A MICROBIAL HORMONE, A-FACTOR, AS A MASTER SWITCH FOR MORPHOLOGICAL DIFFERENTIATION AND SECONDARY METABOLISM IN *STREPTOMYCES GRISEUS*

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1. ABSTRACT

The Gram-positive, soil-inhabiting, filamentous bacterial genus *Streptomyces* employs γ-butyrolactones as chemical signalling molecules or microbial hormones, together with their specific receptors, to regulate morphological and/or physiological differentiation. The A-factor regulatory cascade in streptomycin-producing *Streptomyces griseus* commences aerial mycelium formation and production of all the secondary metabolites including streptomycin. The molecular mechanism by which A-factor triggers streptomycin biosynthesis or the A-factor signal is transmitted to the streptomycin biosynthetic gene cluster has been elucidated. A transcriptional activator AdpA at one of the regulatory steps switches on many genes required for both morphological development and secondary metabolism. Most of the gene cluster for secondary metabolite biosynthesis appear to receive the A-factor signal at the respective pathway-specific transcriptional activator genes via AdpA. Accumulating evidence has shown that a pair of genes encoding a probable γ-butyrolactone biosynthetic enzyme (AfsA-like protein) and its specific receptor (ArpA-like protein) is contained in a number of biosynthetic gene clusters for secondary metabolites in various *Streptomyces* species and controls the biosynthesis of the respective metabolites by activating the pathway-specific regulatory genes. Some strains contain multiple pairs of *afsA-arpA*. It is conceivable that, because of the almost same sites bound by various receptor proteins, the multiple ArpA-like proteins with the same or different ligand specificity in a cell competitively bind the same target sites, thus allowing the cell to grow healthy and to produce antibiotics in response to environmental conditions.

2. INTRODUCTION

The genus *Streptomyces*, which has probably evolved separately from *Bacillus subtilis* for more than 700 million years, comprises Gram-positive, soil-inhabiting, filamentous bacteria. It shows complex morphological differentiation resembling that of filamentous fungi, which makes this genus one of the model prokaryotes to study multicellular differentiation (1, 2). On agar medium, one or more substrate hyphae formed from a germinating spore branch frequently and grow rapidly by cell wall extention at the hyphal tips (Figure 1). Subsequently aerial hyphae emerge by reuse of material assimilated into the substrate mycelium, such as DNA, proteins, and storage compounds. Many cells in substrate hyphae thus lyse and die (3). When
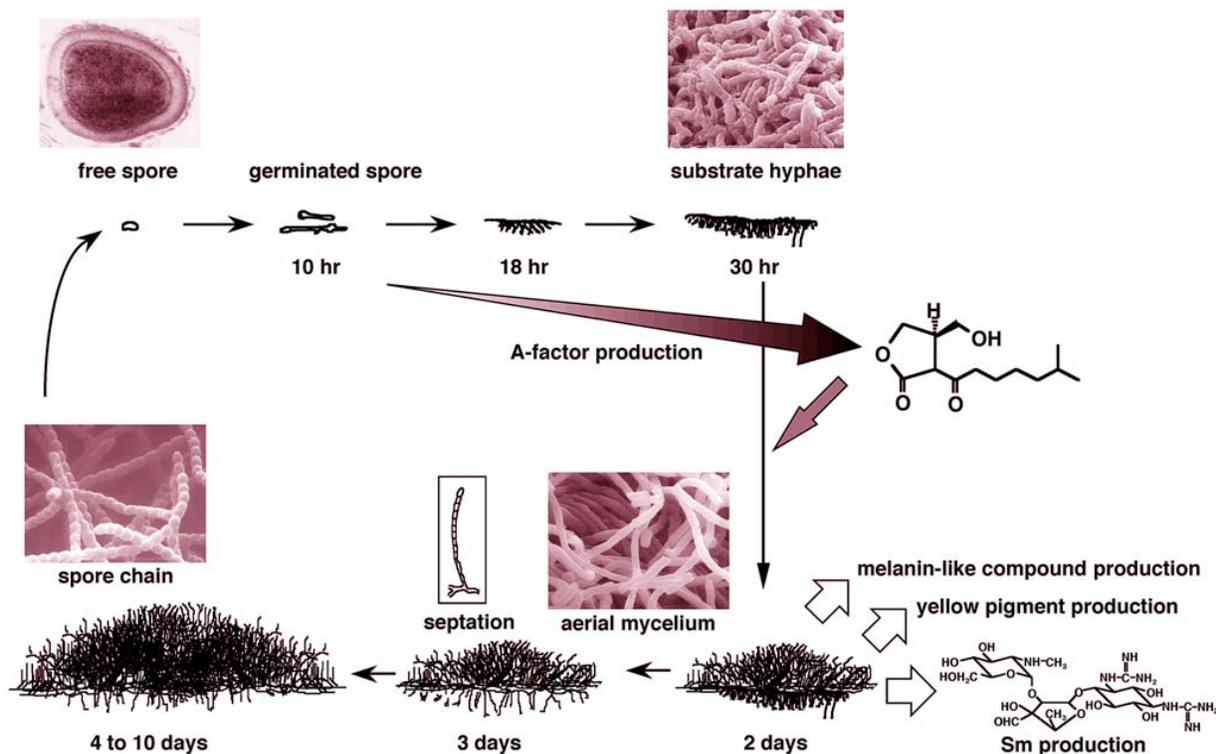
Figure 1. Life cycle of *S. griseus*. Substrate hyphae formed from a germinating spore branch frequently and grow by cell wall extension at the hyphal tips. Aerial hyphae erect from substrate mycelium by reuse of material assimilated in substrate hyphae, and septa are formed to create many unigenomic compartments, which then developed into chains of spores. Scanning electron micrographs at each of the growth stages are shown. A-factor, produced in a growth-dependent manner, switches-on the progression from substrate mycelium to aerial mycelium, and at the same time the production of secondary metabolites including streptomycin, a yellow pigment, and a melanin-like compound.

apical growth of aerial hyphae stops, in contrast to substrate mycelium, septa are formed at regular intervals along the hyphae to form many unigenomic compartments within a sheath composed of elongated hollow or grooved elements, finer fibrillar elements, and amorphous material. The sporulation septa consist of two membrane layers separated by a double layer of cell-wall material, which permits the eventual separation of adjacent spores (4, 5). Spore chains usually consist of many tens of spores. The aerial spores thus formed are resistant to heat-treatment and lysozyme-digestion. Another characteristic of the genus *Streptomyces* is the ability to produce a wide variety of secondary metabolites, including antibiotics and biologically active substances. 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.

