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AmfS, an Extracellular Peptidic Morphogen in \textit{Streptomyces griseus}

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Received 24 September 2001/Accepted 29 November 2001

The \textit{amf} gene cluster was previously identified as a regulator for the onset of aerial-mycelium formation in \textit{Streptomyces griseus}. The nucleotide sequences of \textit{amf} and its counterparts in other species revealed a conserved gene organization consisting of five open reading frames. A nonsense mutation in \textit{amfS}, encoding a 43-amino-acid peptide, caused significant blocking of aerial-mycelium formation and streptomycin production, suggesting its role as a regulatory molecule. Extracellular-complementation tests for the aerial-mycelium-deficient phenotype of the \textit{amfS} mutant demonstrated that AmfS was secreted by the wild-type strain. A null mutation in \textit{amfR}, encoding HlyB-like membrane translocators, abolished the extracellular AmfS activity without affecting the wild-type morphology, which suggests that AmfRA is involved not in production but in export of AmfS. A synthetic C-terminal octapeptide partially induced aerial-mycelium formation in the \textit{amfS} mutant, which suggests that an AmfS derivative, but not AmfS itself, serves as an extracellular morphogen.

The filamentous, soil-inhabiting, gram-positive bacterial genus \textit{Streptomyces} is characterized by the ability both to undergo complex cellular differentiation resembling that of filamentous fungi and to produce a wide variety of secondary metabolites (4--6). The morphological and physiological development of the organism is controlled by complex regulatory circuits consisting of various regulatory proteins. In the streptomycin-producing species \textit{Streptomyces griseus}, both morphological differentiation and antibiotic production are regulated by an autoregulatory hormonal substance, A-factor (2-isocapryloyl-3-R-hydroxymethyl-\(\gamma\)-butyrolactone) (9--11). The autoregulator induces the transcription of \textit{adpA}, the central positive regulator, by inactivating ArpA, which acts as a transcriptional repressor for \textit{adpA} (18, 19). \textit{AdpA} then pleiotropically induces the transcription of genes, such as \textit{adsA} (29) and \textit{strR} (25, 26), encoding specific positive transcriptional regulatory proteins for morphogenesis and streptomycin biosynthesis, respectively.

We previously identified the \textit{amf} gene cluster as a positive regulator for the onset of aerial-mycelium formation in \textit{S. griseus} (23, 24). The \textit{amf} region, which induced aerial-mycelium formation in an A-factor-deficient mutant strain, HH1, contained four complete open reading frames (ORFs): \textit{amfR} encoded a response regulator of a two-component regulatory system, which is essential for the initiation of aerial-mycelium formation, and both \textit{amfA} and \textit{amfB} encoded ABC transporters homologous to HlyB of \textit{Escherichia coli} (3). Additionally, a small possible ORF encoding a peptide consisting of 43 amino acids (aa) (ORF6) was present in \textit{amfB}. The presence of \textit{amfA} and \textit{amfR} on a high-copy-number plasmid was sufficient for the restoration of the aerial mycelium in mutant HH1 (23). While \textit{AmfR} was reasonably deduced to be a transcriptional regulator that may control the expression of genes involved in the onset of cell differentiation, the substrate of the transporters AmfA and AmfB was unclear. Here, we report mutational analysis of the small ORF, now named \textit{amfS}. Several lines of evidence suggest that the gene product is modified and secreted by the AmfAB-mediated transport system to act as an extracellular peptidic morphogen.

\textbf{Gene organization of \textit{amf} and its counterparts.} The original clone containing the \textit{amf} cluster was a 9-kb \textit{Sau3A} fragment (Fig. 1A) (23). The nucleotide sequence of the region upstream from \textit{amfS} revealed a large ORF, \textit{amfT}, encoding a transmembrane protein of 891 aa that showed some similarity to Ser/Thr kinases (7). A subsequent homology search in the completed genomic database of \textit{Streptomyces coelicolor} A3(2) (http://www.sanger.ac.uk/Projects/S_coelicolor/) revealed that the genes \textit{amfT}-\textit{amfS}-\textit{amfB}-\textit{amfA}-\textit{amfR} were conserved in this organism (corresponding to \textit{SC5A7.31} to -35) and the homologous genes include the ORFs previously identified as \textit{ram}. The \textit{ram} gene cluster, consisting of \textit{ramABR} (corresponding to \textit{amfBAR} of \textit{S. griseus}), is known to stimulate aerial-mycelium formation in \textit{Streptomyces lividans} when introduced on a high-copy-number plasmid (12, 14). The gene cluster was also conserved in \textit{Streptomyces avermitilis}, whose genome sequence has recently been completed (H. Ikeda and S. Ōmura, personal communication). We therefore assumed that the proteins encoded by the five ORFs have functional relationships in regulating aerial-mycelium formation. Figure 1B shows the sequence alignment of AmfS and its counterpart in \textit{S. coelicolor} A3(2). The peptides carried an N-terminal acidic region and C-terminal repeats of hydrophobic sequences \([\text{V/L}-\text{S-}\text{L/V}-(\text{L/V})-\text{V/L}-\text{C}]\) for AmfS. The two C-terminal Cys residues conserved in the peptides might play a role important for the structure and function of the molecules. AmfS and the homolog were preceded by an identical potential ribosome-binding sequence (GAAAAGGA). These observations led us to assume that the small ORFs are translated into peptides and could be secreted via the function of the translocators to play some role for morphogenesis.

