"Eucaryotic" Signal Transduction Systems in the Bacterial Genus *Streptomyces*

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(Received Sept. 3, 1993)

A microbial hormone, A-factor, controls streptomycin production, streptomycin resistance, and aerial mycelium formation in *Streptomyces griseus*. A-factor exerts its regulatory role by binding to a specific receptor protein which, in the absence of A-factor, acts as a repressor-type regulator for these morphological and physiological differentiation. In the signal relay leading to streptomycin production, the A-factor signal is transmitted from the A-factor receptor to the upstream activation sequence of a regulatory gene, *strR*, in the streptomycin biosynthetic gene cluster via an A-factor-dependent protein that acts as a transcription factor for *strR*. The signal relay leading to aerial mycelium formation includes proteins similar to response regulators of the procaryotic two-component regulatory systems and to a family of membrane translocators engaged in ATP-dependent secretion mechanisms. Accumulating data show that a large family of γ -butyrolactones act as autoregulators in a wide variety of *Streptomyces* spp. In addition to the hormonal control by the autoregulators, secondary metabolism and morphogenesis are controlled by signal transduction systems via both procaryotic and eucaryotic protein kinases. A pair of the AfsQ1 and AfsQ2 proteins constituting a procaryotic-type histidine-aspartate phosphotransfer system globally controls secondary metabolite formation in *Streptomyces coelicolor* A3(2). Another pair of proteins, AfsK and AfsR, which also serves as a global regulator for secondary metabolism in *S. coelicolor*, constitutes an eucaryotic-type phosphotransfer system in which a protein serine/threonine/tyrosine kinase AfsK phosphorylates serine and threonine residues of AfsR. A wide distribution of DNA sequences homologous with the *afsQ1/afsQ2* and *afsK/afsR* genes suggests that both procaryotic and eucaryotic protein kinases control secondary metabolism in general in *Streptomyces*. Inhibition by K-252a and staurosporine, known as eucaryotic protein kinase inhibitors, of antibiotic production and aerial mycelium formation of *S. griseus* and *S. coelicolor* supports this idea.

INTRODUCTION

The genus *Streptomyces*, which have probably evolved separately from *Bacillus subtilis* for more than 700 million years¹⁾, comprises gram-positive filamentous soil bacteria with a complex life cycle²⁾. Spores germinate to form a branched, multinucleoid substrate mycelium which produces an aerial mycelium in response to nutrient limitation. After septa have been formed at regular intervals along the hyphae, long chains of uninucleoid spores are formed. In addition to the complex morphological differentiation, *Streptomyces* has the ability, as its physiological differentiation, to produce a variety of secondary metabolites. *Streptomyces* produces the majority of more than 5,000 anti-

biotics which have so far been isolated from various microorganisms since the discovery of penicillin. Recombinant DNA techniques for gene cloning in *Streptomyces* have made it possible to isolate and analyze the genes responsible for these two characteristic biological aspects. This methodology has contributed to understanding of the mechanisms of regulation involved in these processes.

We have been studying the regulation of morphological differentiation and secondary metabolism in *Streptomyces* by means of recombinant DNA technology. One of the targets is an autoregulatory factor called A-factor that triggers streptomycin (Sm) production, Sm resistance, and aerial mycelium formation in *Streptomyces griseus*. Its remarkable pleiotropic

effects at an extremely low concentration and its requirement of a specific protein receptor comparable to eucaryotic hormone receptors convince us of the identity of A-factor as a microbial hormone. Another target of our study is two types of a phospho-relay controlling globally secondary metabolite formation in *Streptomyces coelicolor* A3(2). Each of the regulatory proteins comprises a signal transduction system with a specific protein kinase. One (AfsQ1/AfsQ2) of the phospho-relays is a procaryotic-type two-component regulatory system involved in various adaptive responses in a variety of procaryotes. The other (AfsR/AfsK) resembles the phospho-relays of eucaryotes, in which AfsK as a protein serine/threonine/tyrosine kinase phosphorylates serine and threonine residues of AfsR protein as a transcriptional activator for antibiotic production genes.

This review article summarizes our recent study on the regulation of morphogenesis and secondary metabolism in *Streptomyces* with emphasis on the eucaryotic-type signal transduction systems exerted by A-factor and protein phosphorylation. Parts of our work have also been summarized in ref. 3~8. Recent discovery of other γ -butyrolactones similar in structure to A-factor in a wide variety of *Streptomyces* spp. indicates the generality of these γ -butyrolactones as autoregulatory factors. A wide distribution of nucleotide sequences homologous with the *afsK/afsR* and *afsQ1/afsQ2* genes among *Streptomyces* spp. indicates that a combination of procaryotic and eucaryotic phospho-relays regulates globally antibiotic biosynthesis in general in *Streptomyces*.

II. A-factor as a microbial hormone

A-factor (2-isocapryloyl-3*R*-hydroxymethyl- γ -butyrolactone) was originally discovered by Khokhlov *et al.*⁹⁾ as a diffusible extracellular molecule that causes sporulation of a bald mutant of *S. griseus*. The pioneer work by

Khokhlov's group revealed that A-factor was an essential regulatory factor for not only aerial mycelium formation but also Sm production^{10,11)}. Hara & Beppu^{12,13)} confirmed their observations with stereochemically synthesized A-factor and further found that Sm resistance via Sm-6-phosphotransferase was also dependent on A-factor. In addition to these phenotypes, we found that A-factor induced production of a yellow and diffusible pigment containing a sugar moiety in its structure⁵⁾. Thus we can illustrate the pleiotropic effect of A-factor as shown in Fig. 1. In order to elucidate the A-factor regulatory cascade at the molecular level, we have characterized the genes and proteins that transfer the A-factor signal to the downstream genes involved in Sm production and aerial mycelium formation.