A close relationship between the morphological differentiation and physiological differentiation has been well recognized through empirical observations that spontaneous loss of production of a certain antibiotic is frequently accompanied by the loss of sporulation in some *Streptomyces* spp. In addition, morphological development, as well as secondary metabolism, is under the control of various nutritional environments, such as carbon,

nitrogen, and phosphorous nutrients, and trace elements. It is therefore reasonable to assume that multiple genes involved in both the complex processes are regulated by a common regulatory gene or substance. The combination of recombinant DNA techniques and morphological mutants has enabled us to clone various genes that control both morphological and physiological differentiation (1, 6). Examples are *bld* mutants defective in both antibiotic production and aerial mycelium formation, depending on the carbon source, and *whi* mutants defective in spore formation. The *bld* and *whi* genes in *Streptomyces coelicolor* A3(2) have been most extensively characterized.

Chemical signaling molecules having a γ-butyrolactone also control both morphological and physiological differentiation of *Streptomyces* spp. The pioneer work of Khokhlov *et al.* (7, 8) on an autoregulatory factor (A-factor), which induces both sporulation and streptomycin biosynthesis in a mutant of *Streptomyces griseus*, revealed an exact link between secondary metabolism and morphological differentiation. Subsequent studies from several laboratories have indicated that A-factor and its derivatives in various *Streptomyces* spp. are actually autoregulators or microbial hormones that switch on morphological differentiation or antibiotic production, or both. Former studies on A-factor and its homologues
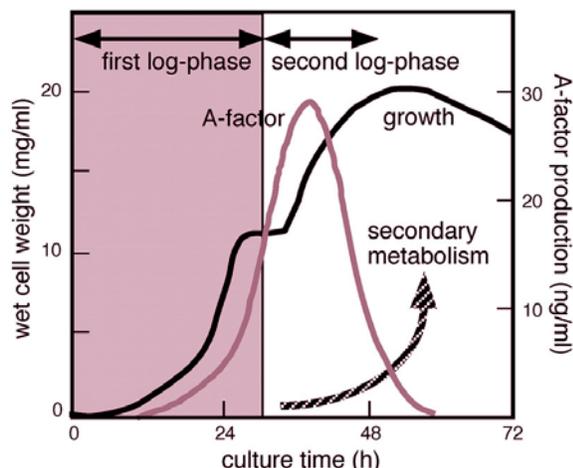
Figure 2. A-factor production and decision point in *S. griseus*. A-factor is produced in a growth-dependent manner and accumulated gradually until the decision point. Streptomycin production starts after the decision point which can be noticed by a transient cessation of cell growth.

were reviewed (9-11). In this review article, I focus on the A-factor regulatory cascade that leads to aerial mycelium formation and streptomycin biosynthesis in *S. griseus*. A-factor is the representative of the γ-butyrolactone-type regulators that control both morphological and physiological differentiation, although many other γ-butyrolactones serve as a regulator only for secondary metabolism, as discussed later.
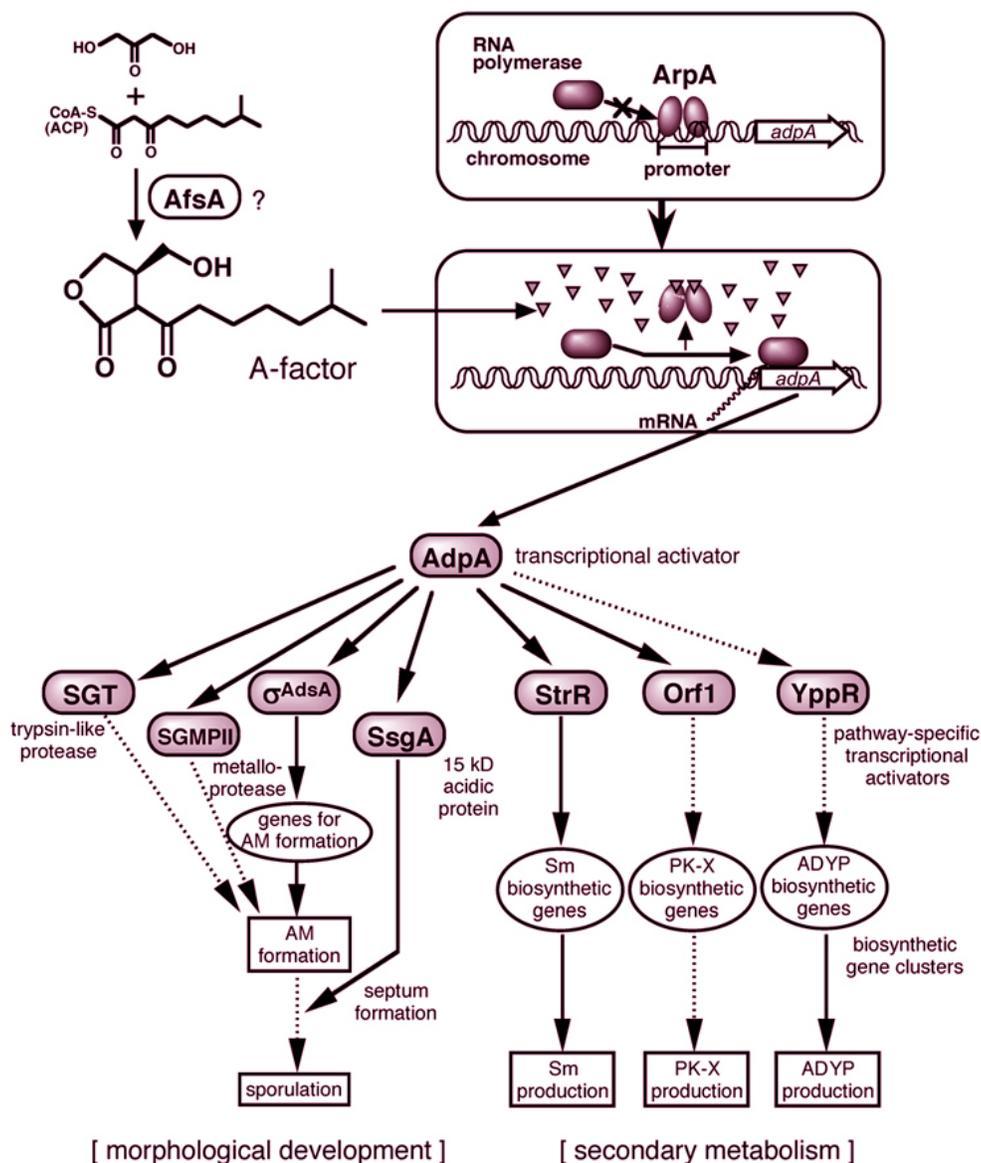
3. A-FACTOR AND DECISION POINT

