An AfsK/AfsR system involved in the response of aerial mycelium formation to glucose in *Streptomyces griseus*

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In *Streptomyces coelicolor* A3(2), a protein serine/threonine kinase (AfsK) and its target protein (AfsR) control secondary metabolism. AfsK and AfsR homologues (AfsK-g and AfsR-g) from *Streptomyces griseus* showed high end-to-end similarity in amino acid sequence with the respective *S. coelicolor* A3(2) proteins, as determined by cloning and nucleotide sequencing. AfsK-g and a fusion protein between AfsK-g and thioredoxin (TRX–AfsK-g) produced in high yield as inclusion bodies in *Escherichia coli* were solubilized with urea, purified by column chromatography and then refolded to an active form by dialysis to gradually remove the urea. AfsR-g was also fused to glutathione S-transferase (GST–AfsR-g); the fusion product in the soluble fraction in *E. coli* was purified. Incubation of AfsK-g or TRX–AfsK-g in the presence of \( \gamma^32P\)ATP yielded autophosphorylated products containing phosphoserine and phosphothreonine residues. In addition, TRX–AfsK-g phosphorylated serine and threonine residues of GST–AfsR-g in the presence of \( \gamma^32P\)ATP. Disruption of chromosomal afsK-g had no effect on A-factor or streptomycin production, irrespective of the culture conditions. The afsK-g disruptants did not form aerial mycelium or spores on media containing glucose at concentrations higher than 1%, but did form spores on mannitol- and glycerol-containing media; this suggests that afsK-g is essential for morphogenesis in the presence of glucose. Introduction of afsK-g restored aerial mycelium formation in the disruptants. The phenotype of afsR-g disruptants was similar to that of afsK-g disruptants; introduction of afsR-g restored the defect in aerial mycelium formation on glucose-containing medium. Thus the AfsK/AfsR system in *S. griseus* is conditionally needed for morphological differentiation, whereas in *S. coelicolor* A3(2) it is conditionally involved in secondary metabolism.

Keywords: *Streptomyces griseus*, serine/threonine kinase, signal transduction, protein phosphorylation, aerial mycelium formation

**INTRODUCTION**

The Gram-positive bacterial genus *Streptomyces* is characterized by complex morphological differentiation resembling that of filamentous fungi, and by the ability to produce a wide variety of secondary metabolites including antibiotics (Chater, 1984, 1989). In eukaryotes, many protein kinases control cellular processes that respond to a plethora of environmental cues via signal transduction networks and response mechanisms. It is conceivable that protein kinases control morphological differentiation and secondary metabolism in soil-living, filamentous *Streptomyces* spp.

Definitive evidence for involvement of ‘eukaryotic’-type protein kinases in the regulation of secondary metabolism in *Streptomyces* was obtained from a study of the AfsK/AfsR system in *Streptomyces coelicolor* A3(2) (Horinouchi & Beppu, 1992b; Matsumoto et al., 1994).
Recombinant AfsK produced in Escherichia coli cells autophosphorylated its serine and tyrosine residues and phosphorylated serine and threonine residues of AfsR, a regulatory protein involved in secondary metabolism in S. coelicolor A3(2) (Horinouchi et al., 1990; Hong et al., 1991). Disruption of the chromosomal afsK gene reduced actinorhodin production, but caused no detectable change in morphogenesis. An afsK-null mutant formed spores like the parental strain, suggesting that afsK is concerned with secondary metabolism but not with morphological differentiation. In this paper, the afsK and afsR genes of S. coelicolor A3(2) are referred to as afsK-c and afsR-c, respectively. The presence of multiple afsK-c homologues in Streptomyces griseus (afsK-g) was predicted by Southern hybridization experiments with a sequence encoding the kinase catalytic domain of AfsK as the probe (Matsumoto et al., 1994).

These observations prompted us to determine the role of the AfsK-c homologue in S. griseus. Since this work revealed that an afsK-c homologue mediated the response of aerial mycelium formation to glucose, we next cloned and characterized a probable afsK-c homologue from S. griseus (afsK-g) on the assumption that AfsR-g, a putative target of AfsK-g, would be concerned with the same biological function. This paper deals with the cloning, nucleotide sequencing and characterization of afsK-g and afsR-g in S. griseus. Biochemical studies showed autophosphorylation of serine and threonine residues in AfsK-g and phosphorylation by AfsK-g of serine and threonine residues of AfsR-g. Genetic studies, including gene disruption experiments, demonstrated that both AfsK-g and AfsR-g are involved in the response of aerial mycelium formation to glucose.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The wild-type strain S. griseus IFO13350 and an A-factor-deficient mutant strain S. griseus HH1 were described previously (Horinouchi et al., 1984). Plasmid pUC19 and E. coli JM109 (Yanisch-Perron et al., 1985) were used for DNA manipulation. For expression of afsK-g in E. coli BL21(DE3) (Studier & Moffatt, 1986), pGEMEX-1′ (Onaka et al., 1995) containing the IPTG-inducible T7 RNA polymerase promoter and PET-32a(+) containing the thioredoxin (TRX) gene (Novagen) were used. For expression of afsK-g, pGEX-5X-1 containing the glutathione S-transferase (GST) gene and E. coli BL21(DE3)pLysS (Novagen) were used. S. griseus was routinely cultured at 28 °C in YMPP medium containing the following (g L−1): yeast extract (Difco), 2; meat extract (Wako Pure Chemicals), 2; Bacto-peptone (Difco), 4; NaCl, 5; MgSO4, 7H2O, 2; glucose, 10 (pH 7.2), supplemented with 20 μg thiotrepton ml−1, if necessary. To investigate sporulation of S. griseus strains, this medium was modified to contain various amounts of glucose or other carbon sources. S. coelicolor A3(2) was grown in Trypoto-Soya broth (Nissui).

