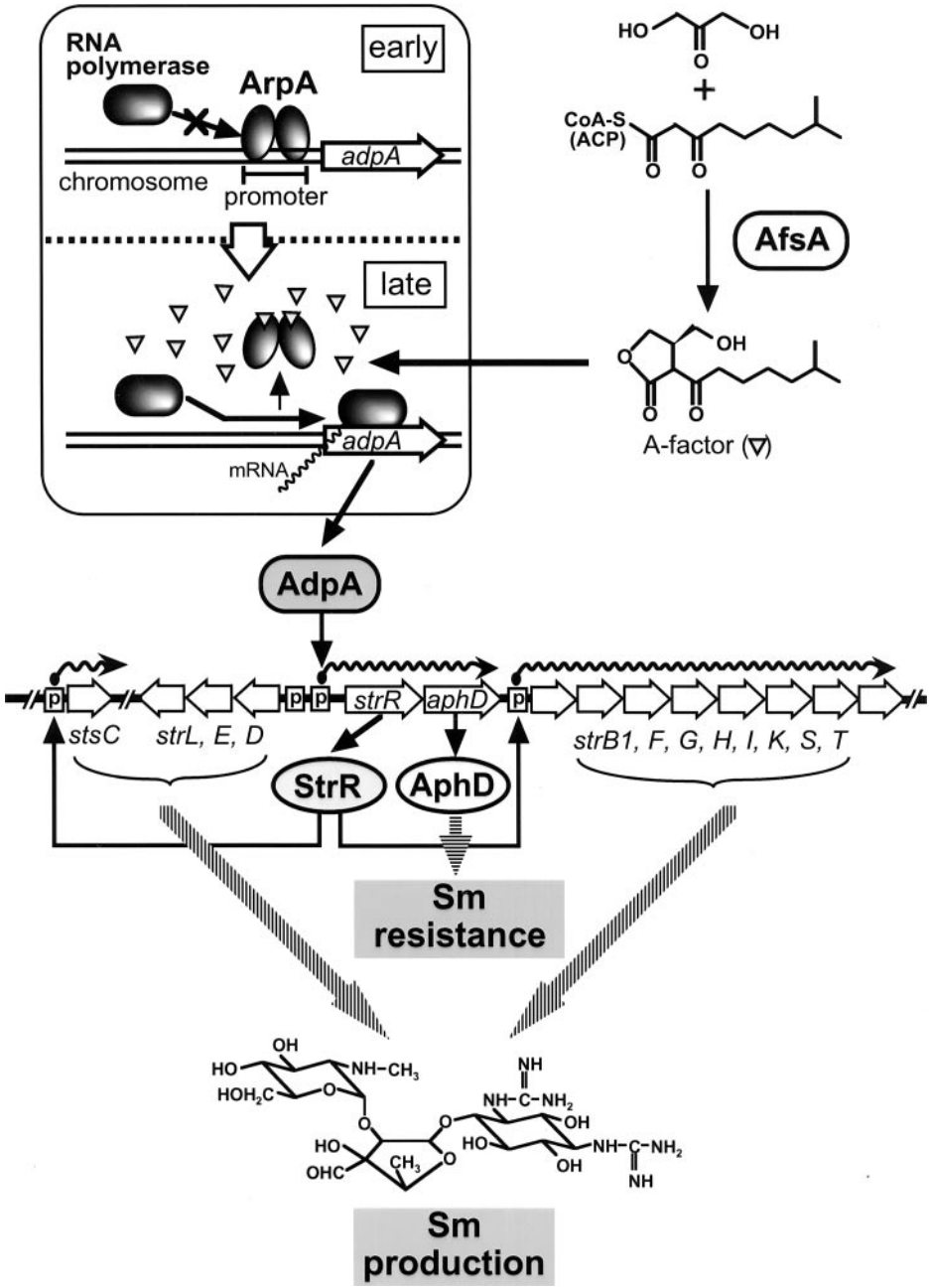

Figure 1 The A-factor regulatory cascade. A-factor is biosynthesized in a growth-dependent manner from a glycerol derivative (C3 unit) and a β-keto acid (C10 unit) by the action of AfsA. The A-factor signal is transmitted to ArpA to AdpA to StrR to streptomycin production genes. *aphD* encoding the major streptomycin resistance determinant, streptomycin-6-phosphotransferase, is cotranscribed with *strR*, which accounts for the dependence of streptomycin resistance on A-factor. Reproduced in a modified form from Ohnishi et al. [21] with permission of Blackwell Science Ltd.