1. Genetics of A-factor production

A-factor-negative mutants of *S. griseus* that are phenotypically deficient in Sm production and sporulation can be obtained at very high frequency by so-called plasmid-curing treatments, such as an acridine orange treatment and protoplast regeneration. Consistent with this, genetic mapping of the A-factor determinant (*afsA*) of *S. griseus* by means of protoplast fusion did not give a fixed position on the chromosome linkage map, predicting an extrachromosomal behavior of the *afsA* gene¹⁴⁾. In addition, A-factor-negative mutants obtained by plasmid-curing treatments were found to have a deletion of a long DNA sequence containing the *afsA* gene, as determined by Southern blot hybridization with the cloned *afsA* gene as probe¹⁵⁾. Recently, a plasmid of more than 1,500 kilobase pairs in length that carries the *afsA* gene was found (H. Kinashi, A. Lezhava, S. Horinouchi, T. Beppu & O. Nimi, presented at the Annual Meeting of SAJ, July, 1993, Tokyo). In contrast to the linear form of giant plasmids in *Streptomyces*, this plasmid appears to be a covalently closed circular form. The

Fig. 1. The A-factor regulatory cascade.

location of the *afsA* gene on the plasmid accounts for the genetic instability of A-factor production in *S. griseus*.

By analogy with the biosynthetic pathway of virginiae butanolides structurally similar to A-factor¹⁶⁾, A-factor is supposedly synthesized from one glycerol derivative and one β -keto acid. The β -keto acid is synthesized from three acetate and one isobutyrate in the same way as the elongation of carbons in polyketide biosynthesis. In relation to the proposed biosynthetic pathway of A-factor, we speculate that the above-described *afsA* gene codes for an enzyme

catalyzing the condensation between the β -keto acid and the glycerol derivative, because a trimmed 1.2 kb fragment encoding a single open reading frame of 301 amino acids confers A-factor production with a marked gene dosage effect to all the *Streptomyces* strains tested¹⁷⁾. It is well conceivable that all *Streptomyces* strains contain the glycerol derivative as a common primary metabolite and a polyketide biosynthetic pathway that endows the β -keto acid as a common metabolite as well.

The transcriptional start point of *afsA* is the adenine residue, the first position of the transla-

tional start codon ATG¹⁸). This presents a striking contrast to the conventional interaction between ribosomes and a ribosome-binding sequence complementary to the 3' end of the 16S rRNA. This coincidence of transcriptional and translational start sites has been found in several actinomycete genes, including *ermE* mediating erythromycin resistance in *S. erythraeus*¹⁹, *sta* mediating streptothricin resistance in *S. lavendulae*²⁰, and *myrA* mediating micinamicin resistance in *Micromonospora griseorubida*²¹. These genes conferring self-resistance on the hosts and the *afsA* gene responsible for biosynthesis of A-factor acting as a trigger of Sm production are supposedly expressed just before the production of the respective antibiotics. It is therefore possible that translation of leaderless transcripts is associated with differential expression of the genes at a specific stage of growth. On the other hand, the cholesterol dehydrogenase gene from a *Nocardia* sp. that is also translated in the same way is expressed from the very early to the late growth phase in *S. lividans*²². These observations suggest that this type of unusual transcription-translation coupling is present in a rather wide variety of actinomycete genes.

2. A-factor receptor protein

The similarity in the functional features of A-factor to eucaryotic hormones prompted us to examine the presence of a specific receptor protein in *S. griseus*. We searched for a binding protein with tritium-labeled A-factor, [1'-³H]-3*R*-hydroxymethyl-2-(6-methylheptanoyl)-4-butanolide, as the probe. An A-factor-binding protein thus detected in the cytoplasm had an apparent molecular weight of about 26,000, as determined by gel filtration chromatography²³. Scatchard analysis showed that the protein bound A-factor in the molar ratio of 1:1 with a dissociation constant, K_d , of 0.7 nM. The number of the protein was roughly estimated to be 30 to 40 per genome. The extremely small

K_d value is consistent with the extremely low effective concentration of A-factor, 10^{-9} M, *in vivo*, and is comparable to those of eucaryotic hormone receptors. The virginiae butanolide-binding protein detected by Kim *et al.*²⁴ shows similar properties as those of the A-factor-binding protein.

Evidence to prove the functional role of the A-factor-binding protein thus detected was obtained from the genetic study of mutants deficient in the binding protein²⁵. *S. griseus* 2247 is a derivative obtained from strain IFO 3237 by UV-irradiation by Dr. R. Nomi, Hiroshima University, as a stable and high-level Sm producer. Strain 2247 required no A-factor for Sm production or sporulation and had a defect in the A-factor-binding protein. This observation implied that the A-factor repressed the expression of both phenotypes in the wild-type strain. We therefore screened for mutants producing Sm and sporulating in the absence of A-factor among mutagenized A-factor-deficient *S. griseus* colonies and isolated three such mutants. As expected, all the three such mutants were found to be deficient in the A-factor-binding protein. Reversal of the defect in the binding protein of these mutants led to the simultaneous loss of Sm production and sporulation. These mutants began to produce Sm and sporulate at an earlier stage of growth than did the wild-type strain. These data proved the functional involvement, as a true receptor, of the A-factor-binding protein physically detected with [³H]A-factor. On the basis of these observations, we speculate a simple model as follows. At an early stage of growth when A-factor has not yet been produced, the receptor protein binds some specific region, probably a promoter sequence, of a still unidentified gene(s) and represses the expression of the genes. When the amount of A-factor reaches a certain critical level at a specific stage of growth, the receptor binds A-factor, as a result of which the receptor is released from the DNA

sequence and the gene(s) repressed is open for transcription. The timing of expression of the gene(s) in question may be determined by the intracellular concentration of A-factor. In relation to this, A-factor is produced just before the cells begin to produce Sm and sporulate¹². The Sm production and sporulation of the mutants deficient in the receptor at an earlier stage than those of the wild-type strain supports this idea.