**General recombinant DNA techniques.** All DNA-modifying enzymes were purchased from Takara Shuzo. [α-32P]dCTP at 3000 Ci mmol−1 (110 TBq mmol−1) for nucleotide sequencing by the M13 dideoxynucleotide method (Sanger et al., 1977) with M13mp18 and M13mp19 (Yanisch-Perron et al., 1985), and for DNA labelling with the BcaBest labelling kit (Takara Shuzo) was purchased from Amersham Pharmacia Biotech. [γ-32P]ATP at 6000 Ci mmol−1 (220 TBq mmol−1) for in vitro phosphorylation and for making 32P-probes for S1 nuclelease mapping was also purchased from Amersham. DNA was manipulated in E. coli and Streptomyces spp. as described by Maniatis et al. (1982) and by Hopwood et al. (1985), respectively.

**S1 nuclease mapping.** Transcriptional start points of afsK-g and afsR-g were determined by S1 nuclease mapping with mRNA prepared as described previously (Horinouchi et al., 1987). To prepare the probe for afsK-g, two primers, 5′-GGTGCAAGAGACATCCCTTCGCC-3′ (corresponding to the sequence −212 to −189, numbering the G residue of the initiation codon GTG as +1) and 5′-GGCGCATCCGGTCTCCTGGGTGTG-3′ (corresponding to +43 to +23) were used to amplify the probe sequence by PCR. The +43 to +23 primer was 32P-labelled at the 5′ end with T4 polynucleotide kinase before PCR. To prepare the probe for afsR-g, 5′-GGTACCGCGCCATCCGGATGC-3′ (corresponding to −121 to −100, numbering the A residue of the initiation codon ATG as +1) and 5′-CGGTATGAAGAGGTCCGGTGGT-GCTG-3′ (corresponding to +60 to +40) were used for PCR, and similarly 32P-labelled at the 5′ end. To prepare the probe for hrdB, 5′-TGGGCCCATTTTCGTGACATAGG-3′ (corresponding to −243 to −220, numbering the G residue of the initiation codon GTG of hrdB as +1; Shinkawa et al., 1995) and 5′-TCATGAGGCCCATCAACGACTCG-3′ (corresponding to +71 to +48) were used as PCR primers with chromosomal DNA of S. griseus IFO13350 as the template, and similarly 32P-labelled at the 5′ end. For high-resolution S1 mapping, protected DNA fragments were analysed on DNA sequencing gels by the method of Maxam & Gilbert (1980).

**Construction of afsK-g expression plasmids in E. coli.** To produce AfsK-g in the intact form, we first constructed pGEMEX-AfsK-g as follows. The ATG codon was included in a Ndel cleavage sequence, CATATG. To place afsK-g under the control of the T7 promoter, the nucleotide sequence (CAAGTG) covering the GTG start codon of afsK-g was first changed to CATATG by PCR. For this purpose, two primers, 5′-GGAGTTGCCAGCATATTGATGCATACGCTG-3′ (italic letters indicate the bases to be replaced) and 5′-GGCGAGCTCTGCTCCGACGGTGCTTGATGC-3′ (a sequence based on the region covering a SacI cleavage site, shown in italic letters, within the coding sequence; see Fig. 1a) were synthesized on an ABI 380A DNA synthesizer. As the target DNA, a 787 bp SalI fragment containing this region (see Fig. 1a) was inserted into M13mp19. After PCR under standard conditions, the mutation was checked by nucleotide sequencing and the Ndel–SacI fragment was recovered. A 3.6 kb SacI–SalI fragment containing the remaining C-terminal portion of AfsK-g was inserted into pUC19 and the 3.6 kb fragment was recovered as a SacI–HindIII fragment. The Ndel–SacI and SacI–HindIII fragments were then inserted between the Ndel and HindIII sites of pGEMEX-1′ by three-fragment ligation to give pGEMEX-AfsK-g for directing the synthesis of AfsK-g in response to IPTG.