tional activator for *strR*, encoding a pathway-specific regulator, by binding an upstream activation sequence, about 270-bp upstream from the transcriptional start point of *strR* [32]. The pathway-specific transcriptional activator StrR then induces transcription of other streptomycin production genes by binding multiple sites in the gene cluster [26], leading to biosynthesis of streptomycin from glucose. The timing of streptomycin production is therefore determined by the intracellular concentration of A-factor. We have also identified a gene encoding an extracytoplasmic function (ECF) sigma factor, σ^{AdsA}, as a target gene of AdpA for morphological development in the regulatory cascade [34].

The A-factor/ArpA system acts as a switch for both physiological and morphological differentiation in an all-or-none fashion in *S. griseus*, whereas the virginiae butanolide (VB)/BarA system controls the timing of virginiamycin production in *Streptomyces virginiae* [16]. We assume that during evolution *Streptomyces* species have developed γ-butyrolactones as "hormonal regulators" in conjunction with their specific receptors to control different stages of physiological and/or morphological differentiation in the regulatory hierarchy. A-factor may control an early developmental stage common to both secondary metabolite formation and morphogenesis in the hierarchy, and VB may control a downstream stage only for secondary metabolism. We speculate that the genes for VB-type regulators are in the gene cluster for the respective secondary metabolite so that production of VB and virginiamycin is synchronized. In fact, VB is produced immediately before virginiamycin biosynthesis [16]. It is also conceivable that most *Streptomyces* species contain redundant γ-butyrolactone regulatory systems, some of which control both physiological and morphological development either as an all-or-none switch or as a tuner and others of which control one of the two processes. *S. coelicolor* A3(2) contains six different γ-butyrolactones [4] and at least three *arpA*-like genes [24] (MJ Bibb, personal communication).

Identification of a eukaryotic-type adenylate cyclase gene in *S. griseus*

Because of the repressor function of ArpA, *arpA* mutants produce streptomycin and form spores in the absence of A-factor. We cloned a gene that suppresses streptomycin production and sporulation by an *arpA* mutant HO2 [15]. Subcloning and nucleotide sequencing identified the cloned gene as an adenylate cyclase (CyaA) that catalyzes cAMP formation from ATP. Culture broths prepared from mutant HO2 harboring *cyaA* on a high-copy-number plasmid accumulated up to 92 pmol cAMP/ml in a growth-dependent manner until mid-exponential phase. The cAMP then rapidly disappeared, probably due to cyclic phosphodiesterase activity. The peak of cAMP production coincided with the decision point serving as a checkpoint for commencement of physiological and morphological development [3,18]. Mutant HO2 harboring the vector pIJ486 produced only 36 pmol cAMP/ml in the same time frame. The profile of cAMP production is the same as that reported by Ragan and Vining [25]. These findings suggested that cAMP at high concentrations inhibited streptomycin production and sporulation. *cyaA* on the high-copy-number plasmid also inhibited these functions in other independently obtained *arpA* mutants. Because this CyaA and that from *S. coelicolor* A3(2) [6] show sequence similarity to CyaAs from *Saccharomyces cerevisiae* and *Shizosaccharomyces pombe*, they can be called eukaryotic-type CyaA. A computer-aided search showed that an actinobacterium, *Brevibacterium liquefaciens*, also contains a similar CyaA.

Effects of cAMP on sporulation and streptomycin production

When a paper disc containing 15 μmol cAMP was placed on medium seeded with *S. griseus* HO2, aerial mycelium formed in a donut-like area around the paper disc. Aerial mycelium formation was inhibited in the donut area, indicating that cAMP at high concentrations inhibited aerial mycelium development and at lower concentrations stimulated aerial mycelium formation. Consistent with this idea, adding 5 μmol cAMP on a paper disc resulted in rapid and abundant aerial mycelium formation around the disc. Thus, stimulation of morphological development by cAMP was concentration-dependent. The same phenotypic changes were observed on medium supplemented with 25 mM TES, which excluded the possibility that the effects of cAMP resulted from a decrease in pH. Effects of cAMP on streptomycin production were also observed; 2.5 mM cAMP in liquid culture inhibited streptomycin production completely, although 0.25 mM cAMP gave an almost undetectable effect.