The structure of A-factor molecule is very specific and practically any alteration of its structure led to decrease or total disappearance of the regulatory activity¹¹. This can be explained in terms of the strict ligand specificity of the A-factor receptor protein. In vitro binding assays showed that the A-factor receptor was unable to bind virginiae butanolide C²⁶ structurally similar to A-factor. Consistent with this, virginiae butanolide C exerted almost no *in vivo* effect on an A-factor-negative mutants of *S. griseus*. The ligand specificity appears to differ from strain to strain even among *S. griseus* strains, since several A-factor analogs with different acyl chain lengths or a hydroxyl group at the 6-position are still active to some extent in an anthracycline-producing *S. griseus* that requires A-factor as an intrinsic autoregulator for morphogenesis and antibiotic production^{27,28}. The A-factor homolog having a hydroxyl group at the 6-position, obtained by reduction of A-factor, shows almost no biological activity on the *S. griseus* strain with which Khokhlov's group has been working¹¹.

3. Regulation of streptomycin biosynthesis by A-factor

A-factor serving as a trigger of Sm biosynthesis does not enhance the yield of Sm since exogenous supplementation of an excess of A-factor or overproduction of A-factor through the gene amplification of *afsA* by use of a multicopy plasmid does not enhance the yield. However, Sm production begins one day earlier than that by the wild-type strain²⁹. This is consistent

with the idea that the timing of Sm production is determined by the intracellular concentration of A-factor, as described above in relation to the earlier Sm production by the mutants deficient in the A-factor receptor.

Concerning A-factor as a trigger of Sm biosynthesis, the question we raised was by what protein the A-factor signal was received and transmitted to the Sm production genes. In the absence of A-factor, neither Sm nor any biosynthetic intermediates were detected in the culture broth¹². Our preliminary data and a detailed study by Distler *et al.*³⁰ on the transcriptional organization of part of the Sm biosynthetic gene cluster showed that at least three mRNAs covering the Sm-6-phosphotransferase gene (*aphD*), a regulatory gene (*strR*), and an aminocyclitol amidinotransferase gene (*strB*) were detectable only in the presence of A-factor (Fig. 2). We then conducted experiments to determine which promoter(s) in this part is directly responsive to A-factor³¹. For this purpose, we constructed a promoter-probe plasmid with a low copy number by using the extremely thermostable malate dehydrogenase (MDH) gene from *Thermus flavus* as the reporter. This *mdh* gene containing no TTA codon is useful as a reporter gene for *Streptomyces*³², because it reflects promoter activities without any influence by the *bldA* gene², when examined with a strong vegetative promoter³³. DNA fragments containing each of the *strR*, *aphD*, *strB1*, and *strD* were inserted at the multicloning site upstream of the promoterless MDH gene. An A-factor-negative mutant of *S. griseus* transformed independently with each of the four plasmids was grown in the presence and absence of A-factor and heat-stable MDH activities were measured after a heat-treatment. These experiments showed that only the promoter of *strR*, a regulatory gene, was directly controlled by A-factor. The promoters of *aphD*, *strB1* and *strD* were not affected. S1 nuclease mapping confirmed a 4 to 5-fold enhancement of *strR*

Fig. 2. Organization of part of the streptomycin biosynthetic gene cluster of *S. griseus* (upper) and DNA-binding proteins that specifically bind to the region upstream of the *strR* promoter (bottom).

Upper The directions and locations of the genes in the cluster are taken from Distler *et al.*³⁵⁾ Under the restriction map of the region containing *strD*, *strR*, *aphD*, and *strB1*, the directions and extents of the transcripts are shown. Among these four promoters, only the promoter for *strR* is directly responsive to A-factor. The *aphD* gene encoding the major Sm resistance determinant is transcribed mainly by read-through from the A-factor-inducible *strR* promoter, which accounts for the rapid induction of Sm resistance by A-factor. **Bottom** The transcriptional start points and the directions of transcripts are indicated by arrows. A protein shown by a shaded wedge is the A-factor-dependent protein that binds to the UAS region 293 to 242 bp upstream of the transcriptional start point of *strR*. The binding of this protein is greatly enhanced by Mg^{2+} . In addition to the A-factor-dependent protein, three other proteins, shown by a hexagon, a circle, and an oval, also bind specifically to this region. The protein shown by a hexagon binds to both sides of the UAS bound by the A-factor-dependent protein, and its binding is abolished in the presence of Mg^{2+} and ATP. Both the 206 bp *TaqI* fragment and the 214 bp *TaqI* fragment show slow electrophoretic mobilities, suggesting that each of these fragments contains a static DNA bending.

transcription by A-factor. Further subcloning experiments indicated that the region 371 to 241 bp upstream of the transcriptional start point of *strR* is essential for its A-factor dependence. These findings implied that a regulatory protein recognized a specific sequence (upstream activation sequence, UAS) for the *strR* promoter to cause general stimulation of Sm biosynthesis. The following observations led us to conclude that the putative regulator was an activator. First, increase in the copy number of the *strR* promoter did not lead to a corresponding increase in the total promoter activity, probably due to titration of an activator that binds to the UAS. Second, the wild-type *S. griseus* strain containing the *strR* promoter on a high copy number plasmid produced no Sm or much less than the wild-type strain, probably titration of the putative activator reduced transcription of the chromosomal *strR* gene.

