Identification of *sas*, a conserved gene cluster involved in the regulation of aerial mycelium formation in *Streptomyces griseus*

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We cloned a DNA fragment that suppressed the aerial-mycelium-deficient phenotype in an *amfS* mutant of *Streptomyces griseus* when it was introduced into the cells via a high-copy-number plasmid. The *sasABCD* gene cluster was identified as being responsible for this suppressive activity. The proteins encoded by *sasABCD* were of unknown function, but the operon structure was found to be conserved in all the strains of *Streptomyces* spp. and related organisms whose genomes have been sequenced. *sasR*, the flanking opposite coding sequence, encoded a putative DNA-binding protein. Subcloning revealed that the presence of all five coding sequences was essential for complete suppression. Scanning electron microscopy of *Streptomyces griseus* strains carrying the *sas* gene cluster at a high copy-number revealed that bundle-like structures consisting of several aerial hyphae were often formed. S1 nuclease protection analyses were performed to identify the transcriptional start site in the promoters preceding *sasA* and *sasR*. The promoter preceding *sasA* was highly active during vegetative growth. Null mutants for *sasABCD* among the *S. griseus* and *S. coelicolor* A3(2) cells exhibited bald phenotypes; this suggested a positive regulatory role of this gene cluster in the onset of morphogenesis in these two phylogenetically distinct *Streptomyces* species.

Received 24 June 2008

Revised 21 August 2008

Accepted 15 September 2008

INTRODUCTION

Organisms of the Gram-positive bacterial genus *Streptomyces* are characterized by the ability to perform complex morphological differentiation in a manner similar to filamentous fungi. Early in their life cycle, these organisms develop branched multinucleoid hyphae that grow into the substrate (substrate mycelium). In response to nutrient limitations, the substrate mycelium produces aerial mycelium that culminates in long spore chains via the formation of septa at regular intervals (Chater, 1993). Extensive studies conducted on *Streptomyces coelicolor* A3(2), the model organism for genetic manipulation, have provided a deep understanding of cell development in *Streptomyces* spp.

We study the genetic regulation of the onset of aerial mycelium formation in *Streptomyces griseus*, the species that produces streptomycin. This organism is well characterized with regard to the role of the γ -butyrolactone autoregulatory factor called A-factor. S. Horinouchi and co-workers have precisely characterized the A-factor signalling cascade and identified multiple A-factor-dependent regulators as the master switches for the initiation of cell differentiation (Horinouchi, 2007).

In this study, we performed screening for a gene that suppresses the deficiency of aerial mycelium formation in

an *amfS* mutant of *S. griseus*. It is assumed that AmfS, a morphogenic peptide equivalent to SapB/RamS of *S. coelicolor* A3(2), plays an important role in the erection of aerial hyphae under the control of A-factor (Ueda *et al.*, 2002, 2005). Recently, the understanding of aerial mycelium formation in *Streptomyces* has greatly progressed based on the precise characterization of SapB in *S. coelicolor* A3(2) (reviewed by Willey *et al.*, 2006). To date, however, no information is available with regard to suppressors of the phenotypic effects of a deficiency of this kind of morphogenic peptide. We anticipated that isolation of such suppressors will provide a new insight into morphogenesis in this group of bacteria. A DNA fragment responsible for the formation of aerial hyphae (albeit morphologically abnormal) in the *amfS* mutant was found to contain a conserved gene cluster of unknown function.

METHODS

Bacterial strains, plasmids and culture conditions. The wild-type strain of *S. griseus* (IFO13350) was obtained from the Institute of Fermentation, Osaka, Japan. Mutants for *amfS* (small coding sequence encoding a morphogenic peptide) and *amfR* (gene encoding a positive transcriptional regulator of *amfTSBA* operon) of *S. griseus* have been described previously (Ueda *et al.*, 1998, 2002). The *bld4* strain, a bald mutant of *S. griseus*, was obtained by performing