On the other hand, an exogenous supply of cAMP to the wild-type *S. griseus* strain gave little change in the timing and abundance of aerial mycelium formation, or in the timing and yield of streptomycin production. We could notice a subtle change in aerial mycelium formation around the cAMP-containing paper discs only by careful examination. Consistent with this, disruption of the chromosomal *cyaA* gene caused little change. Nevertheless, these observations implied that cAMP does cause subtle effects on physiological and morphological development in the wild-type strain. Together with the positive effects of cAMP on germination, antibiotic production and morphogenesis in *S. coelicolor* A3(2) [27] and on tylosin production in *S. fradiae* [28], all of these findings suggest a role of cAMP in general in a wide variety of *Streptomyces* species.

Effects of cAMP on metabolic functions

ppGpp synthesis

Morphological and physiological differentiation is often a response to nutrient limitation. ppGpp is one of the compounds responsible for the stringent response that couples regulatory systems with nutrient limitation. An and Vining [1] reported its presence in *S. griseus* and Ochi [19,20] pointed out its importance in triggering streptomycin biosynthesis and aerial mycelium formation. The importance of ppGpp accumulation for initiating both physiological and morphological development was also recognized in *S. coelicolor* A3(2) [5]. *S. griseus* mutant HO2 began ppGpp synthesis with the onset of aerial mycelium formation and continued it until the onset of sporulation; this is the same time course and yield as in the wild-type strain. Mutant HO2 harboring *cyaA* on the high-copy-number plasmid (plasmid pHCYA1) accumulated a severely reduced amount of ppGpp. This could mean that the failure of mutant HO2 to produce streptomycin in the presence of pHCYA1 and probably in the presence of a large amount of exogenously supplied cAMP is caused by insufficient accumulation of ppGpp.

