A Streptomyces griseus gene (sgaA) suppresses the growth disturbance caused by high osmolality and a high concentration of A-factor during early growth

Noriko Ando, Kenji Ueda† and Sueharu Horinouchi

Author for correspondence: Sueharu Horinouchi. Tel: +81 03 3812 2111 ext. 5123. Fax: +81 3 5802 2931.

INTRODUCTION

A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone), produced in a growth-dependent manner, switches on secondary metabolite formation and morphological differentiation in Streptomyces griseus, presumably by binding to the A-factor receptor protein (ArpA)–DNA complex and releasing the repression caused by ArpA. In the A-factor-deficient mutant strain S. griseus HH1 a large deletion includes afsA which is required for A-factor production. Growth and aerial mycelium formation of strain HH1 on media containing high concentrations of sucrose, sorbitol, mannitol, KCl or NaCl was disturbed by the presence of a large amount of A-factor supplied either exogenously or by a high-copy-number plasmid carrying afsA. This disturbance did not occur on media of normal osmolality and was observed only when A-factor was supplied during the very early stage of growth, about 8 h after inoculation. In addition, neither the wild-type strain nor S. griseus KM7 defective in ArpA exhibited the disturbance. These observations suggest that the presence of a large amount of A-factor during the very early stage of growth, probably during the A-factor-sensitive stage, triggered abrupt and disordered expression of some genes. The effect was apparently mediated through ArpA in the A-factor regulatory cascade and disturbed the physiology of strain HH1 under high osmolality. A gene that suppressed the disturbance was identified 55 kb upstream of the afsA locus in the wild-type strain. The gene, named sgaA, encoded a protein of 264 aa with a calculated molecular mass of 28 kDa.

Keywords: A-factor, afsA, Streptomyces griseus, A-factor-sensitive stage, sgaA

INTRODUCTION

A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) is a chemical signalling molecule, or a microbial hormone, that controls secondary metabolism and cell differentiation in Streptomyces griseus (Khokhlov, 1982, 1988; Eritt et al., 1982; Horinouchi & Beppu, 1992, 1994). It acts as a switch for streptomycin production, streptomycin resistance, yellow pigment production and aerial mycelium formation at an extremely low concentration. Identification of an A-factor-specific receptor protein, ArpA (Miyake et al., 1989; Onaka et al., 1995), of 276 aa and a calculated molecular mass of 29.1 kDa, and subsequent studies with the cloned arpA gene, have led to the idea that A-factor produced at a critical concentration binds to DNA-bound ArpA and releases ArpA from the DNA, thus switching on expression of key genes leading to the onset of secondary metabolite formation and aerial mycelium formation. Consistent with the repressor-like behaviour of ArpA and the A-factor-mediated dissociation of ArpA from DNA, ArpA binds to a 22 bp palindromic nucleotide sequence in the absence of A-factor and exogenous addition of A-factor to the ArpA–DNA complex induces immediate release of ArpA from the DNA (Onaka et al., 1997; unpublished data).

Because of the essential role of A-factor in secondary metabolism and aerial mycelium formation, both exogenous addition of A-factor and introduction of afsA on a plasmid into the A-factor-deficient mutant S.
**METHODS**