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis in our laboratory. The genetic background of this mutant is not known; it lacks the ability to produce AmfS and forms abundant aerial hyphae upon the addition of exogenous AmfS peptide. A mutant for *adpA* (gene encoding the global transcriptional regulator for the onset of morphological and physiological development) (Ohnishi *et al.*, 1999) was obtained from Y. Ohnishi (University of Tokyo, Japan). *S. coelicolor* A3(2) M130 was obtained from the John Innes Centre, Norwich, UK. *Escherichia coli* JM109 (Maniatis *et al.*, 1982) was used as the host strain for DNA manipulation. pUC19 (Maniatis *et al.*, 1982) was used for general DNA manipulation. TA cloning was performed for PCR-generated DNA fragments with the help of pT7Blue (Novagen). The enzymes used for DNA manipulation were purchased from Takara Shuzo. The culture conditions and genetic manipulation protocols used for *E. coli* and *Streptomyces* spp. were as described by Maniatis *et al.* (1982) and Kieser *et al.* (2000), respectively. Strains of *Streptomyces* spp. were grown in Bennett's/sugar medium [containing (g l⁻¹): yeast extract (Difco), 1; meat extract (Kyokuto), 1; NZ amine (Wako Pure Chemical Industries), 2; and an appropriate sugar (Kokusan), 10 (pH 7.2)], YMP/sugar medium [containing (g l⁻¹): yeast extract (Difco), 2; meat extract (Kyokuto), 2; Bacto-peptone (Difco), 4; NaCl, 5; MgSO₄·7H₂O, 2; and an appropriate sugar (Kokusan), 10], ISP4 medium [containing (g l⁻¹): soluble starch (Wako), 10; K₂HPO₄, 1; MgSO₄·7H₂O, 1; NaCl, 1; (NH₄)₂SO₄, 2; CaCO₃, 2; and 1 ml l⁻¹ trace salts solution containing 0.1 g l⁻¹ each of FeSO₄·4H₂O, MnCl₂·4H₂O and ZnSO₄·7H₂O (pH 7.2)] and R2YE medium (Kieser *et al.*, 2000). Agar (1.5%; Kokusan or Difco) was added to the above mixtures to obtain solid media. The plasmid pIJ702 (carrying genes responsible for thiostrepton resistance and melanin biosynthesis) (Katz *et al.*, 1983) possesses a copy number of 40–400 per genome, while the plasmid pXE4 has a low copy number of 1–2 per genome (Kieser *et al.*, 2000). An integration plasmid, namely pKU464 carrying the *phiBT1* integrase gene (Gregory *et al.*, 2003), was obtained from H. Ikeda (Kitasato University). For the selection of transformants from the *E. coli* cells, ampicillin (Wako) and neomycin (Wako) were used at a final concentration of 50 µg ml⁻¹. For selecting the *S. griseus* transformants, thiostrepton (Sigma) and neomycin were used at a final concentration of 20 µg ml⁻¹. A bioassay was performed for streptomycin according to a method described previously (Ueda *et al.*, 2002).

Cloning of the *sas* gene cluster. The gene cluster described in this study was obtained by performing shotgun cloning using an *amfS* mutant as the host. *Bam*HI-digested fragments of *S. griseus* genomic DNA were ligated to *Bgl*II-digested pIJ702 DNA and used to transform the host organism. A transformant, which grew as white colonies due to the formation of aerial mycelium, harboured a plasmid, pTT1, carrying a 7.2 kb fragment that contained coding sequences corresponding to SGR4456–4462 in the genome sequence database (<http://www.genome.ad.jp/>) (Ohnishi *et al.*, 2008). The SGR4456 sequence in this plasmid lacked the 5'-terminal region corresponding to the four amino acids at the N-terminus. This truncated coding sequence occurred as an in-frame fusion with *melC1*, a small coding sequence carried by pIJ702. The cloned fragment was then subcloned in the following manner. To construct pTT1-2, a DNA fragment that was amplified by PCR using the primers sasA-F and sasB-R (Table 1) was first TA cloned onto pT7Blue. Then, the fragment was excised as a *Bgl*II fragment and inserted at the *Bgl*II site of pIJ702. Similar procedures were used to subclone the other plasmids. The inserts of pTT1-3, pTT1-4 and pTT1-5 were amplified by using sasA-F (Table 1) as the forward primer, and sasC-R, sasD-R and sasR-F, respectively (Table 1), as the reverse primers. The insert of pTT1-6 was amplified using sasR-R and sasR-F.