A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone, for the structure see Figure 1) was originally discovered by Khokhlov *et al.* (7) as a diffusible extracellular molecule that causes sporulation of a bald mutant of *S. griseus*. They also found that A-factor is essential for not only aerial mycelium formation but also streptomycin biosynthesis in this mutant. Hara and Beppu (12, 13) confirmed their observations with stereochemically synthesized A-factor at a concentration as low as 10^{-9} M and further found that streptomycin resistance via streptomycin-6-phosphotransferase was also dependent on A-factor. In addition to these phenotypes, we found that A-factor induced production of a diffusible, yellow pigment (9). Thus, one can be easily convinced that A-factor is akin to eukaryotic hormones in its regulatory function.

A-factor is produced in a growth-dependent manner (Figure 2). The concentration of A-factor reaches 25 to 30 ng/ml at the late exponential growth phase and rapidly disappears thereafter (14). Since A-factor is transported freely across the membrane, the intra- and extracellular concentrations of A-factor are the same. The rapid decrease in the amount of A-factor after the exponential growth phase may be ascribed to no active transcription of *afsA* (see below) in the stationary phase and

chemical instability of A-factor. The half-life of A-factor is several hours in water or in fresh medium. The growth-dependent accumulation of A-factor seems to be important for programmed development, because exogenous addition of A-factor at a higher concentration to early culture inhibits growth and aerial mycelium formation (14). The idea that timing is critical for the switching function of A-factor is consistent with the observations that sporulation (15) and streptomycin biosynthesis (16) are determined at an early stage of the life cycle. After this time, A-factor exogenously supplemented to an A-factor-deficient mutant of *S. griseus* can no longer influence physiological or morphological differentiation (14, 16, 17). Neumann *et al.* (16) called this A-factor-sensitive period the "decision phase". The presence of a decision phase was also suggested in *Streptomyces hygroscopicus* (18). The decision point is usually observed at the middle of the exponential growth, which coincides with growth hesitation for 2 to 3 h (Figure 2). We call the growth phases before and after the decision point the first and the second exponential growth phases, respectively. During the decision phase, A-factor is thought to initiate an ordered sequence of metabolic events for programmed, healthy development. An abrupt increase of the A-factor concentration by exogenous addition to the very early culture presumably disturbs the ordered sequence of switching and set off an anomalous sequence of metabolic events. The switching function by A-factor can be followed in terms of a marked alteration in protein turnover, as determined by SDS-polyacrylamide gel electrophoresis (9, 19). Some proteins are produced in response to A-factor, and other proteins are decreased or repressed in production.

As a candidate of a gene encoding an A-factor biosynthetic enzyme, we cloned *afsA* as a gene that conferred A-factor production on an A-factor-deficient mutant of *S. griseus* (20, 21). AfsA of 301 amino acids conferred A-factor production on *Escherichia coli* as well as all the *Streptomyces* strains tested (22), suggesting that AfsA synthesizes A-factor from precursors commonly present in almost all bacteria. We speculate that AfsA condensates a glycerol derivative (C3 carbon unit) and a β-keto acid (C10 carbon unit) derived from the fatty acid biosynthetic pathway (Figure 3). The A-factor synthesis in *E. coli* harboring *afsA* was inhibited by cerulenin that is an inhibitor of β-ketoacyl-acyl carrier protein synthetase catalyzing the condensation of fatty acid biosynthesis (22). The presumptive β-keto acid may contain either acetyl-CoA or acyl carrier protein at the end. Homoserine lactones, which are structurally similar to A-factor and involved in quorum sensing in a variety of bacteria (23, 24), are synthesized from a β-keto acid derived from the fatty acid biosynthetic pathway and *S*-adenosylmethionine (25, 26). However, an *in vitro* experiment with purified AfsA is necessary to determine its role, because Kawachi *et al.* (27) reported that BarX, an AfsA homologue, seemed to be a co-repressor of BarA, an A-factor-receptor homologue.