\textbf{Inactivation of \textit{amfS}.} To assess the role of \textit{amfS}, we disrupted it by the homologous-recombination technique. The...
mutant was generated by using plasmid pDIS-S, which contained a mutated amfS sequence on pUC19 (Fig. 1A). The mutagenized construct, carrying both a nonsense mutation at AAGCTT and a high-copy-number plasmid, pIJ487 (27), were introduced into E. coli (Fig. 1C), was generated by combining the fragments 700-bp region at the amfT site and the po-

dIII fragment. For fragment

mutants generated by the second crossover were screened for

amfS mutant was generated by using plasmid pDIS-S, which con-
tained a mutated amfS sequence on pUC19 (Fig. 1A). The

mutant was recovered as an EcoRI and BamHI fragment by

the standard transformation procedure, and the inte-
grants carrying the plasmid-derived DNA inserted at the

amfS locus via a single-crossover event were screened for

among the resultant kanamycin-resistant colonies. The inte-
grants isolated were checked for the correct recombination by

Southern hybridization, and one of the true recombinants was

then cultured in YMP-glucose liquid medium (containing [in

grams per liter] yeast extract [Difco], 2; meat extract [Kyokuto],

2; Bacto Peptone [Difco], 4; NaCl [Kokusan], 5; MgSO4

[Kokusan], 2; and glucose [Kokusan], 10 [pH 7.2]) without

kanamycin. The cells were then plated onto YMP-glucose solid

medium (containing 1.5% agar [Kokusan] in YMP-glucose)

after appropriate dilution, and kanamycin-sensitive recombi-
nants generated by the second crossover were screened for

among the resultant colonies. Consequently, three amfS mu-
tants were isolated among the kanamycin-sensitive colonies, all

of which showed the phenotype described below. The correct

replacement of the wild-type amfS with the mutated construct

was confirmed by Southern hybridization. The standard mo-

cular recombination techniques in E. coli and Streptomyces

spp. were described in the protocols by Maniatis et al. (15) and

Hopwood et al. (8), respectively.

The resultant amfS mutant showed a bald phenotype (Fig. 2);

on YMP-glucose solid medium, the amfS mutant was unable to

form aerial mycelia. The deficiency was partially recovered on

YMP-maltose medium (containing 1% maltose instead of the

glucose in YMP-glucose medium), but aerial-mycelium forma-

tion was still significantly reduced (not shown). Streptomycin

production was also markedly reduced on all the solid media

tested. The phenotype of the mutant clearly indicates that

amfS plays an important role in cellular differentiation and

antibiotic production in S. griseus. The A-factor production

assay using an A-factor-deficient mutant and Bacillus subtilis

as indicators (11) showed that the amfS mutant normally pro-
duced A-factor. The deficiencies of the amfS mutant were

rescued by the introduction of pSL12 (containing the whole

gene cluster on a low-copy-number plasmid, pIJ922 (8). On

the other hand, pSL12 containing the amfS coding region alone

(Fig. 1A) partially restored the aerial mycelium, which suggests

that amfS is mainly transcribed by the promoter preceding

amfT. pSL12 was constructed by cloning the amfS-containing

700-bp BamHI fragment (corresponding to nucleotides 6925 to

7608) at the amfT site of pIJ922. (B) The amino acid sequence of AmfS was aligned with

the homologous sequence identified in S. coelicolor A3(2). The C-
terminal hydrophobic repeats are indicated by arrows. Identical amino

acids are indicated by asterisks. (C) Nucleotide sequence of the N-
terminal hydrophobic repeats are indicated by arrows. Identical amino

acid positions are indicated by asterisks. (D) Potential site for ribosome binding is underlined. The mutated sequence used for the disruption of amfS (ΔamfS) is indicated below the wild-type sequence. The italic lowercase letters represent the recognition site for HindIII created to check for true recombination.

amfS (the italic and underlined letters represent the HindIII cleavage site and the nonconserved codon, respectively) for fragment a and b [Fig. 1A] (corresponding to nucleotides 7214 to 8093 and 6236 to 7217 of the submitted nucleotide sequence, respectively).

