**Streptomyces** Species as Boundary Microorganisms: Eucaryotic Regulatory Systems for Secondary Metabolism and Morphological Differentiation

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The genus *Streptomyces* is characterized by its ability to produce a wide variety of secondary metabolites and complex morphological differentiation resembling that of fungi. The regulation controlling these two characteristic aspects include "eucaryotic" systems, such as a hormonal control represented by the A-factor regulatory system and protein phosphorylation represented by the afsK/afsR system. A-factor is a chemical signaling molecule that triggers both secondary metabolite formation and aerial mycelium formation in *Streptomyces griseus*. Characterization of the A-factor-specific receptor gene suggests that the receptor acts as a repressor-type regulator for secondary metabolite formation and morphogenesis during the early stage of growth and A-factor at a certain critical intracellular concentration releases the derepression, thus leading to the onset of secondary metabolism and aerial mycelium formation. The A-factor signal is then transferred, via one or more steps, to a transcriptional activator for the strR gene, a transcriptional activator for the whole streptomycin biosynthetic genes. Involvement of protein serine/threonine kinases in signal transduction leading to antibiotic production and morphogenesis, either as a member in the A-factor regulatory cascade or as a regulator in the pathway independent of the cascade, has also been demonstrated by the studies with afsK/afsR of *Streptomyces coelicolor* A3(2) and their homologs of *S. griseus*.

The ability to produce a wide variety of secondary metabolites and a mycelial form of growth that develops into spores are two aspects characteristic of the Gram-positive bacterial genus *Streptomyces*. A-factor (2-isocaproyl-3R-hydroxymethyl-γ-butyrolactone) is a microbial hormone controlling secondary metabolism and cell differentiation in *S. griseus*. It acts as a switch for streptomycin production, streptomycin resistance, yellow pigment production, and aerial mycelium formation at a concentration as low as $10^{-9}$ M (Fig. 1). Although A-factor itself seems to exert its regulatory function in a limited group including *S. griseus* and *Streptomyces actinosporus*, the presence of γ-butyrolactones structurally similar to A-factor in a wide variety of *Streptomyces* spp. suggests that these compounds serve as hormonal regulators for secondary metabolism or morphogenesis, or both, in general in *Streptomyces*.

On the other hand, in eucaryotes, many protein kinases control cellular processes to respond to a plethora of environmental cues via signal transduction networks and response mechanisms. It is conceivable that protein kinases control morphological differentiation and secondary metabolism in soil-living *Streptomyces* spp. We have found a two-component regulatory system typical of procaryotes; the afsQ1/afsQ2 system controlling secondary metabolism in *S. coelicolor* A3(2) and the strR gene controlling aerial mycelium formation in *S. griseus*. In addition to these procaryotic systems, protein phosphorylation reactions in vitro with cell-extracts of *S. griseus* and *S. coelicolor* A3(2) in combination with K-252a and staurosporine, inhibitors of eucaryotic protein serine/threonine kinases, have suggested
Fig. 1. The A-factor regulatory cascade. The afsA gene probably encodes a key enzyme for the biosynthesis of A-factor\(^{23}\). The A-factor signal is transferred via the A-factor receptor protein to the Adp (A-factor-dependent protein) product that serves as a transcriptional activator for strR and aphD\(^{23}\). See Fig. 3 for details on a hypothetical model for the role of the A-factor receptor. The aphD gene encoding a streptomycin resistance determinant is transcribed mainly by the read-through from the A-factor-dependent strR promoter\(^{23}\), which ensures prompt induction of the resistance in response to A-factor. The A-factor signal is also transmitted to the yellow pigment production genes and the genes for aerial mycelium formation. The expression of amfR encoding a protein similar to response regulators of prokaryotic two-component regulatory systems is controlled by A-factor\(^{23}\). The kinase able to phosphorylate Asp-54 of AmfR has not yet been identified. A Ser/Thr kinase and its target protein similar to the S. coelicolor A3(2) afsK/afsR system probably control aerial mycelium formation, because afsK suppresses the aerial mycelium-negative phenotype of an A-factor-deficient mutant of S. griseus\(^{23}\).
the presence of multiple protein kinases sensitive to these inhibitors. More direct evidence for the presence of proteins with phosphotyrosines was reported by Waters et al. by using anti-phosphotyrosine antibody. Possible involvement of protein phosphorylation in the regulation of both morphological differentiation and antibiotic production in Streptomyces has been suggested by the observations that K-252a and staurosporine inhibit aerial mycelium formation and antibiotic production in S. griseus and S. coelicolor A3(2).