Plasmid pTRX-AfsK-g encoding a fusion protein between TRX and AfsK-g was also constructed as follows. pUC19-Kg, in which the 3 kb Aor51HI–HindII fragment (see Fig. 1) encoding the whole AfsK-g was contained at the HindII site, was used as the starting material. To place an EcoRI site in front of the ATG start codon of afsK-g, an N-terminal portion from the start codon to the SacI site was amplified with the following primers: 5′-ggcaggctcatGTTGGAGAGCTG-3′ (capsitals correspond to the afsK-g sequence
and the italicized ATG represents the start codon; the underlined sequence represents an EcoRI site and 5'-gcaaatgccGAGCTCGGTCCAGCCGTCGTC-3' (capital letters correspond to the afsK-g sequence and the italicized and underlined sequences represent SacI and HindIII sites, respectively). Absence of amplification errors was confirmed by nucleotide sequencing. The fragment was digested with EcoRI plus SacI, and cloned between the EcoRI and SacI sites of pUC19. The EcoRI–SacI fragment encoding an N-terminal portion and the 3'6 kb SacI–HindIII fragment encoding the remaining portion of AfsK-g were inserted between the EcoRI and HindIII sites of pET-32a(+) by three-fragment ligation. pTRX-AfsK-g constructed in this way directed synthesis of the fusion protein TRX-histidine tag-enterokinase cleavage site-AfsK-g.

Expression and purification of recombinant AfsK-g and TRX–AfsK-g. To produce AfsK-g, 10 ml of an overnight culture of *E. coli* BL21(DE3) containing pGEMEX-AfsK-g was used to inoculate 1 l Luria broth (Maniatis *et al.*, 1982) supplemented with 100 µg ampicillin ml⁻¹. After incubation at 37 °C for 2.5 h, IPTG (0.5 mM) was added to induce the T7 promoter and incubation was continued for 3 h. Cells harvested by centrifugation were washed once with buffer A [50 mM Tris/HCl (pH 8.3) and 10 mM 2-mercaptoethanol], suspended in 3 ml buffer A and disrupted by sonication. Because AfsK-g was produced as inclusion bodies, the pellet obtained from the sonicate by centrifugation at 5000 g for 5 min was used as the starting material for purification. It was suspended in buffer A containing 4 M urea and the mixture was centrifuged at 5000 g for 5 min. Buffer B (buffer A containing 6 M urea) was mixed with the pellet to solubilize AfsK-g. The supernatant obtained by centrifugation at 20000 g for 30 min contained most of the AfsK-g protein. The solubilized AfsK-g protein was applied to a DEAE-Toyopearl 650M column (4 × 19 cm) previously equilibrated with buffer B and proteins were eluted with 100 ml of a linear gradient of 0–0.5 M NaCl in buffer B. Elution was monitored by SDS-PAGE. Peak fractions (4 ml) were pooled and applied to a Pharmacia Superose 12 HR10/30 FPLC column equilibrated with buffer B. Proteins were eluted with buffer B containing 0.2 M NaCl. Fractions (0.5 ml) containing AfsK-g giving a single protein band on SDS-PAGE were pooled. To refold the denatured AfsK-g, the urea was removed by successive dialysis.

For purification of TRX–AfsK-g, a crude lysate of *E. coli* harbouring pTRX–AfsK-g was used. Because the fusion protein was also produced mainly as inclusion bodies, it was similarly solubilized with 6 M urea. The buffer used was G buffer containing 70 mM Tris/HCl (pH 8.2) and 20% (v/v) glycerol. The solubilized protein was purified as described above by chromatography on a DEAE-Toyopearl 650M column. Protein concentrations were measured with a dye-
binding protein assay kit (Bio-Rad) using bovine serum albumin as the standard.

**Construction of AfsR-g expression plasmid.** An EcoRI site was generated in front of the ATG start codon by PCR with 5'-ggcaggtctATGGACGGTCGACAGGCGAC-3' (the afsR-g sequence is capitalized, with the ATG start codon in italics; an EcoRI site is underlined) and 5'-ggcaggtctACGCCTCCTGGTATCCTGAGAC-3' (the underlined and italicized sequences indicate HindIII and Aor51HI sites, respectively; the Aor51HI site is located in the coding sequence; see Fig. 1b). A XhoI site was generated immediately after the stop codon by PCR with 5'-ggcaggtctCTGGAGATATTTCGGAGAGCCGG-3' (the italicized and underlined sequences are PstI and EcoRI sites, respectively; the PstI site is located in the coding sequence) and 5'-ggcaggtctCTCGAGATTCGCCCACGCCCTGGCGG-3' (the underlined and italicized sequences indicate HindIII and XhoI sites, respectively; the afsR-g stop codon is in bold letters). The sequences amplified by PCR were checked by nucleotide sequencing. The EcoRI–Aor51HI fragment designed for the N terminus, the Aor51HI–PstI internal fragment and the PstI–XhoI fragment designed for the C-terminal portion were assembled correctly in pUC19 by standard DNA manipulations. The EcoRI–XhoI fragment encoding the whole AfsR-g protein was then inserted between the EcoRI and XhoI sites of pGEX-SX-1 to give pGST-AfsR for directing synthesis of a GST–AfsR-g fusion protein.