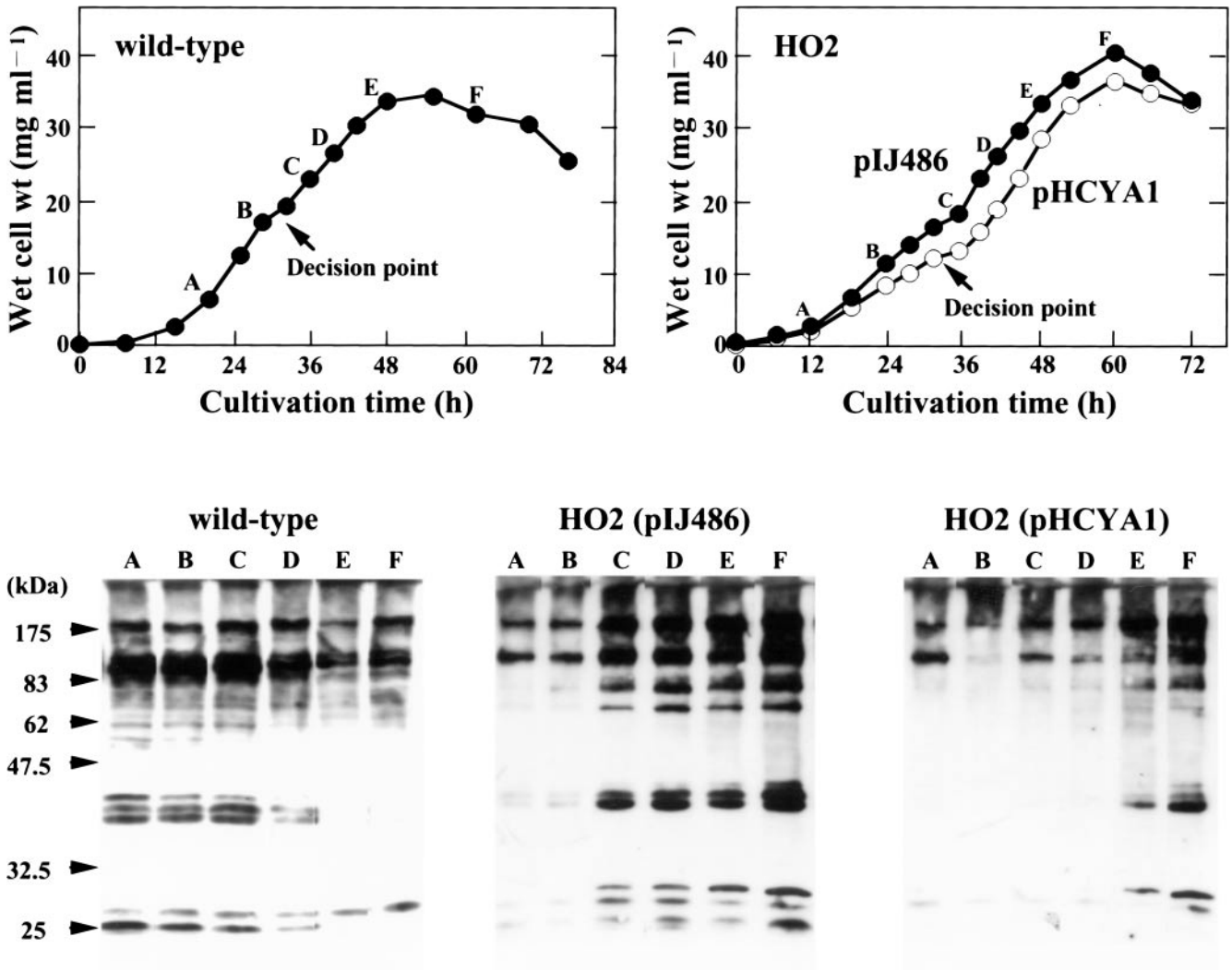

Figure 2 Changes in the profiles of proteins with phosphotyrosine residues in *S. griseus* wild-type strain, mutant HO2 harboring the vector plasmid pIJ486, and mutant HO2 harboring pHCYA1. Strains were grown in liquid medium and mycelium was taken at the indicated times (points A–F) to detect proteins with phosphotyrosine residues by Western blotting with anti-phosphotyrosine antibody. The decision point apparently corresponds to point C. Reproduced in a modified form from Kang et al. [15] with permission of the publisher.

Phosphotyrosine proteins

In *S. griseus*, the protein tyrosine phosphorylation pattern depended on the growth phase (Figure 2A), as was observed in other *Streptomyces* species [33]. Western blotting with anti-phosphotyrosine antibody detected more than nine proteins in the wild-type *S. griseus* strain, all of which were clearly detected before the decision point. Some of them rapidly disappeared after the point, indicating that phosphorylation of these proteins was strictly under decision-point control. We observed that phosphorylation patterns of the wild-type strain and mutant HO2 were totally different (Figure 2B). This implied that mutant HO2 differentiated physiologically and morphologically in an aberrant protein tyrosine phosphorylation background, although little is known about the relationship of proteins with phosphotyrosines to morphogenesis and secondary metabolism. We then examined protein tyrosine phosphorylation in mutant HO2 harboring pHCYA1 (Figure 2C). In this mutant in the presence of pHCYA1, phosphorylation of most proteins detected in the wild-type strain was repressed until the end of exponential growth. Because adding cAMP exogenously to mutant HO2 at a final concentration of 10 mM also prevented phosphorylation, repression of phosphorylation throughout the exponential growth phase can be attributed, directly or indirectly, to overproduction of cAMP. A protein tyrosine kinase in *Acinetobacter calcoaceticus* has been reported to be directly activated by cAMP [8], although modulating activities of cAMP are observed exclusively with protein serine/threonine kinases in eukaryotes.