Interestingly, *afsA* is located very near one end of the linear chromosome of *S. griseus* (28). The location of

Figure 3. The A-factor regulatory cascade in *S. griseus*. A-factor synthesized from a glycerol derivative and a β-keto acid by the action of AfsA, which is accumulated to a critical concentration at or just after the decision point, binds and dissociates the A-factor receptor protein (ArpA) from the promoter region of *adpA* encoding a transcriptional activator. AdpA then binds the promoter regions of many genes required for morphological development and secondary metabolism. For streptomycin biosynthesis, for example, AdpA activates *strR* encoding the pathway-specific transcriptional activator for other streptomycin production genes in the gene cluster. See text for details.

afsA accounts for the instability of streptomycin production and sporulation in this species; *afsA* is readily deleted on UV irradiation and acridine orange treatment of spores and on protoplast fusion of mycelium (20, 29). Since *afsA* encodes a protein essential for the synthesis of A-factor, the deletion of *afsA* results in abolishment of streptomycin production and aerial mycelium formation. This is the molecular basis of the simultaneous loss of streptomycin production and sporulation that has been empirically observed by those who are engaged in streptomycin fermentation.

4. A-FACTOR RECEPTOR PROTEIN (ArpA) AS A TRANSCRIPTIONAL REPRESSOR

The extremely low effective concentration of A-factor and its pleiotropic regulatory function prompted us to search for a specific receptor. By using the optically active

3R form of tritium-labeled A-factor, we identified a protein in the cytoplasmic fraction of *S. griseus* (30, 31). Because of free transport of A-factor across the membrane, *S. griseus* requires no membrane receptor. The A-factor-specific protein, named ArpA, bound A-factor in the molar ratio of 1:1 with a dissociation constant, K_d, of 0.7 nM. The small K_d value is consistent with the effective concentration of A-factor (10^{-9} M *in vivo*) and comparable to eukaryotic hormone receptors. ArpA was purified and partial amino acid sequences were determined to prepare oligonucleotides for cloning of the gene by PCR (32). The nucleotide sequence of *arpA* cloned in this way encoded a 276-amino-acid protein with a calculated molecular mass of 29.1 kDa. A-factor-binding assay with [^{3}H]A-factor showed that a homodimer of ArpA bound A-factor.

We selected oligonucleotides specifically bound by ArpA because it contained a helix-turn-helix DNA-binding motif at its NH$_2$-terminal portion. The method used for binding-site selection was to recover specific oligonucleotides from a pool of random-sequence oligonucleotides by rounds of a binding/immunoprecipitation/PCR amplification procedure with histidine-tagged ArpA (H-ArpA) and anti-ArpA antibody (33). The oligonucleotide pool contained a randomized region of 44 bp. Further binding-site selection experiments on the basis of the initially isolated oligonucleotide identified a 22 bp palindromic binding site, with the sequence 5'-GG(T/C)CGGT(A/T)(T/C)G(T/G)-3' as one-half of the palindrome, as a consensus sequence recognized and bound by ArpA. The nucleotides at positions 1, 2, 4, 5, 6, 7, and 10 were absolutely or almost absolutely conserved, and those at positions 3, 8, 9, and 11 preferred two nucleotides. The dyad symmetry of the binding site is in agreement with the idea that ArpA in the form of a homodimer binds the DNA; one subunit recognizes and binds to one-half of the palindrome and the other subunit binds to the other half.

Genetic studies showed that the A-factor receptor acted as a repressor for streptomycin production and sporulation (31). *S. griseus* mutant 2247, which had no ArpA activity but still produced a large amount of streptomycin, suggested that ArpA represses streptomycin production and sporulation. The screening for mutants producing streptomycin and forming spores in the Δ*afsA* background led to the isolation of four such mutants. As expected, these mutants were deficient in both A-factor-binding activity and A-factor production. Furthermore, introduction of *arpA* even on a low-copy-number plasmid into these mutants completely abolished the ability to produce streptomycin and to form spores, indicating that *arpA* served as a repressor for both streptomycin production and sporulation (32). In support of the repressor-type regulation of ArpA, the mutants deficient in ArpA begin to produce streptomycin 1 day earlier than the wild-type strain (31).

ArpA bound the DNA in the absence of A-factor, and did not bind the DNA in the presence of A-factor at concentrations more than 32 nM (33). In addition, exogenous addition of A-factor to the ArpA-DNA complex caused immediate dissociation of ArpA from the DNA. A-factor is produced in a growth-dependent manner and reaches 100 nM or 25 ng/ml in culture broth (Figure 2). It is therefore apparent that the target sites become free from ArpA gradually as the intracellular concentration of A-factor increases in a growth-dependent manner. This A-factor-mediated dissociation of ArpA from DNA accounts for the switching function of A-factor (Figure 3). It is also clear why streptomycin production starts after the decision point (Figure 2).

Functional amino acid residues of ArpA were identified by analysis of the nucleotide sequence of *arpA* in *S. griseus* mutant HO1 (34) and by sire-directed mutagenesis of *arpA* (35). Mutant HO1 had a Δ*afsA* genetic background but still produced streptomycin and formed spores. The nucleotide sequence of *arpA* in this mutant revealed an amino acid replacement at Pro-115 by Ser (34). The mutant ArpA (P115S) was still able to form a homodimer and possessed A-factor-binding ability but lost the ability to bind DNA. Site-directed mutagenesis identified Trp-119 as an essential amino acid residue for A-factor-binding but not for DNA-binding (35). An amino acid replacement at Trp-119 to Ala generated a mutant ArpA that was unable to bind A-factor, thus resulting in an A-factor-insensitive mutant that bound normally to its target DNA in both the presence and absence of A-factor. Val-41 in the helix-turn-helix DNA-binding domain at the NH$_2$-terminal portion of ArpA was found to be essential for DNA-binding but not for A-factor-binding. These data suggest that ArpA consists of two functional domains, one for helix-turn-helix DNA-binding at the NH$_2$-terminal portion and one for A-factor-binding at the COOH-terminal portion. As a model for A-factor-mediated dissociation of ArpA from DNA, we suppose that A-factor binding to the COOH-terminal domain causes a conformational change in the distal DNA-binding domain, resulting in dissociation of ArpA from the DNA.