**Expression and purification of GST–AfsR-g.** The tac promoter in pGST-AfsR-g in *E. coli* BL21(DE3)pLysS was induced by IPTG in the same way as for pGEMEX-AfsK. The *E. coli* cells were harvested by centrifugation, washed once with PBS buffer containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 1.8 mM KH2PO4 (pH 7.3) and disrupted by mild sonication. Triton X-100 was then added to give a final concentration of 1% and the mixture was gently mixed for 30 min to solubilize the fusion protein. The lysate was cleared of debris by centrifugation at 12,000 g for 10 min. The supernatant, after filtration through a 0.45-μm filter, was applied to a glutathione Sepharose 4B column (Amersham Pharmacia Biotech) previously equilibrated with PBS buffer. Fractions (1 ml) containing GST–AfsR were collected and dialysed against 50 mM Tris/HCl (pH 8/0)/1 mM DTT.

**Phosphorylation protocols and phosphoamino acid analysis.** For autophosphorylation of AfsK-g (10 μg) or TRX–AfsK-g (2.5 μg), the refolded proteins were incubated with 10 μCi (370 kBq) [γ-32P]ATP in 20 mM HEPES (pH 7.2), 10 mM MgCl2, 10 mM MnCl2, at 30 °C for 15 min in a total volume of 20 μl. For phosphorylation of GST–AfsR-g by TRX–AfsK-g, GST–AfsR-g (5-μg protein) was also added to the reaction mixture. The reaction was terminated by boiling for 2 min after adding 4 μl 375 mM Tris/HCl (pH 6.8), 60% glycerol, 12% SDS, 6% 2-mercaptoethanol and 0.003% bromophenol blue. The stopped reaction mixture was fractionated by 0.1% SDS-6% PAGE. Phosphorylated proteins were transferred to a PVDF membrane and detected by autoradiography. 32P-labelled AfsK-g, TRX–AfsK-g and GST–AfsR-g proteins were recovered from the membrane and hydrolysed in boiling 6 M HCl for 90 min (Kamps & Sefton, 1989). The hydrolysates were analysed by onedimensional electrophoresis on a cellulose thin-layer plate (Cooper et al., 1983).

**Gene disruption.** For disruption of afsK-g, the 3018 bp Aor51HI–HincII fragment encoding AfsK-g (see Fig. 1a) was cloned in the EcoRI site of pUC19 after an EcoRI linker had been attached to both ends. The 1.3 kb Smal fragment containing *aphII* (Beck et al., 1982) was inserted between the SacII sites to replace the fragment encoding Pro-83 to Ala-388. The recombinant plasmid was purified, digested with EcoRI, denatured with 0.2 M NaOH and introduced into *S. griseus* IFO13350 to obtain afsK-g disruptants by double-crossover (Oh & Chater, 1997). Correct insertion of *aphII* at the afsK-g locus was checked by Southern hybridization against the NcoI-digested chromosomal DNA with the Aor51HI–SphI fragment and the *aphII* sequence as 32P-labelled probes. For disruption of afsR-g, the 4 kb *SpII* fragment containing most of the afsR-g sequence was cloned in pUC19 (plasmid pUC19SphI). The 194 bp *MluI* fragment encoding Pro-7 to Asp-72 of AfsR-g was replaced with the *aphII* sequence. The pUC19 plasmid containing the disrupted afsR-g was linearized with DraI, denatured and introduced into the wild-type strain IFO13350, as described above. Correct gene replacement was checked by Southern hybridization against the SphI-digested chromosomal DNA with the SacI fragment from pUC19SphI and the *aphII* sequence as probes.

**Construction of plasmids.** The afsK-g sequence was divided into Aor51HI–BanII and BanII–HincII fragments (see Fig. 1a) and these were separately cloned in pUC19 by standard DNA manipulations. The multicloning sites in pUC19 facilitated assembly of the two fragments as an EcoRI fragment, which was then inserted into pKU209, resulting in pKU209-Kg. Similarly, the afsR-g sequence was divided into HincII–HincII and Aor51HI–Aor51HI fragments (see Fig. 1b), which were cloned in pUC19, assembled as an EcoRI fragment and cloned in pKU209 to give pKU209-Rg.

**RESULTS**

**Cloning and nucleotide sequence of afsK-g from *S. griseus***

Southern hybridization of *KpnI*-digested chromosomal DNA of *S. griseus* with part of the DNA fragment encoding the kinase catalytic domain of AfsK from *S. coelicolor* A3(2) (as the probe) showed a single strong signal and two weak signals (Matsumoto et al., 1994). To clone DNA giving the strong signal, a similar Southern hybridization of *BamHI*-digested *S. griseus* chromosomal DNA was performed with an about 800 bp *SpII*–*SphI* fragment encoding an N-terminal portion (Met-1 to Met-248) of the kinase as the probe. Consistent with the previous observation, a single strong signal of 4 kb, in addition to two weak signals, were detected. We cloned the 4 kb fragment on pUC19 in *E. coli* by standard recombinant DNA techniques, including colony hybridization. Because the 4 kb fragment lacked the 3' portion of afsK-g, as determined by nucleotide sequencing, we further cloned a 4 kb SalI fragment by similar DNA manipulation. The restriction map of the region cloned in these two experiments is shown in Fig. 1(a), together with the location of ORFs predicted by FRAME analysis (Bibb et al., 1984) of the nucleotide sequence.