In the case of *aphD* encoding Sm-6-phosphotransferase, read-through from the *strR* promoter appeared to play the major role in the induction of synthesis of the major Sm resistance determinant by A-factor. This is consistent with the observation that the addition of A-factor to the culture of an A-factor-deficient mutant strain induced synthesis of Sm-6-phosphotransferase 3 to 4 hr after the addition, irrespective of the timing of addition⁵). Such direct dependence of the transcription of *aphD* on the A-factor-inducible *strR* promoter accounts for the induction of Sm resistance and would help achieve a rapid increase in self-resistance just before induction of Sm biosynthesis.

The search for the putative activator protein by DNA-binding assays using mobility shift polyacrylamide gel electrophoresis led to the detection of a protein able to bind to a restriction fragment 288 to 191 bp upstream from the transcriptional start point of *strR*³⁴). The binding was detected with the protein source only from the wild-type strain and not from an A-factor-deficient mutant strain. The exogenous sup-

plementation of A-factor to the culture of the A-factor-deficient mutant caused the appearance of the protein in the DNA-binding assay. A synthetic 52 bp in length (region 293 to 242 bp upstream from the transcriptional start point of *strR*), which was synthesized on the basis of the data from the subcloning experiments and the DNA-binding assays with dissected DNA fragments, was retarded by the A-factor-dependent protein. It is most likely that this binding protein, shown by a checked wedge in Fig. 2, plays a role in transmitting the A-factor signal to the Sm biosynthetic gene cluster. During these experiments, we also detected additional proteins that bound to the upstream region of the *strR* promoter (Fig. 2). These proteins are produced, irrespective of A-factor, and their roles are still unclear.

On the basis of the above data, we can figure out that the positive A-factor signal is transmitted to the Sm production genes in such a way that transcription of the *strR* gene is activated by the A-factor-dependent protein, leading to formation of the StrR product, which, in turn, stimulates transcription of the other genes in the cluster (Fig. 3). Consistent with this, introduction of a multicopy plasmid containing the *strR* gene into an A-factor-deficient mutant caused Sm production. This means that all the Sm biosynthetic genes are activated when the amount of the StrR protein reaches a certain critical level, either through the activation of the *strR* promoter by A-factor or by means of gene amplification even in the absence of A-factor. According to Distler *et al.*³⁵), the StrR protein stimulates the transcription of *strB* by an anti-termination mechanism. They assume that the *strB* transcript is prematurely terminated in the absence of the StrR protein, and an increase in the amount of the StrR protein suppresses the premature termination. Concerning the upstream steps in the regulatory cascade, we assume that there is one or more regulatory steps responsible for transmitting the A-factor

Fig. 3. A model for regulation of streptomycin biosynthesis.

signal from the A-factor receptor to the A-factor-dependent binding protein. Our previous study suggested that the binding protein is concerned with only Sm production and not with aerial mycelium formation. On the other hand, the combination of A-factor and its receptor controls both the phenotypes. In Fig. 3, the putative additional regulatory protein is designated as X, which must affect both Sm production and sporulation.

The DNA region bound by the above-described proteins showed a slow mobility on agarose and polyacrylamide gel electrophoresis. A slow electrophoretic mobility of a DNA fragment is a hallmark of bent DNA³⁶⁾. There have been observations to support the idea that DNA bending is an important feature of sites

recognized by specific proteins that are involved in gene expression and initiation of DNA replication. It is therefore possible, by analogy with these examples, that the bent DNA structure is one of the important factors for these proteins to recognize and bind to their own binding sites.

4. A gene cluster involved in aerial mycelium formation in *S. griseus*

In *S. coelicolor* A3(2), two mutant classes defective in the differentiation process have been isolated; *bld* mutants defective in the formation of aerial mycelium and *whi* mutants defective in the formation of spores²⁾. These mutants have been successfully used as hosts for cloning the genes complementing the re-

spective defect. Since A-factor-deficient mutant strains show a Bld phenotype, it seemed possible that the overexpression of A-factor-controlled genes in the absence of A-factor might lead to sporulation in an A-factor-deficient mutant host. In fact, as described above, overexpression of the *strR* gene caused Sm production in an A-factor-deficient mutant. Such cloning experiments yielded four DNA fragments which induced aerial mycelium formation and sporulation. One of these was previously cloned and characterized by Babcock and Kendrick^{37,38}. I will describe another one in detail.

A gene bank of the chromosome of an A-factor-deficient mutant strain, *S. griseus* HH1, was constructed with a multicopy plasmid pIJ487. We then transformed the same strain

with the gene bank. Our expectation was that an increase in the copy number of a regulatory gene involved in aerial mycelium formation would result in an increase in the amount of gene product thereby suppressing the sporulation-negative phenotype of strain HH1. Repeated transformation experiments brought forth four different DNA fragments that induced sporulation to varying extents. None of these restored Sm production. Subcloning and nucleotide sequence analysis of one of these four fragments revealed that two open reading frames, named AmfR and AmfA (Fig. 4), were required to suppress the sporulation-negative phenotype³⁹. The AmfR product with 201 amino acids showed significant end-to-end sequence similarity to the response regulators of bacterial two-component regulatory

Fig. 4. Organization of a gene cluster involved in aerial mycelium formation in *S. griseus*.

Because of significant end-to-end sequence similarity of the AmfR product to the response regulators of two-component regulatory systems, it is most likely that the AmfR protein in the cytoplasm is phosphorylated at its Asp-54 residue by a sensory histidine kinase in the membrane, and serves as a transcription factor for a still unidentified gene(s). The promoter in front of these three genes is repressed by A-factor. AmfA and AmfB are supposed to assemble to form a pore through which some compound is transported by the energy of ATP-hydrolysis. By analogy with the hemolysin B⁷¹, each of the domains with the COOH-terminal half containing the ATP-binding site is localized to the cytoplasm.

systems^{40,41}). The AmfA product with 656 amino acids significantly resembled those of a family of membrane proteins engaged in ATP-dependent secretion mechanisms^{42,43}).