In this short review, we summarize the complex A-factor regulatory cascade with emphases on the characterization of the A-factor receptor protein and on the involvement of a protein serine/threonine kinase in the regulatory cascade. We also describe the afsK gene encoding a serine/threonine kinase that controls antibiotic production in S. coelicolor A3(2) and an afsK homolog controlling aerial mycelium formation under conditions of high osmolality in S. griseus.

I. The A-factor regulatory cascade

1. Properties of A-factor receptor protein

Recent identification of specific receptor proteins for A-factor, virgines butanolides, and IM-2 has provided a substantial clue to the mechanism by which these γ-butyrolactone-type autoinducers are involved as chemical signal molecules. The experiments with [3H]A-factor and a cell-extract of S. griseus showed that approximately 40 molecules of the A-factor receptor protein per genome were present in the cytoplasmic fraction. The dissociation constant was calculated to be 0.7 nM, in agreement with the extremely low effective concentration of A-factor. The finding that the A-factor receptor protein (ArpA) acts as a repressor-type regulator for streptomycin production and aerial mycelium formation has led to the idea that A-factor binds to ArpA as an early event in the A-factor regulatory cascade, resulting in derepression of a still unknown key gene(s) that is required for secondary metabolism and aerial mycelium formation in S. griseus. The repressor-like behavior of ArpA was also confirmed by the experiments with the cloned arpA gene (see below). The step in which ArpA is involved is therefore of central importance for transmitting the A-factor signal to the downstream genes leading to streptomycin production and aerial mycelium formation in the regulatory cascade.

2. Cloning and characterization of the A-factor receptor gene

For cloning of the A-factor receptor gene, we purified the receptor from the mycelium of S. griseus IFO 13350 by ammonium sulfate fractionation followed by five steps of column chromatography. The NH2-terminal amino acid sequences of ArpA and lysyl endopeptidase-generated fragments were determined for the purpose of preparing oligonucleotide primers for cloning arpA by the polymerase chain reaction (PCR) method. The arpA gene cloned in this way directed the synthesis of a protein having A-factor-specific binding activity, when expressed in Escherichia coli under the control of the T7 promoter. The arpA gene was thus concluded to encode a 276-amino-acid protein with a calculated molecular mass of 29.1 kDa, as determined by nucleotide sequencing. Molecular mass determination of ArpA by gel-filtration column chromatography showed that a homodimer of ArpA bound A-factor. The NH2-terminal portion of ArpA (amino acids 1 to 70) contained an α-helix-turn-α-helix DNA-binding motif that showed great similarity to those of many DNA-binding proteins (Fig. 2), which suggests that it exerts its regulatory function for the various phenotypes by directly binding to a certain key gene(s).

The amino acid sequences between ArpA and the virgines butanolide receptor protein
Fig. 2. Amino acid comparison of DNA-binding domains. These contain an α-helix-turn-α-helix motif in the NH₂-terminal portion. Betl, a repressor involved in choline regulation of the osmoregulatory pathway; ORF188, a repressor for parA encoding a drug resistance determinant.