The deduced amino acid sequence of AfsK-g and alignment with other kinases are shown in Fig. 2. A possible RBS, GGTGG, is located 7 nucleotides up-stream of the putative translational start codon, GTG (Fig. 1a). AfsK-g (807 aa) showed 77-4% overall identity in amino acid sequence to AfsK-c (799 aa). As in AfsK-
Fig. 2. Amino acid sequence alignment of AfsK-g and AfsK-c. Identical amino acids are indicated by asterisks. Dashes represent gaps introduced to optimize the alignment. Also shown is an alignment of the N-terminal portions of AfsK-c and AfsK-g with those of other serine/threonine kinases: β-adrenergic receptor kinase (BARK; Benovic et al., 1989), a serine/threonine kinase of Myxococcus xanthus (PKN1; Munoz-Dorado et al., 1991) and a cellular homologue of an oncogene product from Rous avian sarcoma virus (SRC; Anderson et al., 1985). BARK and PKN1 are serine/threonine-specific kinases and SRC is a tyrosine-specific kinase. Highly conserved amino acids in conserved subdomains I–XI in eukaryotic kinases (Hanks et al., 1988) are boxed.

c, the N-terminal portion of AfsK-g, representing the kinase catalytic domain, showed significant sequence similarity to eukaryotic protein serine/threonine kinase domains (Hanks et al., 1988; Soderling, 1990). Alignment of the catalytic domains of both serine/threonine-specific and tyrosine-specific protein kinases showed eleven major conserved subdomains, including Gly-X-Gly-(X)_{3}-Gly (X is a nonconserved amino acid) forming the ATP-binding site (Hanks et al., 1988) and Arg-Asp-Leu-[X]_{5}-Asp-Phe-Gly-(X)_{2}-Gly-Thr-Pro-(X)_{2}-Ala forming a triad of amino acids responsible for recognizing the correct hydroxyamino acid, and for enzyme catalysis (Taylor, 1989). As in AfsK-c, the C-terminal portion of AfsK-g is separated from the catalytic domain by an Ala/Pro-rich region. Sequence similarity in the C-terminal regions of AfsK-c and AfsK-g is low relative to that in the N-terminal portion containing the kinase catalytic domain.

We sequenced upstream and downstream of afsK-c to detect ORFs similar to those found near afsK-c of S. coelicolor A3(2). In S. coelicolor A3(2), afsR-c is located just downstream from afsK-c (Matsumoto et al., 1994). ORF-D (232 aa) lies upstream of AfsK-c, and ORF-B (176 aa), AfsS (63 aa) and AfsR (993 aa) lie downstream of it (Matsumoto et al., 1994, 1995). ORF-1 (269 aa) is present upstream of AfsK-g (Fig. 1a), and is preceded by a possible RBS, GGAAGGA. ORF-1 shows 42%–9% identity in amino acid sequence to ORF-D of S. coelicolor A3(2). However, the region as far as about 2 kb downstream from afsK-c has no ORFs similar to ORF-B, AfsS or AfsR. Thus the organization of genes downstream of afsK in S. griseus and S. coelicolor A3(2) appears to be totally different.

Cloning and nucleotide sequence of afsR-g from S. griseus

Southern hybridization of the SphI-digested chromosomal DNA of S. griseus with part of the DNA fragment encoding two ATP-binding consensus sequences of S.
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Fig. 3. Amino acid sequence alignment of AfsR-g and AfsR-c. Identical amino acids are indicated by asterisks. Dashes represent gaps introduced to optimize the alignment. Two ATP-binding consensus sequences are boxed.

coelicolor A3(2) afsR as the probe gave a strong signal of 40 kb. We cloned this fragment by standard DNA manipulations, including colony hybridization. Because nucleotide sequencing of the 4 kb fragment did not detect the C terminus of AfsR-g, we also cloned a 6.5 kb BamHI fragment that gave a positive signal in similar Southern hybridizations. As a result, we cloned a total of 8 kb containing the whole afsR-g gene (Fig. 1b). A probable RBS, AGG, is present 8 nucleotides upstream of the translational start codon of afsR-g (Fig. 1b). Of the ORFs deduced from the flanking nucleotide sequences, Orf1, Orf2 and Orf4 showed no similarity in amino acid sequence to proteins in the EMBL or GenBank databases, indicating that the gene organization in the regions neighbouring afsR in S. griseus and S. coelicolor A3(2) is totally different.

afsR-g encodes a 974 aa protein that shows high end-to-end similarity to AfsR-c (993 aa; Fig. 3). Like AfsR-c, AfsR-g contains two types of amino acid sequence that resemble a consensus sequence for an adenosine-binding fold (Walker et al., 1982); these are an A-type consensus sequence which contains a flexible loop formed by the Gly-rich sequence followed by Lys, probably interacting with the z-phosphate, and a B-type sequence that contains a hydrophobic b sheet at the back of the adenine nucleotide pocket and Asp for binding a magnesium ion.