Electron microscopy. The hyphae of *S. griseus* and *S. coelicolor* A3(2) strains that were grown at 28 °C for 6 days on ISP4 medium and YMP/glucose medium, respectively, were observed by scanning electron microscopy. To prepare the specimens, agar blocks were fixed with 2% osmium tetroxide for 30 h and were then dehydrated by freeze-drying. Each specimen was sputter-coated with palladium/gold and examined using a VE8800 scanning electron microscope (Keyence).

S1 nuclease mapping. Transcription at the promoters preceding *sasR* and *sasA* was studied by S1 protection analysis. RNA was extracted from *Streptomyces* cells grown on cellophane-covered agar medium, and S1 nuclease mapping was performed, both using previously described methods (Kelemen *et al.*, 2001; Kieser *et al.*, 2000). A 285 bp hybridization probe was prepared by PCR using the primers sasR-F(S1) and sasR-R(S1) (Table 1) for both high- and low-resolution analysis of the *sasR* promoter. Similarly, the *sasA* promoter was analysed using a 278 bp hybridization probe prepared by PCR using the primers sasA-F and sasA-R(S1) (Table 1). All downstream primers were labelled at their 5' end with [γ -³²P]ATP using T4 polynucleotide kinase. The protected fragments were analysed on 6% polyacrylamide gels. A 100 bp ladder marker (Takara) that was labelled with [γ -³²P]ATP using T4 polynucleotide kinase was used as a standard to estimate the transcript sizes in the low-resolution assay. In the high-resolution assay, Maxam–Gilbert sequencing ladders (A+G and C+T reactions) were generated using the ³²P-labelled probe DNA fragment for the identification of the transcription start sites. Quality of RNA was assessed by performing a control assay for *hrdB*, which encodes a major sigma factor (Shinkawa *et al.*, 1995).

Gene disruption. We generated *sasABCD*-knockout mutants by a homologous recombination technique that was based on Redirect technology (Gust *et al.*, 2003). The cosmid clone used for achieving knockout in *S. griseus* cells [containing the region corresponding to nucleotides 5202636–5245708 in the genome sequence database (<http://www.genome.ad.jp/>)] was available in our laboratory. SCF15, the cosmid used for achieving the knockout in *S. coelicolor* A3(2) cells, was obtained from the John Innes Centre. A cassette of an apramycin-resistance gene, *aac(3)IV*, was prepared by PCR, using the primer sets DisG-F/DisG-R (for *S. griseus*) and DisC-F/DisC-R (for *S. coelicolor*) (Table 1). The *sasABCD* region in each cosmid clone was replaced with the apramycin-resistance gene cassette by performing *in vivo* recombination using the λ Red system. The resulting apramycin-resistant cosmid was then transformed into each organism. Subsequently, the apramycin-resistant recombinants were screened, and assessed for true recombination by performing PCR using appropriate primers. The integration plasmids used for genetic complementation of these knockout mutants were prepared in the following manner. The *sas*-coding region was amplified by PCR, using the primer sets sasA-F/sasD-R (for *S. griseus*) and sasA-F(SC)/sasD-R(SC) (for *S. coelicolor*) (Table 1) and was cloned at the *Bam*HI site of pKU464. The resultant plasmid was used to transform each organism to generate a kanamycin-resistant transformant harbouring the plasmid integrated at the BT1 site (Gregory *et al.*, 2003). The mutant of *sasR* of *S. griseus* was obtained similarly as above, using primer sets DisGR-F/DisGR-R (Table 1).

RESULTS

Cloning of the *sas* gene cluster

We screened a library constructed in the high-copy-number plasmid pIJ702 for inserts that suppressed the aerial-mycelium-deficient phenotype of an *amfS* mutant of *S. griseus*, and obtained the plasmid pTT1 (see Methods).