**Bacterial strains, plasmids and media.** The A-factor-deficient mutant *S. griseus* HH1 (Horinouchi et al., 1984) is derived from the wild-type strain IFO 13350. This strain neither produces streptomycin nor forms aerial mycelium. For the A-factor assay the A-factor-deficient mutant *S. griseus* FT-1 no. 2 was used. It is derived from a high-level streptomycin producer, strain FT-1 (Hara & Beppu, 1982). *S. griseus* KM7 (Miyake et al., 1990), derived by NTG mutagenesis from strain HH1, has a defect in the A-factor receptor protein. *Escherichia coli* strains JM109 [recA1 thi-1 endA1 supE44 gyrA96 relA1 hsdR17 Δ(lac–proAB) F− traD36 proAB lacZΔM15] (Yanisch-Perron et al., 1985) and DH5α [F− 80 lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1] (Gibco-BRL) were used in most of the DNA manipulations. The *Streptomyces* vectors used as high-copy-number plasmids (40–100 copies per genome) were pIJ385 carrying thiostrepton and neomycin resistance genes (Ward et al., 1986) and pIJ702 carrying thiostrepton resistance and the melanin genes (Katz et al., 1983); both were obtained from D. A. Hopwood, John Innes Institute, Norwich. The low-copy-number *E. coli–Streptomyces* shuttle vector, pKU209, carrying the thiostrepton resistance gene and the SCP2*-replication origin (Kakinuma et al., 1991) was obtained from H. Ikeda, Kitasato University, Tokyo. Plasmids pAFBl and pAFB2, containing afsA on pIJ385 and pIJ702, respectively (see Fig. 1), were described previously (Horinouchi et al., 1984). Plasmid pAFB15 contained afsA and its promoter from the wild-type strain IFO 13350. This strain neither produces streptomycin nor forms aerial mycelium. For the A-factor assay the A-factor-deficient mutant *S. griseus* FT-1 no. 2 was used. It is derived from a high-level streptomycin producer, strain FT-1 (Hara & Beppu, 1982). *S. griseus* KM7 (Miyake et al., 1990), derived by NTG mutagenesis from strain HH1, has a defect in the A-factor receptor protein. *Escherichia coli* strains JM109 [recA1 thi-1 endA1 supE44 gyrA96 relA1 hsdR17 Δ(lac–proAB) F− traD36 proAB lacZΔM15] (Yanisch-Perron et al., 1985) and DH5α [F− 80 lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1] (Gibco-BRL) were used in most of the DNA manipulations. The *Streptomyces* vectors used as high-copy-number plasmids (40–100 copies per genome) were pIJ385 carrying thiostrepton and neomycin resistance genes (Ward et al., 1986) and pIJ702 carrying thiostrepton resistance and the melanin genes (Katz et al., 1983); both were obtained from D. A. Hopwood, John Innes Institute, Norwich. The low-copy-number *E. coli–Streptomyces* shuttle vector, pKU209, carrying the thiostrepton resistance gene and the SCP2*-replication origin (Kakinuma et al., 1991) was obtained from H. Ikeda, Kitasato University, Tokyo. Plasmids pAFBl and pAFB2, containing afsA on pIJ385 and pIJ702, respectively (see Fig. 1), were described previously (Horinouchi et al., 1984). Plasmid pAFB15 contained afsA and its promoter
(Horinouchi et al., 1985). Plasmid pARPL1 containing arpA on pKU209 was previously described (Onaka et al., 1995).

Growth conditions for E. coli were as described by Maniatis et al. (1982). S. griseus strains were grown on the following agar media: R medium [0.3 M sucrose, 1% glucose, 0.25% K2SO4, 1% MgCl2, 0.01% Casamino acids (Difco), 0.2% trace element solution (Hopwood et al., 1985), 0.3% l-proline, 0.2% l-asparagine, 0.005% KH2PO4, 0.294% CaCl2, 0.025 M TES, pH 7.2, and 2% agar], SD-R medium (sucrose-depleted R medium), YMPS medium [0.2% yeast extract (Difco), 0.2% meat extract (Difco), 0.4% Bacto-Peptone (Difco), 0.5% NaCl, 0.2% MgSO4, 7H2O, 1% glycan, 1% glucose and 2% agar, pH 7.2], Bennett-glucose medium [0.1% yeast extract, 0.1% meat extract, 0.2% N.Z. amine (Wako), 1% glucose and 2% agar, pH 7.2] and Bennett-maltose medium (containing maltose instead of glucose).

General recombinant DNA techniques. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase I (Klenow fragment) and Taq DNA polymerase were purchased from Takara Shuzo Co. (α-[32P]dCTP at 111 TBq mmol-1 for nucleotide sequencing by the M13-dideoxy-nucleotide method (Sanger et al., 1977) with M13mp18 and M13mp19 (Yanisch-Perron et al., 1985) was purchased from Amersham International. Thiostrepton was a gift from Asahi Kasei Co. Ltd. DNA manipulations in E. coli were as described by Maniatis et al. (1982) and those in Streptomyces were as described by Hopwood et al. (1985).