Transcriptional analyses of afsK-g and afsR-g

mRNA was purified from S. griseus IFO13350 mycelium and used to determine transcriptional start points for afsK-g and afsR-g. Low-resolution S1 nuclease mapping identified two transcriptional start points for afsK-g and a single point for afsR-g (Fig. 4a). Both genes were transcribed throughout growth, in this respect resembling brdB which encodes one of the major f factors (Shinkawa et al., 1995). In Fig. 4(a), results obtained with mRNA prepared from S. griseus grown in a medium containing 1–5% glucose are also shown (see below).

High-resolution S1 mapping determined the two 5' ends of the transcripts of afsK-g (Fig. 4b). The hexameric sequences TTCGCA and AAGAAT, separated by 18 bp, are present in front of the upstream one (Fig. 1a). These
hexamers show similarity to those (TTGACA for $-35$ and TATAAT for $-10$ with a 17 bp space) of other prokaryotic promoters, including one type (TTGACA for $-35$ and TAGGAT for $-10$ with a 18 bp space) of a Streptomyces promoter believed to be active during vegetative growth (Hopwood et al., 1986). The downstream transcript is preceded by the putative $-10$ hexamer GAGAAC, but shows no sequence similar to the $-35$ consensus sequence.

At an appropriate position in front of the transcriptional start point of afsR-g (Fig. 4c), there is a hexamer, CATACG, similar to the $-10$ consensus sequence (Fig. 1b), but no sequence similar to the $-35$ consensus sequence is present.

**Autophosphorylation of serine and threonine residues in AfsK-g**

We first expressed the intact afsK-g sequence in *E. coli*, produced AfsK-g and purified it. Briefly, afsK-g was placed under the control of the IPTG-inducible T7 RNA polymerase promoter in such a way that the second GTG codon of afsK-g was connected to the ATG start codon of the T7 gene 10 in pGEMEX-1. *E. coli* harbouring pGEMEX-1 produced AfsK-g of the expected size (83 kDa) in a very large amount in response to IPTG, but as inclusion bodies. The product was solubilized with 6 M urea and purified in a urea-denatured form by ion-exchange chromatography and gel filtration, giving a single protein band by SDS-PAGE. The purified, denatured protein was refolded by dialysis to remove urea gradually. Incubation of the refolded protein with $[\gamma^{32P}]$ATP and Mg$^{2+}$ led to the incorporation of $^{32P}$. Phosphoamino acid analysis showed that $^{32P}$ was incorporated into serine and threonine residues (data not shown).

We then produced AfsK-g as a fusion product with TRX. afsK-g was inserted into pET32a(+) so that the whole AfsK-g sequence was fused to the C terminus of TRX via the linker amino acids. The fusion protein produced in response to IPTG in *E. coli* was still mainly in the insoluble fraction (Fig. 5a). We solubilized the TRX–AfsK-g fusion protein with 6 M urea and purified it to give a single 110 kDa band on SDS-PAGE gels. Incubation of TRX–AfsK-g with $[\gamma^{32P}]$ATP yielded a
product autoprophorylated at threonine and serine residues (Fig. 5c), as determined by phosphoamino acid analysis (Fig. 5d). The efficiency of $[^{32}\text{P}]$P incorporation into TRX-AfsK-g was much higher than into the refolded AfsK-g protein described earlier.

**Phosphorylation of serine and threonine residues of AfsR-g by TRX–AfsK-g**

We inserted afsR-g in pGEX-5X-1 to produce GST–AfsR-g, in which AfsR-g was fused to the C terminus of GST. Induction with IPTG of *E. coli* harbouring the recombinant plasmid yielded fusion protein in both soluble and insoluble forms (Fig. 5b); we purified GST–AfsR-g from the soluble fraction by affinity chromatography to give a single band of 135 kDa on SDS-PAGE gels. Incubation of GST–AfsR-g with TRX–AfsK-g in the presence of $[^{32}\text{P}]$ATP yielded a phosphorylated form of GST–AfsR-g, in addition to an autoprophorylated form of TRX–AfsK-g (Fig. 5c). Phosphoamino acid analysis established that the phosphorylated GST–AfsR-g contained phosphoserine and phosphothreonine (Fig. 5d). TRX–AfsK-g did not phosphorylate GST alone when a crude lysate of *E. coli* containing pTRX-AfsK-g was similarly examined.

**Involvement of afsK-g and afsR-g in aerial mycelium formation on glucose-containing medium**

We disrupted afsK-g and afsR-g and examined the phenotype of the disruptants to determine whether these genes function in cell differentiation and secondary metabolism. Three afsK-g and three afsR-g disruptants checked by Southern hybridization for correct replacement (data not shown) were investigated. These strains grew to almost the same cell mass in liquid culture as the wild-type and thus had no severe growth defects. We first examined their production of streptomycin and A-factor on various media, since afsK-c and afsR-c are involved in secondary metabolism in *S. coelicolor* A3(2) (Horinouchi et al., 1990; Matsumoto et al., 1994). However, streptomycin titres measured by using *Bacillus subtilis* as an indicator and A-factor levels measured by the streptomycin cosynthesis method (Hara & Beppu, 1982) were comparable to those in the wild-type (data not shown), which excludes the possibility that afsK-g and afsR-g influence secondary metabolism.