5. A-FACTOR SIGNAL TRANSFER FROM ArpA TO STREPTOMYCIN BIOSYNTHETIC GENES VIA AdpA

Transcription of the streptomycin biosynthetic genes are supposedly controlled by A-factor because A-factor switches-on streptomycin production. The data from our laboratory (36) and a detailed study of Distler *et al.* (37) on the transcriptional organization of part of the streptomycin biosynthetic gene cluster showed that one mRNA species covering a regulatory gene (*strR*) and the streptomycin-6-phosphotransferase (*aphD*) gene were dependent on A-factor, as determined by S1 nuclease mapping. We then tried to detect and purify a protein that might bind the promoter region of *strR-aphD*, on the assumption that the A-factor signal is transmitted via ArpA and some other regulatory proteins to the A-factor-dependent promoter. As a result, an A-factor-responsive protein (AdpA) able to bind the upstream activation sequence, about 270 bp upstream of the transcriptional start point of *strR*, was detected by gel mobility shift assay (38). StrR was later found to be a pathway-specific

transcriptional activator for all the streptomycin biosynthetic genes (39). AdpA was detected only in the presence of A-factor. After purification of AdpA and determination of partial amino acid sequences, the *adpA* gene was cloned by PCR (40). AdpA encoding a 405-amino-acid protein with a helix-turn-helix DNA-binding motif at the central portion showed sequence similarity to transcriptional regulators belonging to the AraC/XylS family.

The -35 and -10 regions of *adpA* contained a 22 bp palindrome, caggcAGGAACGGACC*GCGCGGTACGCt (the underlines indicate the -35 and -10 promoter elements; * indicates a dyad axis), which showed similarity to the consensus sequence of the ArpA-binding site, (A/C)C(A/G)(T/A)ACCC(A/G)CC*GG(T/C)CGGT(A/T)(T/C)G(T/G). As expected, ArpA bound the promoter region of *adpA* in the absence of A-factor but did not in the presence of A-factor. In addition, exogenous addition of A-factor to the ArpA-DNA complex immediately dissociated ArpA from the DNA. Thus, the promoter of *adpA* turned out to be a target of ArpA. Consistent with this, S1 nuclease mapping showed that *adpA* was transcribed only in the presence of A-factor and *strR* was transcribed only in the presence of intact *adpA*. Furthermore, *adpA* disruptants produced no streptomycin and overexpression of *adpA* caused the wild-type *S. griseus* strain to produce streptomycin at an earlier growth stage in a larger amount.

The signal relay from A-factor to the streptomycin biosynthetic genes has at last established; from *afsA* to *arpA* to *adpA* to *strR* to the streptomycin production genes (Figure 3). A-factor produced probably by the action of AfsA in a growth-dependent manner binds ArpA that has bound and repressed the promoter of *adpA* in an early growth stage. When the concentration of A-factor reaches a critical level, it binds the DNA-bound ArpA and dissociates ArpA from the DNA, thus causing transcription of *adpA*. AdpA thus induced binds the upstream activation sequence of *strR* and activates its transcription. The pathway-specific transcriptional activator StrR then induces transcription of other streptomycin biosynthetic genes by binding multiple sites in the gene cluster, thus leading to biosynthesis of streptomycin from glucose.

The mRNA starting at the promoter of *adpA* extends into *ornA* encoding an oligoribonuclease, which shows that the amount of the oligoribonuclease is also enhanced by A-factor (41). Because *ornA*-disruptants grew slowly and scarcely formed aerial mycelium, it plays an important role in vegetative growth and in the initiation of differentiation by degrading mRNA and supplying monoribonucleotides to the cells during the second exponential growth period. The major streptomycin resistance determinant, *aphD*, downstream of *strR* is also transcribed by read-through from the A-factor-dependent *strR* promoter. The co-transcription of *strR* and *aphD* accounts for the prompt induction of streptomycin resistance by A-factor and achieves a rapid increase in self-resistance just before induction of streptomycin biosynthesis.

6. AdpA REGULON: MULTIPLE TARGETS OF AdpA

adpA disruptants also failed to produce a yellow pigment, suggesting that the biosynthetic genes for the pigment was under the control of AdpA (40). Furthermore, disruption of *adpA* caused the host to show a bald phenotype. These observations implied that AdpA controls multiple, unlinked genes necessary for physiological and morphological differentiation. We started isolating multiple genes as DNA fragments bound by AdpA. For this purpose, a library of fragmented chromosomal DNA of about 300 to 500 bp from *S. griseus* was constructed either by *Hae*III digestion or by sonication, followed by attachment of a linker at the ends. The library was incubated with AdpA to allow formation of protein-DNA complexes. The complex was separated from free DNA by gel mobility shift and the DNA in the complexes was purified by extraction from gel pieces. The purified DNA was then amplified by PCR with the linkers as primers. For enrichment of the DNA fragments, the PCR product was subjected to the second and further cycles of the gel mobility shift-PCR procedure. Repeated experiments yielded 60 DNA fragments that were specifically bound by AdpA (42, 43). The presence of many genes, all of which are simultaneously activated by AdpA at a specific point in the growth phase, means that the signal from A-factor is greatly amplified at this regulatory step via AdpA as an amplifier. We have so far analyzed several of these isolated fragments.

6.1. An extracytoplasmic function sigma factor AdsA

The DNA fragment most abundantly obtained was located in front of a gene, named *adsA*, encoding an extracytoplasmic function (ECF) σ factor belonging to a subgroup of the primary σ^{70} family (42). As expected, transcription of *adsA* depended on A-factor and *adpA*. DNase I footprinting identified the AdpA-binding site of the antisense strand to be positions from +7 to +41 with respect to the transcriptional start point of *adsA*, and that of the sense strand to be positions from +12 to +46. A weak palindrome was found in the binding site. The unusual position of the binding site of AdpA as an activator suggests the presence of a mechanism by which AdpA activates transcription of *adsA* in some unknown way. The *in vivo* function of *adsA* was determined to be involved only in aerial mycelium formation and not in secondary metabolite formation, since Δ*adsA* mutants failed to form aerial mycelium but still produced streptomycin and a yellow pigment. σ^{AdsA} also serves as an amplifier of the A-factor signal since it supposedly transcribes multiple genes required for ordered development.