Construction of plasmids. Plasmids constructed in this study are shown schematically in Fig. 1. pAFB1L was constructed by inserting the 9.1 kb PstI fragment in the PstI site of the low-copy-number plasmid pKU209. For construction of pAFB10, an internal PvuII fragment was deleted from pAFB1. pAFB15L was constructed by first cloning the indicated EcoRI–BamHI fragment between the EcoRI and BamHI sites of pUC19. The fragment was then excised with EcoRI and SalI and inserted between the EcoRI and XhoI sites of pKU209. Deletion of the indicated MluI–EcoRI fragment from pAFB1 yielded pAFB18. For construction of pAFB19L, the EcoTI41–MluI fragment was inserted into the EcoRI site of pKU209 after an EcoRI linker had been attached to both ends. The EcoTI41–MluI fragment with an EcoRI linker at both ends was inserted into the EcoRI site of pAFB15L to give pAFB18L.

An afsK homologue together with a region encoding OrfX just upstream of the afsK homologue in S. griseus was cloned in E. coli (unpublished results) and registered in the EMBL/GenBank/DDJB databases under the accession number D45246. The afsK gene in S. coelicolor A3(2) was reported previously (Matsumoto et al., 1994). For construction of pAFK1L, a 2 kb SacI fragment covering the entire orfx and its upstream region of about 770 bp was cloned in the SacI site of pKU209.

A-factor assay by the streptomycin-cosynthesis method. A-factor production by S. griseus strains was assayed by the streptomycin-cosynthesis method described by Hara & Beppu (1982). The amount of A-factor was estimated by measuring the diameter of the growth inhibition zone and comparing it with a calibration curve obtained with authentic A-factor on paper discs. In a semi-log plot, a linear relationship was observed between the diameter of the growth inhibition zone and amounts of A-factor from 1 to 10 ng.

For A-factor assays of S. griseus cultured in liquid medium, strains were grown on a reciprocal shaker in YMPS medium at 28°C for 3 d for the wild-type strain and for 4 d for strains harbouring recombinant plasmids. Thiostrepton (40 μg ml-1) was added to strains harbouring plasmids with the thiostrepton resistance gene as a selection marker. The mycelium was homogenized with a glass homogenizer and 1 ml was used to inoculate 100 ml YMPS medium. The culture broth was sampled at intervals and A-factor was extracted with ethyl acetate to free it from streptomycin, which would interfere with the A-factor assay. The ethyl acetate was evaporated and the A-factor was diluted with ethanol to an appropriate concentration. Thiostrepton in the culture broth had a negligible effect on this assay.

RESULTS

Lack of aerial mycelium formation by S. griseus HH1 containing afsA at a high copy number

We previously cloned a 9.1 kb PstI fragment that restored A-factor production in the A-factor-deficient mutant S. griseus HH1 in the high-copy-number plasmid plJ385 (giving pAFB1; Fig. 1). The cloned fragment also restored aerial mycelium and spore formation and streptomycin production on Bennett-maltose and Bennett-glucose agar media (Horinouchi et al., 1984). Subcloning experiments identified afsA as the gene that conferred these phenotypes, since pAFB15 containing only afsA restored A-factor production in strain HH1 (Horinouchi et al., 1985). We also found that the 9.1 kb region was deleted in strain HH1, because this region was located close to one end of the linear chromosome (Lazhava et al., 1997). During a recent study of afsA we noticed that strain HH1 harbouring pAFB2 was unable to form aerial mycelium or spores on R medium used routinely for protoplast regeneration (Fig. 2). On the other hand, the transformant on R medium produced almost the same amount of streptomycin as strain HH1 harbouring pAFB1. We then examined the formation of aerial mycelium by strain HH1 harbouring various plasmids (Fig. 2) on R medium. Both pAFB1 and pAFB10 conferred aerial mycelium and spore formation, whereas pAFB2 or pAFB15 did not. Because strain HH1 harbouring plJ702, plJ385 or pKU209 responded in the same way to a large amount of A-factor, as described below, the phenotype on R medium did not depend on the vector used for subcloning. These observations suggested that the 0–3.02 kb PstI–PvuII region on pAFB10 suppressed the aerial-mycelium-defective phenotype.