Table 1. Oligonucleotide primers used in this study

Name	Sequence (5'–3')*	Restriction enzyme	Position (nt)†
sasA-F	<u>AGATCT</u> CAACAGGGCGGTGATGAC	<i>Bgl</i> III	5218636–5218655
sasB-R	<u>AGATCT</u> ACCATCAGCTGTCTCCTC	<i>Bgl</i> III	5217181–5217198
sasC-R	<u>AGATCT</u> CTTCAGTGTCTCGGTGTTTC	<i>Bgl</i> III	5216553–5216574
sasD-R	<u>AGATCT</u> CAGACCTCCAGGCCCGC	<i>Bgl</i> III	5216205–5216222
sasR-R	<u>AGATCT</u> TCACTCCACGAAGAGAGCG	<i>Bgl</i> III	5215931–5215949
sasR-F	<u>AGATCT</u> TATCGATGTGCGCCAGACATG	<i>Bgl</i> III	5214823–5214843
sasA-R(S1)	AGACCGCTCGTCCAGTCGACC	–	5218358–5218378
sasR-F(S1)	TGCACTGCTGGCTGGGTCTGCTC	–	5214863–5214884
sasR-R(S1)	TCGAAAAGCCGCTGCAACC	–	5215129–5215147
DisG-F	TTACAGCCGTACGCCACGTCTGGGAGGGGAGATCCGCGTGattccggggat- ccgtcgacc	–	5218441–5218479
DisG-R	TGCGGGGCCGACCGAAGACCTCGCGGCGGCCGGGGTCAgttaggtgg- agctgcttc	–	5216169–5216207
DisGR-F	GACCTTCTGGCTGAATAGAACGGTCGGCGCCGGTCTGattccggggat- ccgtcgacc	–	5215038–5215076
DisGR-R	GCGGCTGAGAATCCGCGCGGGCCGGGAGCCCGCCCTCAgttaggtgg- agctgcttc	–	5215985–5215947
sasA-F(SC)	<u>AGATCT</u> GAGCTTCTTCACGTTGTCCAG	<i>Bgl</i> III	727333–727313
sasD-R(SC)	<u>AGATCT</u> TCAACCTCCAGTTCGCTCTC	<i>Bgl</i> III	724639–724659
DisC-F	GTTCGCGGTACAGCCGTGCAGGGGAGGGGATCCGCGTGattccgggga- tcctcgacc	–	726844–726806
DisC-R	GCGCCGGTCCCGCGGCGGGTGAACGGGCGGCCGGGATCAgttaggtgg- agctgcttc	–	724603–724641

*Sites for restriction are underlined. Sequences corresponding to the apramycin resistance gene are shown in lower case.

†Corresponding position in the genome sequence database of *S. griseus* (<http://streptomyces.nih.go.jp/griseus/>) and *S. coelicolor* A3(2) (<http://gib.genes.nig.ac.jp>).

The *amfS* mutant cells harbouring pTT1 grew as white colonies due to the formation of aerial hyphae (Fig. 1a). Scanning electron microscopy of the white colonies demonstrated abundant growth of aerial mycelium with occasional unique bundle-like structures, reminiscent of fungal synnema structures, due to the adhesion of several hyphae (Fig. 1b). Occasional formation of spore chains was also seen (data not shown). The introduction of pTT1 into the cells of other bald mutants, including an *amfR* mutant (data not shown) and a *bld4* mutant, as well as the wild-type strain (Fig. 1b), also resulted in the growth of unusual aerial mycelium. The introduction of pTT1 did not restore aerial mycelium formation in a knockout mutant of *adpA*, the central regulatory gene of the A-factor cascade that positively controls the transcription of A-factor-dependent regulatory genes including the *amf* cluster (Horinouchi, 2007) (data not shown). These results indicate that some *adpA*-dependent gene other than *amf* is essential for the suppressive effect of pTT1.

A cross-feeding assay revealed that the *amfS* mutant harbouring pTT1 did not induce the growth of aerial mycelium in an *amfS* mutant harbouring an empty plasmid growing in close proximity (Fig. 1c, left panel), while the wild-type strain did (Fig. 1c, right panel). This supported the view that the *amfS* mutants harbouring

pTT1 did not produce the AmfS peptide and that the gene cloned into pTT1 was not the *amfS* gene.