In the A-factor regulatory pathway, *afsA*, *arpA* and *adpA* are common to secondary metabolism and morphological differentiation, because disruption of either one of these genes results in abolishment of both the phenotypes. AdpA therefore serves as a switch for many genes, each of which is necessary for respective phenotypes under the control of A-factor. Although σ^{AdsA} is one of the important proteins required for normal morphological

development, introduction of multicopies of *adsA* or the *adsA*-coding sequence under the control of the *lac* promoter into Δ*adpA* mutants does not restore aerial mycelium and spore formation in these mutants, which indicates the presence of additional genes that are required for aerial mycelium formation and controlled by AdpA. Consistent with this idea, AdpA controls the expression of several protease genes that are concerned with aerial mycelium formation (see below).

The orthologue of *adsA* in *S. coelicolor* A3(2) is *bldN* that has been cloned by means of complementation of a bald mutant (44). In the hierarchy of *bld* genes in *S. coelicolor* A3(2), transcription of *bldN* depends on *bldG* and *bldH*, but not on *bldA*, *bldB*, *bldC*, *bldF*, *bldK* or *bldJ*. *bldN* is also repressed by BldD, a transcriptional factor for aerial mycelium formation, to prevent premature expression during vegetative growth (45). The importance of the hierarchy of the *bld* genes in *S. griseus* is unclear because of lack of genetic studies on *bld* genes in this species. Nevertheless, we can safely say that the A-factor signal is transmitted to *adsA* via AdpA and starts the downstream sequence of the regulatory cascade leading to aerial mycelium formation in a timely fashion. The *bld* genes in *S. coelicolor* A3(2) depend on the carbon source, and dispensable for sporulation on, for example, mannitol-containing medium. However, the switching by A-factor does not depend on any nutritional conditions.

6.2. Metalloendopeptidase and trypsin-like protease

Characterization of the DNA fragments bound by AdpA identified two protease genes (unpublished results). These are metalloendopeptidase (*sgmA*; 46) and trypsin-like protease (*sprT*; 47) genes. Both proteases are extracellular enzymes. Transcription of these genes is developmentally regulated because of the dependence on A-factor and AdpA. Although AdpA bound two sites, positions about -60 and -255, upstream of the transcriptional start point of *sgmA*, it is unclear if both or either one of the two sites is required for transcriptional activation. How are these proteases concerned with aerial mycelium formation or secondary metabolism? We presume that, in consideration of the emergence of aerial hyphae into the air from substrate hyphae, these proteases hydrolyze proteins assimilated into the substrate hyphae for reuse of amino acids for aerial mycelium formation. Consistent with this idea, disruption of *sgmA* results in a delay of growth and aerial mycelium formation. At this stage of development, hydrolyzing enzymes for degradation of proteins, lipids, glucans, and other storage compounds in the substrate hyphae are presumably required. It is possible that AdpA switches on the multiple genes encoding such hydrolyzing enzymes, in addition to SgmA and SprT. The control by AdpA of multiple genes required for healthy development explains why introduction of *adsA* into the Δ*adpA* mutant failed to restore aerial mycelium formation.

6.3. *ssgA* required for septation in aerial hyphae

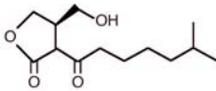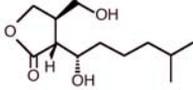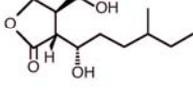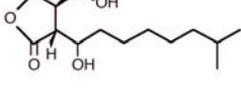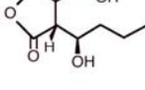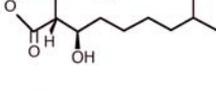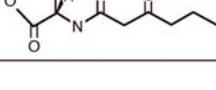We also identified a DNA fragment locating in front of *ssgA*, which had been reported to be essential for septum formation in aerial mycelium in *S. griseus* (48) and *S. coelicolor* A3(2) (49). AdpA bound three sites, positions +60, -110, and -235 with respect to the major transcriptional start point of *ssgA*. Transcription of *ssgA* was almost abolished in the *S. griseus* Δ*adpA* mutant, indicating its dependence on AdpA and A-factor. In *S. griseus*, *ssgA* appears to be controlled in response to nutritional conditions mainly by *ssfR* that is encoded immediately upstream of *ssgA* (48). This means that *ssgA* is controlled by two different regulatory pathways; one by *ssfR* probably sensing nutritional conditions and the other by the A-factor regulatory cascade acting like a clock by sensing the concentration of A-factor. Δ*ssgA* mutants of both *S. griseus* and *S. coelicolor* A3(2) showed a "white" phenotype, indicative of aerial hyphae formation. This is an exceptional example to show that A-factor controls a step for morphological development after aerial hyphae have been formed; all the steps controlled by A-factor hitherto observed are involved in transition from substrate hyphae to aerial hyphae.

6.4. Pathway-specific transcriptional activators

In *S. griseus*, A-factor controls the biosynthesis of all the secondary metabolites, including streptomycin and a yellow pigment. This means that every biosynthetic gene cluster for secondary metabolites contains a receiver of the A-factor signal via AdpA, like *strR* in the streptomycin biosynthetic gene cluster. Among the DNA fragments bound by AdpA, we found a gene encoding a large ATP-binding regulator of the LuxR family (Orf1 for PK-X biosynthetic genes in Figure 3). Examples in this family are PikD for pikromycin biosynthesis in *Streptomyces venezuelae* (50), RapH for rapamycin biosynthesis in *Streptomyces hygroscopicus* (51), and AveR for avermectin biosynthesis in *Streptomyces avermitilis* (52). These regulators are supposed to act as pathway-specific transcriptional activators for the respective macrolide antibiotics (53). Downstream of the regulatory gene probably controlled by AdpA, a type I polyketide synthase gene is present, which strongly suggests that the regulatory gene found as an AdpA-binding site serves as the pathway-specific transcriptional activator gene for the probable macrolide antibiotic. We are currently determining the chemical structure of the macrolide antibiotic.