On the other hand, neither the afsK-g disruptants nor the afsR-g disruptants formed aerial mycelium, and accordingly spores, on glucose-containing medium (Fig. 6a). Scanning electron micrographs of these mutants on
glucose-containing medium (Fig. 7) confirmed macroscopic observations in occasionally showing a short aerial hypha for the afsK-g disruptant, but never for the afsR-g disruptant. The centres of the wild-type and afsK-g disruptant colonies contained only sparse aerial mycelium, probably due to catabolite repression by glucose. The afsK-g and afsR-g disruptants formed spores as abundantly as the parental strain on mannitol- and glycerol-containing media. The effect of glucose on aerial mycelium formation by the disruptants was obvious at concentrations above 1%. On such media aerial mycelium formation by the disruptants was almost no aerial hyphae on glucose-containing medium, whereas they formed abundant aerial hyphae on mannitol- and glycerol-containing media. (b) Complementation of aerial-mycelium-defective phenotypes of afsK-g and afsR-g disrupted mutants on 15% glucose-containing medium by the respective genes. Top row, introduction of pKU209 or pKU209K-g into the wild-type did not affect aerial hyphae formation, whereas pKU209K-g abolished it almost completely; middle row, introduction of pKU209K-g into strain ΔafsK-g restored formation of aerial hyphae; bottom row, introduction of pKU209R-g into strain ΔafsR-g restored formation of aerial hyphae.

**Fig. 6.** Effects of afsK and afsR disruption on aerial mycelium formation by *S. griseus*. All colonies were formed by plating suspensions of spores formed on glucose-free medium. Photographs were taken after growth at 28 °C for 7 d. (a) Wild-type (wt) and disrupted strains of *S. griseus* on YMPE medium containing 1.5% each of glucose, mannitol or glycerol. The wild-type formed aerial mycelium on all media, although with glucose, aerial mycelium was less abundant. Both afsK-g and afsR-g disrupted strains (ΔafsK-g and ΔafsR-g, respectively) formed almost no aerial hyphae on glucose-containing medium, whereas they formed abundant aerial hyphae on mannitol- and glycerol-containing media. (b) Complementation of aerial-mycelium-defective phenotypes of afsK-g and afsR-g disrupted mutants on 15% glucose-containing medium by the respective genes. Top row, introduction of pKU209 or pKU209K-g into the wild-type did not affect aerial hyphae formation, whereas pKU209R-g abolished it almost completely; middle row, introduction of pKU209K-g into strain ΔafsK-g restored formation of aerial hyphae; bottom row, introduction of pKU209R-g into strain ΔafsR-g restored formation of aerial hyphae.

Introduction of afsK-g on the low-copy-number recombinant plasmid pKU209K-g into the afsK-g disruptants restored the aerial mycelium-defective phenotype on 1.5% glucose-containing medium (Fig. 6b), although aerial hyphae were sparse in the catabolite-repressed central area of the colonies.

On the other hand, introducing afsR-g as pKU209R-g (vector copy number of 1–2) into the wild-type strain abolished aerial mycelium formation (Fig. 6b). In addition, streptomycin production was almost abolished. We assume that this is due to disturbance of physiological conditions caused by a slightly larger amount of AfsR-g in the transformant than in the wild-type strain. Even an increase in the copy number of afsR-g of 1–2 might increase the amount of AfsR-g enough to influence metabolic pathways. When pKU209R-g was introduced into the afsR-g disrupted strain, aerial mycelium formation was comparable to that in the afsK-g disrupted strain harbouring pKU209K-g (Fig. 6b).

Since afsK-g and afsR-g seemed to be involved in the response of aerial mycelium formation to glucose, we examined the effects of glucose on transcription. mRNA prepared from mycelium grown for various times on a cellophane sheet laid on the surface of agar medium showed that both genes were transcribed to almost the same levels throughout growth, irrespective of whether glucose was present (Fig. 4a). These results exclude the possibility that glucose influences transcription of these genes.

**Phosphorylation of AfsR-g by an additional serine/threonine kinase**

In *S. coelicolor* A3(2), AfsR-c was phosphorylated on its serine and threonine residues not only by AfsK-c but also by a still unknown kinase (Matsumoto et al., 1994). A crude lysate prepared from the afsK-g disruptant retained the ability to phosphorylate GST–AfsR-g added exogenously to the reaction mixture containing...
Since the phosphorylation of its serine and threonine residues (data not shown). Since the phosphorylation of its serine and threonine residues (data not shown).

**DISCUSSION**

The present study clearly demonstrates that a protein serine/threonine kinase AfsK-g and its target AfsR-g are involved in the response of aerial mycelium formation to glucose. These proteins appear to be concerned only with aerial mycelium formation, and not with secondary metabolism. It is conceivable that they are members of a signal transduction system responding to glucose in the medium. Based on analogy with typical signal transduction systems in prokaryotes and eukaryotes, we speculate that AfsK-g autophosphorylates its serine and threonine residues upon sensing a high concentration of glucose and transfers the signal to AfsR-g by means of phosphorylation; the phosphorylated GST–AfsR-g then activates expression of multiple genes required for aerial mycelium formation under such conditions. Since transcription of afsK-g or afsR-g was not affected by glucose, kinase activity of AfsK-g may be enhanced autonomously on sensing excess glucose or with the aid of some other protein(s) able to sense the glucose concentration. Because A-factor in cooperation with its receptor serves under all conditions as an ‘all-or-none’ switch for aerial mycelium formation in *S. griseus* (Horinouchi & Beppu, 1992a, 1994; Horinouchi, 1996), the AfsK-g/AfsR-g system must function after the switch has been turned on by A-factor. The present study provides clues for investigating how *Streptomyces* spp. respond to carbon sources in the medium.