A single gene, afsA, caused aerial mycelium formation and streptomycin production in strain HH1 on media such as Bennett-maltose, Bennett-glucose and YMPS agar (Horinouchi et al., 1984, 1985). To determine which ingredient in R medium inhibited aerial mycelium formation, we grew the strains harbouring pAFB15 or pAFB2 on various media and established that pAFB2 and pAFB15 conferred the ability to form aerial mycelium and spores on SD-R medium (Fig. 2). We then examined the effect of sucrose in R medium on aerial mycelium formation by strain HH1 harbouring these plasmids. Strain HH1 harbouring pAFB1 and pAFB10 sporulated abundantly on R medium containing 0.3 M sucrose, whereas aerial mycelium formation by strain HH1 harbouring pAFB2 diminished as the concen-
single ORF, named sgaA (see below), we examined the influence of media on the phenotypes of strain HH1 containing various plasmids to determine the relationship between afsA and sgaA.

**Lack of aerial mycelium formation by strain HH1 containing afsA at high copy number under high osmolality**

Because the lack of aerial mycelium formation was observed only on media containing sucrose at a high concentration, we tested other sugars and salts associated with osmolality. Sorbitol and mannitol exerted the same effect as sucrose; strain HH1 harbouring pAFB1, pAFB10 or pAFB18 formed aerial mycelium and spores on the medium containing 0.3 M sorbitol or mannitol, whereas strains harbouring pAFB2 or pAFB15 did not form aerial mycelium when sorbitol or mannitol was higher than 0.3 M (data not shown). Sorbitol and mannitol did not affect streptomycin production by any of these strains.

NaCl and KCl exerted a similar effect to sucrose on aerial mycelium formation by strain HH1 containing afsA at high copy number; strain HH1 harbouring pAFB1, pAFB10 or pAFB18 formed aerial mycelium and spores at 0.15 M, whereas the strains harbouring pAFB2 or pAFB15 did not (data not shown).

To examine the effect of copy number of afsA on aerial mycelium formation by strain HH1 on R medium, we used pKU209 as the vector. The copy number of pKU209 in S. griseus was about 1–2 per genome, as judged by agarose gel electrophoresis of the plasmid band in cleared lysates. Strain HH1 harbouring pAFB15L and pAFB18L (Fig. 1) formed aerial mycelium and spores on R medium containing 0.3 M sucrose (Fig. 2). Furthermore, these transformants formed spores to almost the same level as the wild-type strain on media containing high concentrations of sorbitol, mannitol, KCl or NaCl (data not shown). We concluded that aerial mycelium formation by strain HH1 was disturbed at high osmolality only when afsA was present at high copy number.

**Amount of A-factor produced by strain HH1 containing afsA on multicopy plasmids**

A-factor assays by the streptomycin-cosynthesis method showed that strain HH1 containing afsA on pKU209 (plasmids pAFB1L, pAFB15L and pAFB18L) produced about 1 ng A-factor per colony on R medium and SD-R medium. A-factor production appeared not to be affected significantly by sucrose in the medium. On the other hand, the amounts of A-factor produced on R medium and SD-R medium by the transformants harbouring afsA on the high-copy-number plasmids pIJ385 and pIJ702 (plasmids pAFB1, pAFB2, pAFB10, pAFB15 and pAFB18) were too large to be calculated precisely from a calibration curve obtained with paper discs and A-factor in a range of 1–10 ng. However, the bioassay method showed that the amounts of A-factor...
produced in both media did not differ significantly. Also the timing of A-factor production was consistent in all cases. Fig. 3 shows A-factor production by S. griseus grown in the medium without sucrose. The amount of A-factor produced by the wild-type strain, S. griseus IFO 13350, reached a maximum [25 ng (ml broth)] at 1 d and gradually decreased, as observed previously (Hara & Beppu, 1982). Strain HH1 harbouring pAFB1L produced about 24 ng A-factor (ml broth)$^{-1}$, but with a maximum at 3 d of growth. In contrast, formation of A-factor by strain HH1 harbouring pAFB1 reached 7 mg (ml broth)$^{-1}$ with a maximum at 3 d of growth. In all strains, maximum A-factor production was observed just before cell growth ceased (as determined by wet cell weight), but strain HH1 harbouring pAFB1L or pAFB1 grew more slowly than the wild-type strain. Slower growth was also observed for strain HH1 harbouring plJ385 or pKU209 under these culture conditions and the growth of strain HH1 harbouring pAFB1 was slower still. This may be explained in terms of a growth inhibitory effect of a large amount of A-factor, as described below.