The biosynthetic gene cluster for the yellow pigment, whose production is dependent on A-factor, was cloned by shotgun cloning with a mutant defective in the pigment production as the host. The major yellow pigment was identified to be an actinomycin derivative (unpublished results). A gene encoding a transcriptional activator belonging to an expanding family of regulatory proteins (SARPs, *Streptomyces* antibiotic regulatory proteins) (54), including ActII-ORF4 for actinorhodin biosynthesis (55) and RedD for undecylprodigiosin biosynthesis (56) in *S. coelicolor* A3(2), was present as a member of the gene cluster. We are currently characterizing the regulatory gene, on the assumption that it serves as a receiver of the A-factor signal as the pathway-specific activator gene for the yellow pigment biosynthesis.

Table 1. Gamma-Butyrolactones in *Streptomyces* species

Factors	Producer	Biological activity	References
	S. griseus (A-factor)	Streptomycin Yellow pigment Sporulation	Khokhlov (8); Mori (79)
	S. virginiae (VB-A)	Virginiamycin	Yamada *et al.* (62)
	S. virginiae (VB-B)	Virginiamycin	Yamada *et al.* (62)
	S. bikiniensis, S. cyaneofuscatus	Anthracycline	Gräfe *et al.* (65)
	S. lavendulae (IM-2)	Showdomycin Minimycin	Hashimoto *et al.* (64)
	S. coelicolor A3(2) (SCB1)	Actinorhodin Undecylprodigiosin	Takano *et al.* (66)
	V. fischeri	Bioluminescence	Eberhard *et al.* (80)

6.5. Melanin production genes

During our shot-gun cloning experiments using an A-factor-deficient mutant of *S. griseus* as the host and the chromosomal DNA from the wild-type strain, we happened to clone a DNA fragment that conferred red-brown pigment production on the host (57). This pigment is produced in an almost unnoticeable amount by the wild-type strain. Subsequent studies on the cloned fragment have shown that the production of the pigment depends on A-factor, either directly or indirectly. The cloned fragment encodes a chalcone synthase-related polyketide synthase, named RppA, and a P-450 enzyme, both of which are responsible for pigment production (58, 59). The 372-amino-acid *rppA* product, which was the first enzyme categorized in type III polyketide synthases, selects malonyl-CoA as the starter, carries out four successive extensions and releases the resulting pentaketide to cyclize to 1,3,6,8-tetrahydroxynaphthalene (THN). THN is then converted to a melanin-like compound by the action of the P-450 enzyme (unpublished data). It is still unknown whether AdpA directly controls the expression of these genes. These genes may be transcribed by the RNA polymerase with a certain σ factor that is under the control of AdpA.

7. A-FACTOR HOMOLOGUES

Hara and Beppu (12) screened for compounds having A-factor activity on an A-factor-deficient mutant strain of *S. griseus*, on the assumption that regulatory compounds are not necessarily specific for a given strain, and found a wide distribution of such compounds among actinomycetes including *Streptomyces*, *Actinomyces*, and *Nocardia*. Similar observations were reported by Eritt *et al.* (60) and Efremenkova *et al.* (61). In addition to these compounds, several other A-factor-like compounds have been also reported (10, 11). Examples are shown in Table 1; these include a series of virginiae butanolides (VBs) A to E controlling virginiamycin production in *Streptomyces virginiae* (62, 63), IM-2 controlling production of showdomycin and minimycin in *Streptomyces lavendulae* (64), a regulatory factor controlling both morphological differentiation and anthracycline biosynthesis in *Streptomyces bikiniensis* and *Streptomyces cyaneofuscatus* (65), and SCB1 stimulating antibiotic production in *S. coelicolor* A3(2) (66). One of the homoserine lactones involved in quorum sensing (23, 24) is also shown for comparison of the chemical structures. The wide distribution of A-factor-like compounds is consistent with the observation that sequences homologous to *afsA* encoding a probable A-factor biosynthetic enzyme are present in a wide variety of actinomycetes (20).

Nucleotide sequences homologous to *arpA* encoding the A-factor receptor (32), *barA* encoding the VB receptor (67), *farA* encoding the IM-2 receptor (68), and *scbR* encoding the SCB1 receptor protein (69) have also been found in a wide variety of actinomycetes. BarA and

FarA serve as repressors for production of the respective antibiotics (70, 71), just like ArpA for streptomycin production. The ligands for these four receptors are known, although the ligands for the following ArpA homologues are still not known. CprA and CprB in *S. coelicolor* A3(2) (72), TylP (73) and TylQ (74) both in the tylosin biosynthetic gene cluster in different strains of *Streptomyces fradiae* and an *arpA*-like gene in the biosynthetic gene clusters for nikkomycin in *Streptomyces tendae* (75) and for pristinamycin in *Streptomyces pristinaespiralis* (76) are examples. The genome sequencing of *Streptomyces* strains will reveal a wide distribution of *afsA* and *arpA* homologues in this genus.