The response regulators of two-component regulatory systems are phosphorylated on their aspartate residues and act as transcription factors for genes responsible for adaptive responses. Alignment of the AmfR protein with other regulators in this family predicted that Asp-54 might be phosphorylated. Consistent with this, an amino acid replacement from Asp-54 to Asn resulted in the loss of the ability of *amfR* to induce sporulation in strain HH1. Involvement of the AmfR protein in aerial mycelium formation in *S. griseus* is analogous to the initiation of sporulation in *B. subtilis*, in which a phosphorelay including KinA, Spo0F, Spo0B, and Spo0A is required^{44,45}). Spo0F and Spo0A are members of the response regulators. Transcriptional analysis of the *amfR* gene showed that this gene was transcribed by the promoter located further upstream of the AmfR-coding region. Between the promoter and the coding sequence were present two additional open reading frames, ORF4 and ORF5, as shown in Fig. 4. Interestingly, this promoter appeared to be repressed in the presence of A-factor, suggesting the presence of complex regulation for the expression of this operon. We have not yet identified the kinase gene responsible for the phosphorylation of AmfR.

The AmfA protein seems to be a member of the family involved in the transport of many different compounds, specific for each transport protein. This family includes the mammalian P-glycoprotein (multidrug resistance protein), Ste6 of *Saccharomyces cerevisiae*, and the *E. coli* hemolysin B. We found an additional open reading frame, named AmfB, which was greatly similar to AmfA in size and amino acid sequence. Because the *amfB* gene enhanced sporulation induced by the cooperation of *amfA* and *amfR*, and because some of the proteins

belonging to this family form a tandem duplication giving rise to two homologous halves, we speculate that AmfA and AmfB assemble in the membrane to form a heterodimer rather than a homodimer. Involvement of AmfA and AmfB in aerial mycelium formation reminds us, again, of the Spo0K system necessary for the initiation of sporulation in *B. subtilis*^{46,47}). The Spo0K system consisting of several proteins constitutes a structure similar in topology to the functional domains of the membrane translocators. We therefore speculate that the AmfA/AmfB system is homologous to the *B. subtilis* Spo0K system. The compound transported by the AmfA/AmfB system remain unknown.

I would like to refer to the TTA codon in the AmfR coding sequence. A TTA codon for Leu is confined to a very small number of streptomycete genes engaged in the regulation of morphogenesis and secondary metabolism. In *S. coelicolor* A3(2), *bldA* specifying this tRNA controls both aerial mycelium formation and secondary metabolism at the translational level^{2,48}). This suggests that the translation of *amfR* is regulated by the *bldA* gene, in addition to the possible transcriptional control of *amfR* by A-factor, as described above. Interestingly, an additional gene involved in aerial mycelium formation, which was cloned during our study³⁹) and by Babcock & Kendrick³⁸), was also reported to contain a TTA codon. This means that these two genes are the targets of the *bldA* gene with regard to morphogenesis. Concerning Sm production, I just mention here that the *strR* gene contains a TTA codon.

Some functional redundancy operating during the formation of aerial mycelium has been suggested from a general survey of *bld* mutants of *S. coelicolor* A3(2)²); sporulation of most *bld* mutants is affected by carbon sources and antibiotic production of these mutants is also affected simultaneously. It is possible that there are multiple signal transfer systems involved in aerial mycelium formation, acting

either independently or downstream of the other systems. The existence of four different suppressors of the Bld phenotype caused by A-factor-deficiency suggests that expression of one suppressor at a certain level can bypass a repressed expression of the other. It means that alternative genes, but with different regulation, can fulfill functionally homologous roles.

III. Global regulation of secondary metabolism by protein phosphorylation

The recombinant DNA methodology has led to the cloning and characterization of genes encoding not only the biosynthetic enzymes but also regulatory proteins for antibiotic production. These approaches have revealed that the full complement of genes, including structural synthetic genes as well as regulatory and resistance determinants, necessary for any particular biosynthetic pathway for each antibiotic is organized within a single cluster⁴⁹. Production of secondary metabolites is genetically controlled by the regulatory genes within the cluster, as well as those not closely linked to the cluster. One of the latter is the *afsR* system that globally influences secondary metabolism in *S. coelicolor* A3(2). The *afsR* gene was identified on a DNA fragment that conferred the ability to produce A-factor and the pigmented antibiotics actinorhodin and undecylprodigiosin on *S. lividans*, which does not produce these metabolites under the cultural conditions used^{50,51}. A similar cloning experiments brought forth a DNA fragment containing the *afsQ1/afsQ2* genes⁵². Subsequent genetic and biochemical analyses have shown that the AfsR protein is phosphorylated by a kinase, named AfsK, in the same manner as those in eucaryotic signal transduction systems, indicating that a signal transduction system similar to that found in higher organisms is involved in the regulation of secondary metabolism in *Streptomyces*. In addition to the eucaryotic-type protein

phosphorylation, a procaryotic-type phosphorylation operated by the AfsQ1/AfsQ2 system has also been found to control secondary metabolism.