Interdependent regulation of physiological and morphological development

In addition to the A-factor regulatory cascade and the presumptive cAMP regulatory cascade, *S. griseus* contains a regulatory system via eukaryotic-type protein phosphorylation. The representative of such phosphorylation is the AfsK/AfsR system [17,30] (Figure 3). AfsK containing a kinase catalytic

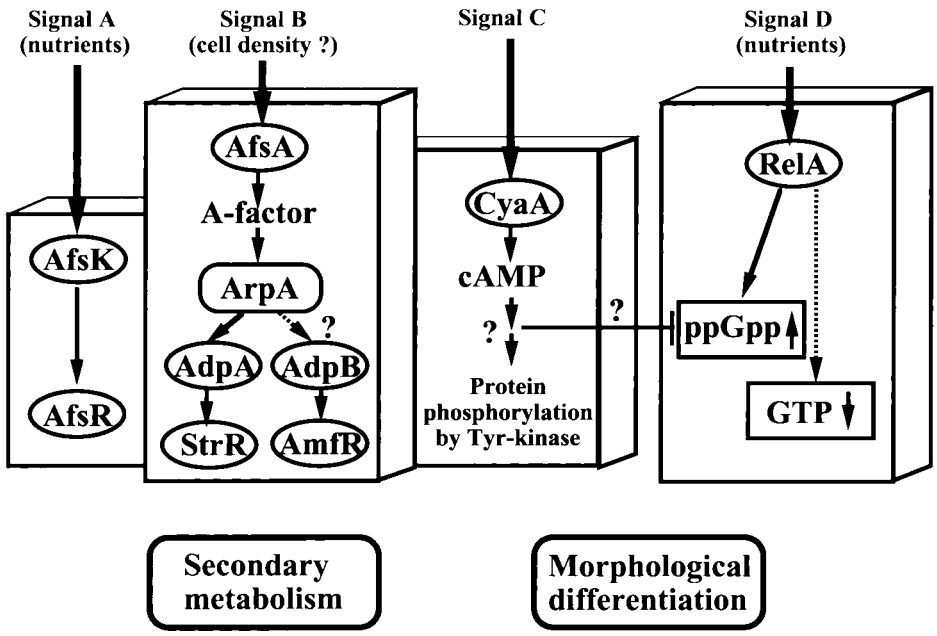

Figure 3 Interdependent regulation of secondary metabolism and morphological differentiation. The A-factor signal cascade is essential for both processes. AdpB and AmfR are members in the A-factor signal cascade leading to morphological differentiation [29]. The AfsK/AfsR protein phosphorylation and the cAMP signal cascade are nonessential but are important for the cells for healthy growth and development in response to various environmental conditions (signals A–D). A-factor production and RelA expression may also be responsive to certain environmental conditions.

domain similar to eukaryotic protein serine/threonine kinases autophosphorylates its serine and threonine residues and phosphorylates serine and threonine residues of AfsR. The phosphorylated form of AfsR activates still unknown genes that are required for the response of aerial mycelium formation to glucose. Disruption of either *afsK* or *afsR* results in the failure of aerial mycelium formation on glucose-containing medium. Thus the AfsK/AfsR system is essential for aerial mycelium formation conditionally, but not essentially. The same AfsK/AfsR system in *S. coelicolor* A3(2) is involved in secondary metabolism depending on nutritional conditions [7]. AfsK supposedly autophosphorylates upon sensing nutritional conditions such as the concentrations of glucose and nitrogen sources, and starts this signal cascade for modulation of morphological differentiation and/or secondary metabolism. Because *Streptomyces* contains multiple serine/threonine kinases [9,31], it is conceivable that multiple cascades like the AfsK/AfsR system function in response to a plethora of environmental cues. Accumulation of ppGpp and a corresponding decrease in the amount of GTP are also important for the development processes, as described above. RelA (Figure 3) catalyzing synthesis of ppGpp may also be activated, directly or indirectly, by an environmental change.

The A-factor cascade is essential for both morphological and physiological development. Once *S. griseus* has commenced development by A-factor, the signal cascades through cAMP, AfsK/AfsR, and ppGpp modulate or tune the A-factor cascade for the purpose of healthy development and growth depending on environmental conditions. Elucidation of these complex regulatory networks is challenging and important for practical purposes, such as yield enhancement of secondary metabolites and shortening of fermentation, in using *Streptomyces* species, which are sometimes called "boundary" microorganisms and "eukaryotic" prokaryotes.

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