Subsequent nucleotide sequencing and search in the genome database revealed that pTT1 carried a 7.152 kb *Bam*HI fragment at the *Bgl*III site of pIJ702. This region contained seven protein-coding sequences (CDSs) corresponding to SGR4456–SGR4462 (Fig. 2); SGR4456 was truncated at the 5' terminus (see Methods). A subsequent subcloning experiment revealed that pTT1-4, containing SGR4457–4460, induced aerial mycelium formation at a slightly lower level in comparison to the original plasmid (Fig. 1a), and that pTT1-2 and pTT1-3, containing SGR4459–4460 and SGR4458–4460, respectively, did not (data not shown). The introduction of pTT1-5, carrying SGR4457–4460 along with the intact SGR4456, induced aerial mycelium formation to a high degree (Fig. 1a). The introduction of the intact SGR4456–4460 region into the cells via a low-copy-number plasmid (pXE4) did not induce the formation of aerial mycelium (data not shown). These results indicated that the introduction of four CDSs corresponding to SGR4457–4460 into the *amfS* mutant via a high-copy-number plasmid was essential to restore the formation of aerial mycelium and they raise the possibility that SGR4456 is also related to this restorative effect. On the basis of these observations, the five CDSs are hereafter

Fig. 1. Phenotypes of *S. griseus* conferred by the introduction of the plasmid obtained in this study. (a) Colonies of the *amfS* mutant harbouring the original plasmid (pTT1) and its derivatives grown on YMP/glucose agar medium. The white colour of the colony is due to the development of aerial mycelium from the substrate mycelium, and the dark (brown) colour is due to the growth of only substrate mycelium. The region introduced into each plasmid is shown in Fig. 2. Patches were photographed on day 4. (b) Scanning electron micrographs of *S. griseus* strains (*amfS* mutant, *bld4* mutant and wild-type) harbouring pTT1. The introduction of the plasmid into the cells often caused the formation of bundle-like structures due to the adhesion of several aerial hyphae. Arrowheads indicate that the bundle-like structures also occurred in the wild-type carrying pTT1. Bars, 10 μ m. (c) A cross-feeding assay showing the secretion of AmfS. In the control assay (right panel), aerial mycelium was formed in the marginal region of the *amfS* mutant colonies (lower row) due to the activity of the AmfS peptide that was secreted by the wild-type colonies (upper row). In contrast, the *amfS* mutant cells that were grown in close proximity to the *amfS* mutant harbouring pTT1 (left panel) did not form aerial mycelium; this indicated that neither AmfS nor a substitute diffusible morphogen was produced by the *amfS* mutant harbouring pTT1.

designated *sasABCD*R (suppressor of *a*erial mycelium deficiency of *amfS* mutant).

The results of a database search (<http://www.genome.ad.jp/>) revealed that proteins encoded by *sasA* and *sasD* did not exhibit distinct homology with any proteins of known function. The SasA (137 aa) and SasD (124 aa) products were homologous to each other (35 % identity). The results of a Pfam motif search revealed that the SasB product (269 aa) contained a helix–turn–helix domain in the C-terminal region. The major part (24–113 aa) of the SasC

product (168 aa) exhibited a distinct similarity (E value = 7.6×10^{-19}) with the proteins of the roadblock/LC7 family. This family has been well characterized in terms of the association with eukaryotic dynein (Song *et al.*, 2005), but it also includes a gliding motility protein (Mgl) of *Myxococcus xanthus* (Stephens *et al.*, 1989). The SasR product exhibited an end-to-end similarity with transcriptional regulators of the Rpi family (Sorensen & Hove-Jensen, 1996). Currently no information is available with regard to the localization of Sas proteins, but none of them retained a distinct N-terminal signal sequence for secretion.

Fig. 2. Schematic representation of the gene cluster carried by pTT1. (a) The *Bam*HI region of *S. griseus* that was cloned onto pTT1. The region contained seven complete coding sequences, including *sasABCD*, but it lacked the 5' region of *sasR*. In this construction, a plasmid-borne promoter involved in melanin biosynthesis (Katz *et al.*, 1983) directed the expression of *sasR*, which was in-frame with a small coding sequence (*melC1*) on this plasmid. The regions corresponding to the subcloned fragment are also shown. SGR4461 and 4462 encode a putative amino acid permease and urocanate hydratase, respectively. pTT1-5 and pTT1-6 contain the intact promoter region preceding *sasR*. (b) The *sas* gene cluster homologues. The *sasABCD* gene cluster was found to be conserved in all *Streptomyces* spp. whose complete genome sequences are known. The cluster was also found in the genome of *N. farcinica*. The *sasR* homologue was located in the same locus as *sasABCD* in *S. avermitilis* but in a different locus in *S. coelicolor* A3(2). The *sasR* homologue was not found in *N. farcinica* and *F. alni*.