(BarA) showed end-to-end similarity. This is conceivable because the chemical structures of their ligands, A-factor and virginiae butanolide, are very similar. Notwithstanding the similarity between ArpA and BarA, their ligand specificities greatly differ; virginiae butanolide did not compete the binding of A-factor to ArpA, and vice versa. In addition, virginiae butanolide had no A-factor activity in vivo. We imagine that the COOH-terminal portions of the two receptor proteins recognize and bind their respective ligands, since their NH₂-terminal portions of these receptors containing an α-helix-turn-α-helix DNA-binding motif seem to serve as a DNA-binding domain. Because of the great similarity between ArpA and BarA not only in amino acid sequence but also in autoregulatory role, we assume that the regulatory steps in which these receptors and ligands are involved are controlled in the same way.

3. arpa as a repressor-type regulator

We introduced arpa on a high-copy-number plasmid pIJ486 (plasmid pARPH1) into the wild-type S. griseus strain IFO 13350 to see possible effects of the overexpression of ArpA on streptomycin production and morphogenesis. The A-factor binding assay with a crude-lysate prepared from this recombinant S. griseus strain revealed about 3.2-folds activity than strain IFO 13350, as expected. However, the timing and abundance of sporulation of the two strains were the same, when grown on YMPG medium or nutrient agar broth (data not shown). In addition, the amounts of streptomycin produced by the recombinant strain and the wild-type strain containing the vector plasmid pIJ486 were the same (data not shown). This can be explained in terms of the extreme difference in the molecular numbers of A-factor as the ligand and its receptor with an extremely small dissociation con-
stant in the cell; because of a overwhelmingly large number of the A-factor molecule, the regulatory role of ArpA in response to A-factor is not affected when the A-factor binding activity and probably the number of the receptor increase 3.2-folds at the most. The wild-type S. griseus strain produced 0.2 μg of A-factor per ml\(^{19}\), which corresponds to \(5 \times 10^{14}\) molecules per ml.

We next introduced pARPH1 into S. griseus strain KM7 which was derived from an A-factor-deficient mutant strain HH1 and deficient in ArpA\(^{10}\). This mutant strain overproduced streptomycin and formed spores earlier than the wild-type strain. The phenotypes of strain KM7 led to the idea that ArpA acted as a repressor-type regulator for secondary metabolism and morphogenesis\(^{14}\). We therefore expected that endowment of the A-factor receptor activity to strain KM7 would result in the repression of streptomycin production and sporulation. Strain KM7 containing arpA on a high-copy-number plasmid pJL486 did not form spores, as expected. In addition, strain KM7 containing a multicopy of arpA produced no streptomycin, when assayed by bioautogram with Bacillus subtilis as the indicator. Plasmid pARPL1, whose copy number was supposedly 1 to 2 in S. griseus as in S. lividans, also repressed sporulation completely and streptomycin production severely. Since the copy number of arpA supplemented in trans by pARPL1 in strain KM7 is supposedly 1 to 2, the genotype of this recombinant strain is almost the same as strain HH1 which shows a Bld and streptomycin-negative phenotypes. These observations are in agreement with the above idea that ArpA acts as a repressor-type regulator for these phenotypes at an early stage of growth (Fig. 3).


A wide distribution of A-factor-like compounds in Streptomyces spp. has been suggested by the fact that many Streptomyces strains produce active compounds triggering streptomycin production and aerial mycelium formation in an A-factor-deficient mutant strain of S. griseus\(^{18}\). In addition to these compounds having A-factor activity, several other A-factor-like compounds as signaling molecules have been also reported\(^ {1,2}\). These observations led us to suppose that receptor proteins for these compounds were distributed among these Streptomyces strains. We employed a PCR method with the same primers as those used for the cloning of arpA from S. griseus to search for an arpA homolog among the chromosomal DNAs from various Streptomyces spp. We tested for four strains that were shown to produce a compound(s) having A-factor activity\(^ {10}\). A 150-bp fragment, together with several other fragments, was amplified with the chromosomal DNAs from all the four Streptomyces strains examined. This size (150-bp) was the same as that of the fragment encoding part of ArpA of S. griseus. The presence of amplified fragments with the same size as that for arpA suggests that almost all Streptomyces strains contain a receptor protein similar to ArpA. In fact, nucleotide sequencing of the region covering the 150-bp fragment amplified with the S. coelicolor A3(2) chromosomal DNA showed that it encoded a protein homologous with ArpA (manuscript in preparation). In addition to the wide distribution of A-factor-like compounds in a variety of Streptomyces spp.,\(^ {1,2}\), the presence of arpA homologs still more leads to the idea that a hormonal control, like the A-factor system in S. griseus, controls morphogenesis and/or secondary metabolism in Streptomyces in general.