1. AfsK/AfsR system

The *afsR* gene was identified because it complemented an *afsB* mutation on the chromosome⁵⁰. The *afsB* gene of *S. coelicolor* A3(2) is a regulatory gene for antibiotic production and *afsB*-negative mutants fail to produce actinorhodin, undecylprodigiosin, and A-factor¹⁴. Although the cloned *afsR* gene was assumed to coincide with the *afsB* gene, subsequent experiments have shown that the *afsR* product is a bypass function with regard to *afsB* complementation⁵³. The AfsR protein has been shown to be important in its own right, since disruption of its function results in a decrease in the amount of actinorhodin produced⁵⁴. Each of the NH₂- and COOH-terminal halves could partially confer pigment production on *S. lividans*, which misled us at the early stage of this study to assume that the cloned fragment contained an additional gene named *afsC* upstream of the *afsR* gene⁵⁵⁻⁵⁷. Nucleotide sequencing revealed that it codes for a single protein of 993 amino acids with a molecular weight of 105,600⁵⁴. The AfsR protein contains A- and B-type ATP-binding consensus sequences at its NH₂-terminal portion (formerly called the *afsC* region) and a helix-turn-helix DNA-binding consensus sequence at its COOH-terminal portion (formerly called *afsB* region). Mutations at either one of the two ATP-binding sequences, that were generated by site-directed mutagenesis, revealed their functional importance. The ability of the AfsR protein to awake the "sleeping" or silent biosynthetic pathway for actinorhodin in *S. lividans* was confirmed to result from transcriptional activation of *act* genes⁵⁷.

For the purpose of preparing the anti-AfsR antibody, we first expressed a fusion gene between the calf prochymosin and *afsR* genes in

Fig. 5. Restriction map of the region downstream of the *afsR* gene and the location of open reading frames predicted from the FRAME analysis⁷²⁾ of the nucleotide sequence.

Between the NH₂- and COOH-terminal portions of the AfsK product is an Ala/Pro-rich sequence. The abnormally high G+C composition of the 1st and 2nd codon positions in the middle of the *afsK*-coding region is due to the presence of the Ala/Pro-rich sequence. Plasmid pAFK19 containing the *afsK* gene downstream of the *lac* promoter was used for expression of AfsK in *E. coli*.

E. coli and the fused protein produced as inclusion bodies was electroeluted by polyacrylamide gel electrophoresis. Starting with a crude lysate of recombinant *S. griseus* containing a multicopy plasmid with the whole *afsR* gene, we purified the AfsR protein to homogeneity through several steps of chromatography, including affinity column chromatography with ATP-agarose, and use of the anti-AfsR antibody for its detection⁵⁸⁾. Because of the presence of ATP-binding motifs in AfsR and its importance for the regulatory function, as determined by means of site-directed mutagenesis, we examined the possible ability of AfsR to interact with ATP. The AfsR protein was found to be phosphorylated through the transfer of the γ -phosphate group of ATP in the presence of the cell lysates of *S. coelicolor* A3(2) and *S. lividans*⁵⁸⁾. This phosphorylation proceeded very rapidly, and most of the AfsR molecules in the reaction mixture appeared to be phosphorylated

within 20 sec. The kinase activity is not generally present in *Streptomyces* spp. but specific to certain species, since the cell lysate of *S. griseus* contains no activity phosphorylating the AfsR protein.

The AfsR protein showed no sequence similarity to any of the regulatory proteins of prokaryotic two-component regulatory systems. In addition, the ³²P-label on the AfsR protein was stable to acid- and alkali-treatments, and highly resistant to pyridine and hydroxylamine, indicating that the ³²P-labeled AfsR contained *O*-phosphates, such as phosphoserine, phosphothreonine, and phosphotyrosine. Besides these features of the AfsR phosphorylation, the *in vitro* phosphorylation of the AfsR protein with the cell lysate of *S. coelicolor* A3(2) as a kinase source was severely inhibited by K-252a and staurosporine, both of which are known to be eucaryotic protein kinase inhibitors. Close location of the kinase gene, named *afsK*, to the *afsR*

gene was predicted from the observation that *S. griseus* harboring a plasmid containing the region downstream of the *afsR* gene showed an activity to phosphorylate the AfsR protein. As mentioned above, *S. griseus* showed no such activity. The phosphorylation activity was mainly localized in the membrane fraction. The nucleotide sequence of this region predicted three open reading frames, ORF-A, -B, and -C (Fig. 5)⁵⁹). Among these, ORF-A with 799 amino acids showed significant sequence similarity to eucaryotic protein serine/threonine kinases^{60,61}). All eleven major subdomains conserved in serine/threonine-specific and

tyrosine-specific kinases were conserved in the AfsK product.

We next produced the AfsK protein by use of an *E. coli* host-vector system. Incubation of the cell lysate from the recombinant *E. coli* strain with [γ -³²P]ATP revealed a self-catalyzed phosphate incorporation into both serine and tyrosine residues. This specificity toward phosphorylated residues indicates that the AfsK kinase is a member of a group of kinases with dual specificity for serine/threonine as well as tyrosine residue. The recombinant AfsK kinase phosphorylated the purified AfsR protein at both serine and threonine residues. Disruption

Fig. 6. A model of phosphorelay by an eucaryotic-type AfsK/AfsR system and a procaryotic-type AfsQ1/AfsQ2 system in signal transduction.

The AfsK protein associated with the membrane autophosphorylates its serine and tyrosine residues upon sensing a certain stimulus, and catalyzes the phosphorylation of serine and threonine residues of the transcriptional regulator AfsR in the cytoplasm. The phosphorylated AfsR protein then activates the transcription of a still unidentified gene(s), leading to activation of antibiotic production genes. The AfsQ2 product integrated in the membrane autophosphorylates its 294th His residue upon sensing a certain external stimulus, and transmits its signal by phosphotransfer to the 52nd Asp residue of the transcription factor AfsQ1.

of the chromosomal *afsK* gene by use of phage ϕ C31KC515 resulted in significant loss of actinorhodin production, which indicates the functional involvement of *afsK* in the regulation of antibiotic production in *S. coelicolor* A3(2)⁵⁹. On the analogy of various protein kinases in eucaryotic cells, we speculate that AfsK activates its own kinase activity by autophosphorylating its tyrosine and serine residues on sensing external stimuli and then phosphorylates AfsR through the transfer of the γ -phosphate of ATP (Fig. 6). Southern hybridization experiments showed a wide distribution of nucleotide sequences homologous to the *afsK* gene as well as the *afsR* gene among many *Streptomyces* spp. It is thus shown that protein phosphorylation by eukaryotic-type protein kinases plays an important role in the regulation of secondary metabolism in a wide variety of *Streptomyces*.