The *sasABCD* cluster was also found in the genomes of *Streptomyces avermitilis*, *S. coelicolor* A3(2) and *Nocardia farcinica*. The short length of the intergenic regions (–10 to 54 bp) supported the view that *sasABCD* constituted an operon structure in these organisms. This also suggested that the four proteins in this operon are functionally related and that they play a general role in this group of bacteria. The *sasABCD* gene cluster homologue was not found in the genomes of other organisms, although homologues of each component were present in the genomes of various bacteria, including cyanobacteria and Gram-negative bacteria such as *Myxococcus* and *Xanthomonas*. The *sasR* homologue of *S. avermitilis* was located in the flanking region of the *sasABCD* cluster, while that of *S. coelicolor* A3(2) was located at a different locus. The *sasR* homologue was not present in the genome of *N. farcinica*. *sasABC* comprised a cluster in *Frankia alni*, but the *sasD* homologue was in a different locus (Fig. 2b).

Transcription of the *sas* gene cluster

The transcriptional activity of the promoters preceding *sasA* and *sasR* was studied by performing S1 nuclease protection analyses. A high-resolution analysis assigned the unique transcriptional start site in both the promoters (Fig. 3a, b). Potential –35 and –10 sequences were found in the *sasR* promoter (TGGACA...CTGAAT), but not in the *sasA* promoter.

The low-resolution S1 analysis revealed that the activity of the *sasA* promoter was relatively high and especially active during the vegetative growth phase (Fig. 3c). On the other hand, the activity of the *sasR* promoter was very low and was observed during the early growth phase (data not shown). These two promoters exhibited similar transcriptional activity in a disruption mutant of *adpA*, the central regulator of the A-factor cascade (Ohnishi *et al.*, 1999) (data not shown).

Fig. 3. S1 nuclease protection analyses of the promoters preceding *sasA* and *sasR*. (a) Nucleotide sequence of the promoter region preceding *sasA* (upper block) and *sasR* (lower block). The transcriptional start positions determined by high-resolution analyses are indicated by the wavy arrows. The possible -35 and -10 regions of *sasR* promoter are underlined. (b) High-resolution analyses for the determination of transcriptional start sites of the promoters preceding *sasA* and *sasR*. Maxam–Gilbert sequencing ladders (C+T and G+A reactions) were generated with the same ^{32}P fragment as the probe DNA. The positions of the S1-protected fragment are shown by arrowheads and the transcriptional start sites were assigned to the residues indicated by the wavy arrows. It is known that the fragments generated by the chemical sequencing reactions migrate 1.5 nucleotides further than the corresponding fragments generated by S1 nuclease digestion of the DNA–RNA hybrids (half a residue from the presence of the 3'-terminal phosphate group and one residue from the elimination of the 3'-terminal nucleotide) (Sollner-Webb & Reeder, 1979). The RNA prepared from the wild-type cells grown for 3 days on YMP solid medium containing glucose was used for hybridization. (c) Low-resolution S1 analysis. The activity of the *sasA* promoter was estimated by the intensity of the hybridization signal. RNA was extracted from the cells of each *S. griseus* strain, grown on YMP solid medium containing 1% glucose for the indicated periods. The wild-type strain grew as substrate mycelium (SM) at day 1, as a mixture of substrate and aerial mycelium (AM) at day 2 and as a mixture of aerial mycelium and spores (Sp) at day 3. Quality of RNA was confirmed by a control assay for *hrdB* (data not shown).

diffusible pigment antibiotic) as did the wild-type cells. The mutant was also defective in the formation of aerial mycelium when cultured on YMP/maltose and R2YE media. Aerial growth was restored in the *sasABCD* mutants of *S. griseus* and *S. coelicolor* by transforming them with an integration vector containing the intact gene cluster (data not shown).

Phenotype of knockout mutants

To study the role of the *sas* gene cluster, null mutants with deletions of the four coding sequences ($\Delta sasABCD$) were generated in *S. griseus* and *S. coelicolor* A3(2) (see Methods). The *sasABCD* mutant of *S. griseus* was defective in the formation of aerial mycelium (bald phenotype) on ISP4 agar (Fig. 4). This mutant phenotype was dependent on the medium composition; the mutant exhibited the wild-type morphotype when cultured on media such as Bennett's/glucose and YMP/glucose. The level of streptomycin produced by the mutant was the same as that produced by the wild-type strain in various media with different compositions (data not shown). We also obtained a null mutant of *sasR* of *S. griseus*, and found that it exhibited a wild-type phenotype on all media tested (data not shown).