II. Protein serine/threonine kinases involved in secondary metabolism and morphogenesis

1. Properties of afsK/afsR

The AfsK kinase of S. coelicolor A3(2) M130 is one of "eucaryotic" protein kinases that
are inhibited by K-252a and staurosporine, inhibitors of serine/threonine kinases. The recombinant AfsK protein produced in E. coli cells autophosphorylated its serine and tyrosine residues and phosphorylated serine and threonine residues of AfsR, a regulatory protein involved in secondary metabolism in this microorganism\(^{19,20}\). Disruption of the chromosomal afsK gene resulted in a reduced level of actinorhodin production, but caused no detectable change in morphogenesis. An afsK-null mutant still formed spores as did the parental strain, suggesting that the afsK gene is concerned with secondary metabolism and not with morphological differentiation\(^{19}\). An afsR-disrupted mutation also resulted in reduced production of actinorhodin but caused no effect on morphological differentiation\(^{21}\). Figure 4 shows the model of the global regulation of secondary metabolism by afsK/afsR in S. coelicolor A3(2).

In Fig. 4, the afsQ1/afsQ2 system that also globally controls secondary metabolism in this microorganism\(^{7}\) is shown. AfsQ1 and AfsQ2 constitute a two-component regulatory system typical of procaryotes. On the analogy of signal transduction in eucaryotes and procaryotes, both AfsK/AfsR and AfsQ2/AfsQ1 phosphorylation systems appear to serve as a switch for the onset of secondary metabolism on sensing respective environmental signals.

2. Suppression by afsK of aerial mycelium
formation of an A-factor-deficient mutant strain of *S. griseus*

We found that introduction of the *afsK* gene of *S. coelicolor* A3(2) into an A-factor-deficient mutant strain, HH1, of *S. griseus* caused sporulation. *S. griseus* HH1 containing *afsK* on pIJ922 (plasmid pIJ922-AFK) with its copy number of 1 to 2 sporulated to almost the same extent as the wild-type strain\(^2\). No A-factor production was detected in this sporulating mutant strain (data not shown), when assayed by the streptomycin-cosynthesis method\(^17\). In addition, a bioassay with *B. subtilis* ATCC 6633 as the indicator showed that pIJ922-AFK caused no streptomycin production. These observations denied the possibility that *afsK* induced sporulation resulting from the reversal of A-factor production. Electron microscopy showed that the spores induced by *afsK* were the same in size and shape as those of the wild-type strain. Since A-factor acts as a switch at the developmental step from substrate myceli-

![Diagram](image)

Fig. 4. Global regulation of secondary metabolism by protein phosphorylation. The membrane-associated *AfsK* kinase autophosphorylates Ser and Tyr residues on sensing some external signal, and activates its own kinase activity. *AfsK* then phosphorylates Ser and Thr residues of *AfsR*, and phosphorylated *AfsR* stimulates transcription of the antibiotic production genes via one or more steps. Expression of *afsR* results in stimulation of the transcription of the actinorhodin biosynthetic gene cluster\(^2\), *AfsQ1/AfsQ2* constituting a two-component regulatory system also triggers antibiotic production. The membrane-spanned *AfsQ2* kinase senses an external signal and autophosphorylates a His residue. The phosphorylated *AfsQ2* then transfers the phosphate to an Asp residue of *AfsQ1*, a response regulator.
um to aerial hyphae, the afsK gene of *S. coelicolor* A3(2) is concluded to suppress the aerial mycelium-negative phenotype of *S. griseus* HH1 without interfering A-factor production. A mutant afsK gene encoding a protein without the ability to autophosphorylate or to phosphorylate AfsR showed no such activity. This implies that the kinase activity of AfsK is essential for the suppression of the aerial mycelium-negative phenotype of *S. griseus* HH1.