2. Multiple protein kinases involved in secondary metabolism and morphogenesis in *Streptomyces*

During the study of the *in vitro* phosphorylation of the AfsR protein by cell lysates from *S. coelicolor* A3(2) and *S. griseus*, we found that many proteins were phosphorylated by incubation of the cell lysates with [γ -³²P]ATP⁶². Furthermore, eucaryotic protein kinase inhibitors, such as K-252a, staurosporine, and herbimycin, inhibited the phosphorylation of these proteins to varying extents. The presence of multiple proteins whose phosphorylation is affected in obviously different ways by kinase inhibitors with different modes of action predicts the presence of multiple protein kinases. Since these kinase inhibitors do not show any significant inhibitory effect on the phosphotransfer between OmpR and OmpZ in *E. coli* (T. Mizuno, personal communication), the kinases sensitive to these inhibitors appear to be those of eucaryotic-type, rather than procaryotic-type comprising two-component regulatory

systems.

Most of the *in vitro* phosphorylation reactions were greatly enhanced by 10 mM manganese. Calcium at 10 mM also enhanced the phosphorylation of several proteins. In several *Streptomyces* spp., Ca²⁺ is required for aerial mycelium formation^{63,64}. Although the concentrations used in the *in vitro* phosphorylation are far higher than physiological concentrations, it is possible that these pervasive trace elements modulate the activities of the respective protein kinases *in vivo*, thereby playing a developmental role. In relation to this, a calmoduline-like protein from *Streptomyces erythraeus* has been reported⁶⁵.

Consistent with the *in vitro* effects of the kinase inhibitors, at concentrations showing no killing effects, they inhibited aerial mycelium formation and yellow pigment production of *S. griseus*⁶². They also exerted a similar effect on *S. coelicolor* A3(2). These inhibitors exerted almost no effect on the growth of substrate mycelium or germination of spores. Figure 7 shows an interesting pattern of inhibition of aerial mycelium formation and pigmentation in *S. griseus* when 230 μ g (50 nmol) of K-252a in a paper disc was placed on medium seeded with spores. At higher concentrations of K-252a (close to the disc), *S. griseus* sporulates normally and produces a yellow pigment. At slightly lower concentrations, K-252a completely inhibited aerial mycelium formation and pigmentation, resulting in the formation of a doughnut zone of inhibition. In other words, K-252a shows an optimum concentration for influencing morphogenesis and pigmentation. We have as yet no reasonable explanation for this interesting phenomenon. In addition to the inhibition of aerial mycelium formation by the kinase inhibitors, they also inhibited Sm production of *S. griseus* in liquid culture and actinorhodin production of *S. coelicolor* A3(2) on solid medium. All these observations clearly show that *Streptomyces* strains possess several protein

Fig. 7. Effects of K-252a on the morphogenesis and pigment production of *S. griseus*.

A paper disc containing 230 μ g of K-252a was placed on agar medium seeded with spores of *S. griseus* IFO 13350. The plate carries a control paper disc containing dimethylsulfoxide used for dilution of K-252a. Photographs were taken after 7 days of growth (left). At higher concentrations of K-252a, strain IFO 13350 sporulates normally and at slightly lower concentrations, K-252a completely inhibits aerial mycelium formation. For clearer observation of the production of pigment, a photograph of the reverse side of the plate is shown (right). Concentration-dependence on K-252a of inhibition of pigment production is the same as that of aerial mycelium formation.

kinases of eucaryotic-type which are essential for morphogenesis and secondary metabolite formation. The discovery of multiple protein kinases probably involved in signal transduction for morphogenesis and secondary metabolism presents a new perspective in the study of *Streptomyces* biology.

3. AfsQ1/AfsQ2 system

The *afsQ1/afsQ2* genes were cloned from *S. coelicolor* A3(2) by the strategy used for cloning the *afsR* gene⁵². The *afsQ1* gene conferred on *S. lividans* phenotypes similar to those of *afsR* when introduced on a plasmid. The AfsQ1 protein with 225 amino acids shows great similarity in amino acid sequence to the response regulators of typical procaryotic two-component

regulatory systems. The termination codon, TGA, of *afsQ1* overlaps the initiation codon, GTG, of *afsQ2* encoding 535-amino-acid protein. The AfsQ2 product shows homology with the sensory histidine protein kinases of two-component systems. In agreement with the assumption that the AfsQ1 and AfsQ2 proteins comprise an aspartate-histidine phosphotransfer system, an amino acid replacement from Asp-52 to Glu, generated by site-directed mutagenesis, resulted in loss of the ability of AfsQ1 to stimulate antibiotic production in *S. lividans*. Alignment of AfsQ2 with other sensor proteins predicted that His-294 was the site of autophosphorylation. We speculate, by analogy with the well-characterized two-component systems^{40,41}, that AfsQ2 located in the mem-

brane is autophosphorylated at His-294 upon sensing a certain environmental signal and then the signal is transferred to AfsQ1 in the cytoplasm by means of phosphotransfer to Asp-52 (Fig. 6). The phosphorylated form of AfsQ1 would then serve as transcriptional activator for antibiotic production genes, probably via one or more intermediate regulatory genes. This type of regulation appears to function in general in *Streptomyces* spp., because DNA sequences hybridizing to *afsQ1* and *afsQ2* are widely distributed. It is thus apparent that procaryotic-type phosphorelays represented by *afsQ1/afsQ2* and eucaryotic-type phosphorelays represented by *afsK/afsR* are both involved in the regulation of secondary metabolism in a given *Streptomyces* spp.