The *sasABCD* mutant of *S. coelicolor* A3(2) also exhibited a bald phenotype (Fig. 5). When cultured on YMP/glucose agar medium, the mutant did not form aerial mycelium but produced the same levels of actinorhodin (the blue

DISCUSSION

In this study, we identified *sas* as a new locus involved in morphological development in *S. griseus*. The wide distribution of this gene cluster suggests that it plays a general role in *Streptomyces* spp. and related bacteria. The information currently available regarding the function of homologous proteins is insufficient for deducing the function of the Sas products. Although our evidence supported the view that Sas proteins take part in a particular process, we could not detect distinct interactions among Sas proteins in a two-hybrid-system analysis that was performed using *E. coli* as a host (our unpublished observation). Hence, we arrived at the assumption that the proteins do not form a complex with strong associations.

The activation of promoters preceding the *sas* genes during the early growth phase suggests that the role of Sas proteins is specific to vegetative cells. The fact that these promoters exhibited wild-type activities in the

Fig. 4. Macroscopic and microscopic observation of solid culture of an *sasABCD* mutant of *S. griseus*. The parental and *sasABCD* mutant strains were grown on ISP4 solid medium for 4 days. Bars, 5 μ m.

adpA mutant indicates that the expression of *sas* genes is independent of the A-factor cascade. The result of subcloning (Figs 1a and 2a) raises the possibility that the SasR product, a putative transcriptional regulator, controls the activity of the promoter preceding *sasA*, but we have not yet confirmed the binding of SasR protein to the promoter region. The fact that knockout of *sasR* did not affect the wild-type phenotype in *S. griseus* supports the view that the role of *sasR* is not significantly related to that of *sasABCD*, although the close location of the coding sequences still implies some functional relationship. Characterization of the transcriptional control of the *sas* gene cluster may provide new information with regard to developmental signalling in *Streptomyces*.

The introduction of the *sas* gene cluster into *amfS* mutant cells via a high-copy-number plasmid induced aerial mycelium formation. We previously found that *amfS* encodes morphogenic peptides essential for the formation of aerial mycelium in *S. griseus* (Ueda *et al.*, 2002). Evidence from a recent study on *S. coelicolor* A3(2) has indicated that AmfS produced by *S. griseus* is a homologue of the morphogenic hydrophobic peptide SapB in *S. coelicolor* A3(2) (Willey *et al.*, 2006). Hence, AmfS is currently assumed to be a hydrophobin that induces the formation of aerial mycelium by reducing the surface tension of the substrate mycelium, based on the knowledge gained from the study of *S. coelicolor* A3(2).

A possible explanation for the suppressive effect of *sas* is that its overexpression induced the production of a hydrophobic morphogen alternative to AmfS. However, the results of a

Fig. 5. Macroscopic and microscopic observation of solid culture of an *sasABCD* mutant of *S. coelicolor* A3(2). The parental and *sasABCD* mutant strains were grown on YMP solid medium containing 1% glucose for 4 days. Bars, 2 μ m (upper) and 20 μ m (lower).

cross-feeding assay (Fig. 1c) did not reveal the secretion of any diffusible morphogenic substance by the *amfS* mutant harbouring pTT1. Another possibility is that the *sas* genes are associated with the cellular responses to hydrophobins. Although the existence of such a mechanism has not yet been confirmed, the possibility that some specific system is involved in or associated with the response of substrate mycelium to exogenous hydrophobins could prove to be a persuasive argument. The marked adhesion of aerial mycelium that was induced by the introduction of *sas* genes in various strains (Fig. 1b) leads to the assumption that it has some relationship with the mechanism of cell-surface activation. Further investigations on the functions of the Sas proteins may reveal other aspects of the complex morphological development in *Streptomyces* spp.

ACKNOWLEDGEMENTS

We thank Y. Ohnishi and S. Horinouchi for providing the *adpA* mutant, and H. Ikeda for providing the integration plasmid. This study was supported by the High-Tech Research Center Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Edited by: M. Paget