It should be noted that each of the receptors has strict ligand specificity. ArpA does not bind IM-2 and BarA does not bind A-factor. Consistent with this, IM-2 or VBs exert no *in vivo* effect on an A-factor-deficient mutant of *S. griseus*. We assume that during evolution *Streptomyces* spp. have developed γ-butyrolactones as "hormonal regulators" in conjunction with their specific receptors to control different stages of morphological and/or physiological differentiation in the regulatory hierarchy. Co-evolution of the receptor and its ligand has been facilitated by the adjacent location of the two genes; a pair of *arpA* and *afsA* homologues, such as *barA* and *barX*, *farA* and *farX*, and *scbR* and *scbA*, are adjacent, although *afsA* and *arpA* in *S. griseus* are encoded by the regions very far from each other (28; H. Kinashi, personal communication). It is also consistent with the fact that a given strain contains multiple *arpA* homologues. *S. coelicolor* A3(2) contains at least four *arpA* homologues, *scbR*, *cprA*, *cprB*, and an *arpA* homologue in the methylenomycin biosynthetic gene cluster, and a series of A-factor homologues including SCB1 (66, 77). A-factor switches-on the very early step common to both morphological and physiological differentiation in the hierarchy, thus controlling both processes. VB and IM-2 switch-on the later step concerned only with secondary metabolism, thus controlling only antibiotic production. This may reflect the course of VB production; VB is produced abruptly just before antibiotic production, which is a contrast to A-factor which is produced in a growth-dependent manner during the first exponential growth phase and at a little accelerated rate during the second phase (Figure 2). In addition, the *barA* and *barX* genes responsible for the VB receptor and probable VB synthetic enzyme (67, 70) and the *farA* and *farX* genes for the IM-2 system (68, 71) are members in the biosynthetic gene clusters for respective secondary metabolites. We therefore expect that an operon containing *afsA* and *arpA* homologues in a biosynthetic gene cluster for a certain secondary metabolite influences the transcription of the gene cluster, thus regulating the biosynthesis of the secondary metabolite. The *arpA* homologues in the biosynthetic gene cluster for tylosin (73, 74), nikkomycin (75), methylenomycin (oral presentation by S. J. O'Rourke & K. F. Chater at ISBA Symposium in Vancouver, August, 2001), and lankacidin and lankamycin (oral presentation by K. Yamada *et al.* at the Annual Meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry in Kyoto, March, 2001) may be categorized in this group.

An exception is *spbR*, encoding an ArpA homologue, in the pristinamycin biosynthetic gene cluster (76). SpbR controls not only pristinamycin production by binding to the promoter region of the pathway-specific transcriptional activator *pap*R1 but also morphological differentiation and expression of a superoxide dismutase. Furthermore, mutations in *spbR* suggest that it serves as a positive regulator for these phenotypes, which is a contrast to *arpA*, *barA*, and *farA*, all serving as negative regulators for morphological and/or physiological differentiation. It may be possible that ArpA-type DNA-binding proteins serve as a transcriptional activator. In fact, gene disruption experiments with *cprA* in *S. coelicolor* A3(2) suggest its positive function for actinorhodin production and aerial mycelium formation (72).

An additional note I would like to make is that the ArpA family recognize and bind the same binding sites. Folcher *et al.* (76) compiled the binding sites and predicted candidates in the DNA database. This is reasonable because the DNA-binding domains including helix-turn-helix motifs of this family are highly conserved. CprA and CprB bind the ArpA-binding sites (35), and ScbR and SpbR share the same binding sites (76). It is therefore conceivable that each of the multiple ArpA proteins in a given *Streptomyces* strain recognizes and binds cognate binding sites at a specific stage under normal growth conditions but under some conditions the concentrations of the ArpA proteins are disturbed and some of them competitively bind additional binding sites, resulting in patterns of gene expression that are different from those of the normally growing cells.

8. CONCLUDING REMARKS AND BIOLOGICAL IMPLICATIONS

Streptomyces is one of the representative genera in the complex environment in the soil. The branched filamentous morphology with differentiated hyphae and spores is akin to that of fungi that contain sex pheromones with functional features similar to γ-butyrolactones. The γ-butyrolactone-type autoregulators produced at a portion of a hypha in response to some environmental stimuli can freely move within the individual hypha and spread into neighboring hyphae. Because of the filamentous growth, *Streptomyces* may have developed the diffusible γ-butyrolactone regulatory systems so that these facilitate communication between the cells at a distance within an individual hypha and between different hyphae. The filamentous mycelia of *Streptomyces* are close enough to communicate to each other. The signaling system between physically separate individual cells in the same mycelium can be termed a hormonal regulation, rather than a quorum sensing found in the Gram-negative single-cell bacteria growing in liquid culture (23, 25). However, A-factor is also important for cell-cell communication between neighboring mycelia, similar to the quorum sensing system. Since a given *Streptomyces* strain contains its own γ-butyrolactone and its receptor with strict ligand specificity, this system also facilitates discrimination of the signal originated from neighboring living things, thus allowing the cell to recognize the neighbor to be a member of the same

species or not. This system is also advantageous to the survival in the ecosystem; A-factor produced by a cell is accepted by several hyphae and causes rapid sporulation of a whole population, which is advantageous compared with piecemeal sporulation of individual hyphae induced by environmental stimuli such as nutritional limitation.

The A-factor and receptor system in *S. griseus* acts as an all-or-none switch for both morphological and physiological differentiation. CprA and CprB act as a tuner for these processes in *S. coelicolor* A3(2). On the other hand, the VB system in *S. virginiae* also controls the timing of antibiotic production but not morphological development. These observations imply that *Streptomyces* has evolved the γ-butyrolactone regulatory system to control different steps in the regulatory hierarchy for health growth, as an all-or-none switch for some phenotypes and as just a tuner for other phenotypes. That may be why a *Streptomyces* strain contains redundant γ-butyrolactone regulatory systems.

It is conceivable that *Streptomyces* employs diffusible low-molecular-weight chemicals, other than γ-butyrolactones, for cell-to-cell communication. Although several such compounds were reported (reviewed in ref. 9, 10), no definitive or sufficient data to be termed autoregulators have been obtained because of lack of genetic studies. In relation to chemical signalling molecules, Ueda *et al.* (78) conducted extensive pairing analyses with various *Streptomyces* species to examine the possibility of interspecific stimulation of antibiotic production and/or sporulation. Surprisingly, various levels of stimulation of antibiotic production and/or sporulation was observed in various combinations at high frequency. *Streptomyces* species may produce a common substance(s) to help each other in the ecosystem, in addition to γ-butyrolactones to discriminate each other.

9. ACKNOWLEDGMENTS

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