Disruption of the chromosomal *afsQ1* or *afsQ2* gene of *S. coelicolor* A3(2) does not result in any detectable change in secondary metabolite formation or morphogenesis. This presents a contrast to the case of *afsR* and *afsK*; disruption of either *afsR* or *afsK* results in significant, but not complete, loss of pigment production. We assume that effects of mutation in *afsQ1* and *afsQ2* might be quite subtle under the usual laboratory cultural conditions. Suppression by *afsQ1* of a class (*absA*⁶⁶) of regulatory mutants deficient in pigment production supports the idea that the *afsQ1/afsQ2* system is involved in regulation of secondary metabolism, dependently on some regulatory genes and independently of other regulatory genes. The *afsR* gene also restores pigment production in the *absA* mutant. In addition to *afsB*, *afsK/afsR*, *afsQ1/afsQ2*, and *absA*, there are two other pleiotropic genes, *absB*⁶⁷) and *abaA*⁶⁸), affecting production of secondary metabolites in *S. coelicolor* A3(2). Clarification of the antibiotic regulation network including these pleiotropic genes requires further work and will be useful for a better understanding of antibiotic regulation in other *Streptomyces* spp.

CONCLUDING REMARKS

There remain questions to be answered about the A-factor regulatory cascade shown in Fig. 1. How is A-factor synthesis controlled in the life cycle? What is the gene(s) controlled by the A-factor receptor protein? How many regulatory proteins are involved in transferring the A-factor signal from the A-factor receptor to the A-factor-dependent binding protein acting as a transcription factor for *strR* and to the *amfR* operon whose expression seems to be repressed by A-factor? Furthermore, the relationship of the A-factor regulatory cascade and protein kinases that are sensitive to K-252a and staurosporine is yet unclear. Some phosphorelays appear to be directly included as intermediate regulatory steps in the cascade, because we have observed a change in the profile of phosphorylated proteins in the presence and absence of A-factor (unpublished data). The AmfR protein, probably a receiver of a phosphate from a still unidentified kinase, is a member of the signal relay specific to aerial mycelium formation in the cascade, although AmfR comprises a procaryotic-type histidine-aspartate phosphotransfer system. Genetic and biochemical approaches will make clear these points and lead to a better understanding of the hormonal control mediated by A-factor in *S. griseus* and by a large family of γ -butyrolactones in other *Streptomyces* spp.

The hormonal control exerted by γ -butyrolactones is a characteristic feature of actinomycetes. These diffusible autoregulators may be products of convergent evolution in filamentous microorganisms and may have evolved to act as chemical signals between the cells at a distance in the hyphae. Rapid sporulation of a whole population induced by the diffusible endogenous factors would be advantageous to the survival in the ecosystem, compared with piecemeal sporulation of individual hyphae induced by environmental stimuli such as nutri-

ent limitation. Absolute requirement of a specific receptor protein with a distinct ligand specificity suggests that the receptors have evolved concomitantly with the autoregulators in the same microorganisms. Such a system composed of different sets of autoregulators and receptor proteins would be useful for discriminating among different chemical signals and for preventing miscommunication among various actinomycetes in the ecosystem. On the other hand, there are two examples showing that a γ -butyrolactone acts as an autoregulator in single cell bacteria other than actinomycetes. One is an autoinducer (3-oxohexanoyl homoserine lactone) of bioluminescence in *Vibrio fischeri*⁶⁹⁾, and the other is a conjugation factor (*N*-acyl-L-homoserine lactone) involved in *Agrobacterium* conjugation via Ti plasmids⁷⁰⁾. The autoinducer in *V. fischeri* at a concentration of about 1 to 2 molecules per cell induces the enzymes for luminescence. The conjugation factor in *A. tumefaciens* controls conjugal transfer of Ti plasmid from *Agrobacterium* donors to bacterial recipients. These findings suggest that the hormonal control by a large family of γ -butyrolactones is present not only in filamentous actinomycetes but also in a rather wide variety of microorganisms.

Involvement of the AfsQ1/AfsQ2 two-component regulatory system in the regulation of secondary metabolism is not very surprising, since a variety of adaptive responses including sporulation in *B. subtilis* and virulence in *Agrobacterium* are controlled by similar phosphorelay systems⁴¹⁾. It is also not strange that filamentous *Streptomyces* spp. have developed the AfsR/AfsK system of eucaryotic-type as the regulator for secondary metabolism, regarded as physiological differentiation, since a large family of protein kinases mediate the responses of eucaryotic cells to external stimuli. This means that the ancestral kinase gene had already been present before eucaryotes appeared. The study of protein phosphorylation in

Streptomyces has just begun, and further work will provide a picture of the signal transduction via phosphorelays. Fundamental knowledges of *Streptomyces* biology and rational genetic breeding of *Streptomyces* spp. as antibiotic-producers will come from the elucidation of the mechanisms of "eucaryotic" signal transduction mediated by chemical signals like A-factor and by AfsK-type protein kinases.

ACKNOWLEDGMENTS

I express my sincere thanks to Dr. Kunimoto Hotta and the other members of SAJ for honoring me with the Award from the Society for Actinomycetes, Japan. My special thanks are to Professor Teruhiko Beppu who has guided and encouraged me throughout the work. The great efforts of all collaborators are also cordially acknowledged.

This work was supported in part by research grants from the Ministry of Education, Science, and Culture of Japan; the Japan Antibiotics Research Association; the Araki Memorial Foundation for Medical and Biochemical Researches; the Sankyo Foundation for Medical and Pharmaceutical Researches; the Mochida Memorial Foundation for Medical and Pharmaceutical Researches; the Kato Memorial Foundation for Bioscience Researches; the Waksman Foundation of Japan; and the Ministry of Agriculture, Forestry and Fisheries of Japan (BMP93-III-2-9).

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