In *S. coelicolor* A3(2), afsK-c and afsR-c seem to be concerned only with secondary metabolism; mutants disrupted in afsK-c or afsR-c form abundant spores on any medium irrespective of carbon or nitrogen source (Matsumoto et al., 1994). Thus, despite their strong similarity, the homologous AfsK/AfsR systems in *S. coelicolor* A3(2) and *S. griseus* control different metabolic processes. By careful examination of the phenotypes of *afsR* mutants of *S. coelicolor* A3(2), Floriano & Bibb (1996) showed that afsR-c is a pleiotropic regulatory gene required for secondary metabolism under some nutritional conditions. Because of their close end-to-end similarity in amino acid sequence, we propose that the AfsKs in both strains sense nutritional conditions, such as carbon and nitrogen concentrations, and activate their kinase activity by autophosphorylating serine and threonine residues. Differences in phenotype of afsK and afsR mutants in the two species presumably depend on genes or gene products controlled by phosphorylated AfsRgs. We postulate that the AfsK/AfsR systems can regulate both morphological differentiation and secondary metabolism in response to nutritional conditions. The possibility that the system in *S. griseus* influences secondary metabolism and that the
system in *S. coelicolor* A3(2) influences morphogenesis, but in both cases to undetectable levels, cannot be excluded. Some phenotypic changes in *afsK* and *afsR* mutants may become apparent under certain circumstances, but not on agar medium in Petri dishes under laboratory conditions.

We previously reported that *AfsK*-c autophosphorylates its threonine and tyrosine residues (Matsumoto et al., 1994). Because the autophosphorylated amino acids of *AfsK*-g were serine and threonine, we constructed a new plasmid to produce *AfsK*-c with a histidine tag in *E. coli*, refolded it rapidly and found that it autophosphorylated its serine and threonine residues (T. Umeyama & S. Horinouchi, unpublished data). We therefore assume that the difference in autophosphorylated amino acids of *AfsK*-c can be ascribed to subtle differences in the conformation of refolded proteins, as suggested by Lindberg et al. (1992). The dual specificity in autophosphorylation of protein kinases observed *in vitro* does not accurately reflect true substrate specificity (Nishida & Gotoh, 1993).

An additional serine/threonine kinase (*AfsX*) able to phosphorylate *AfsR*-g exists in *S. griseus*. *AfsR*-c is also phosphorylated by *AfsK*-c and an additional kinase in *S. coelicolor* A3(2) (Matsumoto et al., 1994). Why then does disruption of *afsK* cause severe defects in aerial mycelium formation and antibiotic production in the respective species? Fine tuning of *AfsR* activity by phosphorylation by *AfsK* and *AfsX* in a concerted way may be important for normal development in response to nutritional conditions. Another possible explanation is that *AfsK* and *AfsX* phosphorylate serine and threonine residues at different positions in *AfsR*. A candidate for *AfsX* is perhaps encoded by one of the two genes giving a weak signal in the Southern hybridization experiment with the *afsK*-c sequence as probe.

That multiple serine/threonine-specific protein kinases play an important role in regulating morphogenesis and secondary metabolism in *Streptomyces* was initially suggested by *in vitro* protein phosphorylation experiments with cell lysates of *S. griseus* and *S. coelicolor* A3(2) treated with staurosporine and K-252a, known eukaryotic-type protein kinase inhibitors (Hong et al., 1993), and by direct screening by PCR for genes encoding a eukaryotic kinase domain (Urabe & Ogawara, 1995). Because of their different modes of action, the inhibitors affect the *in vitro* phosphorylation reactions of multiple proteins in different ways. Important roles for protein kinases sensitive to these drugs were implied from *in vivo* experiments in which the inhibitors affected aerial mycelium formation and antibiotic production (Hong et al., 1993; Hong & Horinouchi, 1998). Proteins with phosphotyrosines, detected by immunoblotting with anti-phosphotyrosine antibody (Waters et al., 1994; Kang et al., 1999) and a protein phosphotyrosine phosphatase (Li & Strohl, 1996; Umeyama et al., 1996) were also reported. In addition to these eukaryotic-type kinases, a two-component regulatory system typical of prokaryotes plays a regulatory role in secondary metabolism in *S. coelicolor* A3(2) (Ishizuka et al., 1992; Brian et al., 1996). Morphogenesis and secondary metabolism of *Streptomyces* spp. are thus controlled by signal transduction systems consisting of both prokaryotic- and eukaryotic-type protein phosphorylation.

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