These fragments were separately amplified by the standard PCR technique with the following primers: 5′-CCGAATTCC TCCGGATCATCCTCG-3′ (the italic letters represent an EcoRI cleavage site for cloning into pUC19-KM) and 5′-GAG AAGCTTGGCTAGATGATGTCCTTTCGG-3′ (the italic and underlined letters represent the HindIII cleavage site and the nonconserved codon, respectively) for fragment a and b of the nucleotide sequence predicted by the FRAME program.

The resultant amfS mutant showed a bald phenotype (Fig. 2); on

YMP-glucose solid medium, the amfS mutant was unable to

form aerial mycelia. The deficiency was partially recovered on

YMP-maltose medium (containing 1% maltose instead of the

glucose in YMP-glucose medium), but aerial-mycelium forma-

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amfT. pSL12 was constructed by cloning the amfS-containing

700-bp BamHI fragment (corresponding to nucleotides 6925 to

7608) at the amfT site of pIJ922, which was amplified by

PCR with primers 5′-GGAGATCCGCTTTCACTCGGCA T-3′ and 5′-GGTGGAGCCAGAGGAGGATCCCGAG-3′ (the italic letters represent BamHI cleavage sites for cloning).

A null mutant of amfBA. An amfBA mutant was also generated as follows. The flanking fragments of amfBA (fragments c and d [Fig. 1A]) were prepared by appropriate restriction of pSP01, the original plasmid carrying the 9-kb Sau3AI fragment at the BamHI site in the multilinker of pJ487 (27), which enabled us to recover the Sau3AI fragment as an EcoRI-HindIII fragment. For fragment c, the Sau3AI-PmaCI region was recovered as an EcoRI-BgII fragment by attaching an
8-mer BglII linker at the PmaCI cleavage end. For fragment d, the NcoI-BamHI region was recovered as a BglII-HindIII fragment by blunt-end formation at the NcoI cleavage end with the Klenow fragment followed by attaching an 8-mer BglII linker. The fragments c and d were combined by cloning them between the EcoRI and HindIII sites of pUC19 by three-fragment ligation. The resultant plasmid was cleaved with BglII and ligated to an aphII cassette prepared as a 0.9-kb BamHI fragment by an appropriate PCR procedure to generate pDIS-BA. The mutagenized construct was then recovered from pDIS-BA as an EcoRI/HindIII-digested fragment, and the linear DNA was introduced into S. griseus IFO13350 by the standard transformation procedure. The mutants were screened for among the resultant kanamycin-resistant colonies that carry the mutagenized construct via a double-crossover event. Subsequent checking by Southern hybridization with appropriate probes confirmed the true disruptant. In contrast to the bald phenotype of the amfS mutant, the resultant null mutant of amfBA showed the wild-type morphology and streptomycin productivity (Fig. 2).

**Extracellular complementation.** Extracellular-complementation tests with the amfS mutant as a recipient demonstrated the putative exogenous activity of the AmfS product (Fig. 3). The activity to form aerial mycelium was restored to the amfS mutant upon growth near the wild-type or the amfS mutant harboring pSL12, which suggests that the extracellular AmfS or its derivative supplied from the donor strains can complement the nonsense mutation in amfS to restore morphogenesis. On the other hand, the extracellular stimulatory activity was produced by neither the mutant for amfR (24) nor that for amfBA (this study). The activities in those mutants were restored by the introduction of pSL1, which confirmed that the deficiencies are linked to each mutation. These results indicate that AmfR and AmfBA are essential for extracellular AmfS activity. The same experiment showed that an adpA mutant (18) also lacked the extracellular activity, suggesting that regulation by AmfS is under the control of A-factor (data not shown).

Since AmfR is a response regulator of a two-component regulatory system, it is most likely that it regulates transcription of amfS. However, we previously observed that an amfR mutant produced streptomycin at the wild-type level (24), which contradicts the streptomycin-negative phenotype of the amfS mutant. If amfR is essential for the transcription of amfS, the amfR mutant should be as defective in both aerial-mycelium formation and streptomycin production as the amfS mutant. There is at present no explanation for the inconsistency. On the other hand, the AmfBA products are probably involved in the export of AmfS or its derivative. The wild-type morphology of the amfBA mutant may be caused by AmfS accumulated intracellularly. The intracellular AmfS could be converted to a putative active form, which is suggested below, by some mechanism that occurs independently of its secretion. Otherwise, the wild-type phenotype of the amfBA mutant could be caused by a very reduced but sufficient level of extracellular AmfS.