Suppression of the aerial mycelium-negative phenotype of *S. griseus* HH1 by the serine/threonine kinase encoded by afsK suggests the involvement of a kinase functionally similar to AfsK in aerial mycelium formation in *S. griseus*, either as a member of the A-factor regulatory cascade or as a regulator controlling a certain step in a pathway independent of A-factor. Although afsK appears to be involved only in secondary metabolism, and not in morphogenesis, in *S. coelicolor* A3(2), it is well conceivable that a kinase with a catalytic domain similar to that of AfsK is involved in morphogenesis in *S. griseus*. This idea is in agreement with our previous findings that K-252a and staurosporine, known as eucaryotic-type protein kinase inhibitors, inhibit many protein phosphorylation reactions with cell-extracts of *S. griseus* and *S. coelicolor* A3(2) to varying extents and also inhibit aerial mycelium formation of these strains.

3. Control of aerial mycelium formation by an afsK homolog in *S. griseus*

The above observations suggested the involvement of an afsK-like gene in aerial mycelium formation in *S. griseus*. The presence of an afsK homolog in *S. griseus* was predicted by Southern hybridization experiments with part of the afsK sequence encoding the kinase catalytic domain as the probe. We therefore cloned and characterized the afsK homolog of *S. griseus* in order to determine whether it was concerned with aerial mycelium formation or secondary metabolism, or both. An afsK homolog (named afsK2) of *S. griseus* was cloned and its nucleotide sequence was determined. The AfsK2 product with 807 amino acids showed 74.5% identity in amino acid sequence to *S. coelicolor* A3(2) AfsK over the entire sequence (unpublished manuscript in preparation). Like *S. coelicolor* A3(2) AfsK, the recombinant AfsK2 protein autophosphorylated its serine and tyrosine residues. Disruption of the chromosomal afsK2 gene with the *E. coli* single-stranded phage vector M13 caused no effect on streptomycin production or sporulation on routine agar medium. However, the *S. griseus* strain with the disrupted afsK2 gene did not form aerial mycelium on agar medium containing a high concentration of sucrose or sorbitol, indicating that afsK2 is essentially required for morphogenesis under the conditions of high osmolality. The afsK2 mutation did not respond to NaCl or KCl in the medium. Despite the great similarity between the two kinases from *S. griseus* and *S. coelicolor* A3(2), these appeared to be different in their substrate specificity, because afsK was unable to complement the afsK2 mutation and, unlike AfsK, the recombinant AfsK2 failed to phosphorylate the AfsR protein. It is thus apparent that a structurally very similar serine/threonine kinase is involved in the regulation of morphological differentiation in *S. griseus* and secondary metabolism in *S. coelicolor* A3(2).

**CONCLUSIONS**

"Eucaryotic" signal transduction systems for morphological and physiological differentiation operate in the bacterial genus *Streptomyces*. One is chemical signaling molecules as microbial hormones, represented by A-factor, and the other is protein phosphorylation via serine/threonine kinases. *Streptomyces* strains resemble fungi not only in their morphological differentiation but also in cellular control through signal transduction. These will shed a new light
on the study of cellular differentiation as basic biology and on secondary metabolite formation as applied microbiology. A better understanding of these regulatory systems will make the genus *Streptomyces* a more fascinating target.

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