**FIG. 2.** Phenotypes of the amfS and amfBA disruptants. For colony morphology (left), patches were photographed after 5 days of growth at 28°C on YMP-glucose medium. For streptomycin productivity (right), colonies were grown for 5 days at 28°C on YMP-maltose medium, overlaid with soft agar containing spores of B. subtilis, and incubated overnight at 37°C. WT, wild type.

**FIG. 3.** Extracellular complementation of the aerial-mycelium deficiency in the amfS mutant. Each donor strain (upper colonies) was inoculated in close proximity to the amfS mutant (lower colonies) on YMP-glucose agar medium, and patches were photographed after 5 days of growth at 28°C. WT, wild type.
We need more precise analyses to show whether AmfBA is directly involved in secretion of AmfS.

To further characterize the above-mentioned extracellular activity, the culture supernatant of the wild-type strain was examined. The *S. griseus* wild-type strain was cultured in 1 liter of YMP-glucose liquid medium for 2 days at 28°C, and 2 volumes of ethanol was added to the culture supernatant. The mixture was incubated for 2 hours at 4°C and centrifuged at 15,000 × g for 20 min. The resultant precipitate was dissolved in 1× TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 7.0]) to obtain a solution containing approximately 3 mg of protein/ml. Fifty microliters of the solution was then used to saturate a filter disk, which was subsequently placed onto a YMP-glucose agar plate inoculated with the *amfS* mutant to form a confluent lawn. After incubation at 28°C, the ethanol-precipitated fraction of the wild-type strain diffusing from the disk restored aerial mycelia in the adjacent colonies of the *amfS* mutant (Fig. 4A). In contrast, the same fraction of the *amfS* mutant did not show aerial mycelium-inducing activity, suggesting that the effective substance contained in the wild-type fraction is the AmfS product or its derivative.

**Synthetic AmfS peptides.** We next carried out the assay described above with chemically synthesized AmfS peptides. First, the full-size 42-aa peptide (from Ala-2 to Pro-43) was prepared by the solid-phase synthesis method (Sawady Technology, Inc.) and dissolved in 1× TE (pH 7.0) containing 1% Triton X-100 to obtain a 1-mg/ml stock solution. The solution was further incubated with various ratios of reduced and oxidized glutathione, with the expectation of obtaining a form with the correct intrachain disulfide bridge. These preparations were subsequently diluted (20 to 200 times) with 1× TE and applied to filter disks that were processed as described above.

The result showed that 50 μg or less of the synthetic peptide did not induce aerial-mycelium formation in the *amfS* mutant (not shown). We then synthesized the C-terminal portion of AmfS containing the second hydrophobic repeat (35-LSVV-LCTP-42) (Fig. 1B). We speculated that the hydrophobic units may be important for the activity and that the peptide containing a single Cys residue can avoid an unwanted intramolecular disulfide bonding. As shown in Fig. 4, the synthetic octapeptide caused stimulation of aerial mycelium in the *amfS* mutant in a dose-dependent manner. Abundant aerial mycelium occasionally culminating in formation of spore chains was clearly observed by scanning electron microscopy. The minimum effective amount of the synthetic C-terminal peptide on a filter disk was 0.3 μg (0.36 nmol). Although the activity of the octapeptide was reproducible, it was irregularly dispersed from the

FIG. 4. Stimulation of aerial-mycelium formation in the *amfS* mutant by the wild-type culture supernatant and synthetic C-terminal octapeptide of AmfS. (A) Disks were soaked with ethanol (EtOH)-precipitated fractions of the wild type (WT), the *amfS* mutant (∆*amfS*), and the synthetic C-terminal octapeptide (AmfSc). The white colonies contrasting with the dark ones were producing aerial hyphae. Patches were photographed after 5 days of growth for the ethanol precipitate and after 7 days of growth for the synthetic peptide. (B) Scanning electron micrograph of the *amfS* mutant with (left) and without (right) induction by the synthetic octapeptide. The AmfSc-treated colonies formed abundant aerial mycelia, most of which differentiated into spore chains via septum formation. In contrast, the untreated mutant grew only substrate hyphae. The photographs were taken after 7 days of growth. Bar, 2 μm.
disks, and the aerial-mycelium formation was delayed by ap-
proximately 2 days, appearing at 7 days, while aerial-mycelium
formation with the ethanol precipitate of the wild-type culture
broth was visible in 5 days. These results suggest that the
octapeptide is not identical but mimetic to the actual active
form of AmfS and that the peptidic molecule derived from the
AmfS product is an extracellular morphogen in S. griseus. It
also could be that the active fraction collected from the wild-
type culture contains a mixture of signals which are sufficient
to cause the distinct induction of aerial-mycelium formation in
the amfS mutant.

Involvement of extracellular peptides in procytotic cellular
development has been well characterized in B. subtilis. The
oligopeptide permease encoded by the spo0K locus is essential for
the onset of endospore formation in this organism and is
known to be involved in global uptake of regulatory peptides
(13, 21). These include PhrA, an inhibitor of the specific phos-
phatase (RapA) for Spo0A, which is produced as a propeptide
of 44 aa and processed into a C-terminal hexapeptide to be
active as an inhibitory molecule (20).

In S. coelicolor A3(2), the hydrophobic peptide SapB is known
as an extracellular morphogen (16, 28). Although the precise
structure of the peptide is unknown, the amino acid content for
the whole 18 aa (28) is different from that of the AmfS ho-
logom of S. coelicolor. While SapB is assumed to be an extra-
cellular surfactant that reduces the surface tension of the sub-
strate mycelium to allow protrusion of aerial hyphae (22),
AmfS is assumed to function intracellularly, as suggested by
the wild-type phenotype of the amfB4 mutant. The presence of
such an intracellular regulatory peptide has been implied by
the study of bldK, which encodes an oligopeptide permease in
S. coelicolor (16, 17). The involvement of AmfS not only in
aerial-mycelium formation but also in antibiotic production
implies its role as a pleiotropic signal molecule. We expect that
future biochemical studies will reveal its intrinsic role in the
regulation and function of AmfS in S. griseus.

Nucleotide sequence accession number. The DDBJ acces-
sion number of the sequence described in this paper is
AB006206.

We thank M. Nishiyama and H. Ikeda for helpful discussions.
This study was supported by the High-Tech Research Center

REFERENCES
tide sequence and exact localization of the neomycin phosphotransferase
between base composition and codon usage in bacterial genes and its use for
the simple and reliable identification of protein-coding sequences. Gene
30:157–166.
B, P-glycoprotein and other members of a novel family of membrane trans-
R. A. Sleekely, and P. Setlow (ed.), Regulation of prokaryotic development:
structural and functional analysis of bacterial sporulation and germination.
American Society for Microbiology, Washington, D.C.
Microbiol. 47:685–713.
Prokaryotic development. ASM Press, Washington, D.C.
conserved features and deduced phylogeny of the catalytic domain. Science
241:72–82.
Genetic manipulation of Streptomyces: a laboratory manual. The John Innes
Foundation, Norwich, United Kingdom.
controls cellular differentiation and secondary metabolism in Streptomyces
nant of A-factor biosynthesis in streptomycin-producing organisms: cloning
2001. The ram-dependence of Streptomyces lividus differentiation is by-
peptide functions intracellularly to contribute cell density signaling in
Streptomyces coelicolor coeII that causes accelerated aerial mycelium formation
laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Har-
bort, NY.
mease responsible for the import of an extracellular signal governing aerial
molecule involved in production of aerial mycelium by Streptomyces coelicolor.
regulatory cascade leading to streptomycin biosynthesis in Streptomyces
griseus: identification of a target gene of the A-factor receptor. Mol. Micro-
biol. 34:102–111.
1995. Cloning and characterization of the A-factor receptor gene from Strep-
phatases deactivate the response regulator components of the sporulation
1991. The oligopeptide transport system of Bacillus subtilis plays a role in
A surface active protein involved in aerial hyphae formation in the filamentous
fungus Schizochytrium commune restores the capacity of a bald mutant of the
filamentous bacterium Streptomyces coelicolor to erect aerial structures. Mol.
Microbiol. 30:595–602.
involved in aerial mycelium formation in Streptomyces griseus encodes pro-
teins similar to the response regulator and membrane translocator. J. Bac-
24. Ueda, K., C.-W. Hohe, T. Tosaki, H. Shinkawa, T. Beppu, and S. Horinou-
essential for onset of aerial mycelium formation in Streptomyces griseus. J.
responsive protein that binds to the upstream activation sequence of strF, a
regulatory gene for streptomycin biosynthesis in Streptomyces griseus. J.
Identification of an A-factor-dependent promoter in the streptomycin bio-
Bibb. 1996. Construction and characterization of a series of multi-copy
promoter-probe plasmid vectors for Streptomyces using the amnycloside
481.
Extracellular complementation of a developmental mutation implicates a
small sporulation protein in aerial mycelium formation by S. coelicolor. Cell
65:491–500.