**Growth inhibition of strain HH1 by exogenous A-factor at high concentration during early growth**

The above observations suggested that a large amount of A-factor disturbed aerial mycelium formation by strain HH1. We then examined the effect of A-factor on aerial mycelium formation by placing paper discs containing varying amounts of A-factor on the agar surface just after spreading mycelium on the agar. As seen in Fig. 4, 0.5 pg A-factor inhibited growth of strain HH1. In the inhibitory zone sparse growth of substrate mycelium was observed, indicating that the inhibition was not complete. In fact, after prolonged incubation the sparse growth of substrate mycelium in the inhibition zone became more apparent. In addition, a doughnut-like zone of sporulation formed around the paper disc, as seen also for strain HH1 harbouring pKU209 or pAFK1L (see Fig. 5). Although growth inhibition was detectable only with large amounts of A-factor under these conditions, it is certainly conceivable that smaller amounts would also disturb the growth of cells but to an extent not detectable by this assay. On the other hand, growth of strain HH1 on SD-R medium was not inhibited even by 5 pg A-factor. On SD-R medium, sporulation of strain HH1 around the paper discs depended on the concentration of A-factor. Similar examination of the effect of A-factor on the wild-type strain IFO 13350 showed no effect of 5 pg A-factor on growth or sporulation on R medium.

A growth inhibition zone was seen around the paper disc containing 1.5 pg A-factor when the disc was placed 0-8 h after the plates were spread with mycelium of strain HH1 (Fig. 4). However, no growth inhibition was observed when discs were placed 12 h or later after the plate was spread. In contrast, a large sporulation zone was observed around discs placed 8-12 h after plates were spread; the size of the sporulation zone decreased.
Fig. 4. Effect of A-factor on the growth of strain HH1. Paper discs containing varying amounts of A-factor were applied immediately after inoculation and the plates were incubated for 5 d at 28 °C. (a) The wild-type strain S. griseus IFO 13350 was spread on both R and SD-R media as a control and a paper disc containing 5 μg A-factor was applied. (b) Strain HH1 was spread on both R and SD-R media and paper discs containing the indicated amounts of A-factor were applied. (c) After inoculation of strain HH1, paper discs containing 1.5 μg A-factor were applied at the indicated times (h). Every patch was separated by a gap to avoid A-factor diffusion.

for discs placed at later times. However, the sporulation caused by A-factor supplied at 12 h and later was less abundant than that of either the wild-type strain or strain HH1 supplemented with a few ng A-factor at the time of inoculation. The time-dependence of the response to A-factor can be explained in terms of an A-factor-sensitive physiological stage during very early growth (Barabas et al., 1994; Neumann et al., 1996). Presumably, excess A-factor disturbs the growth of strain HH1 during this A-factor-sensitive stage. In liquid culture (Fig. 3), A-factor production by strain HH1 harbouring pAFB1 did not begin until 12 h after inoculation, suggesting that in cultures showing a prolonged lag period before growth, the A-factor-sensitive stage is delayed.

All of the data suggested that the aerial-mycelium-defective phenotype of strain HH1 containing afsA on a multicopy plasmid under high osmolality was due to A-factor produced in large amounts early in growth. In fact, the A-factor assay (Fig. 3) showed that despite a time lag of 12 h for growth and A-factor production, strain HH1 containing afsA at high copy number produced 120 ng A-factor ml⁻¹ at 24 h. Because the defect in aerial mycelium formation and growth was not observed with the wild-type strain or strain HH1 harbouring pAFB1, pAFB10 or pAFB18, the 1·72 kb PsI–MluI region probably suppressed the phenotype. Subcloning experiments on the basis of the nucleotide sequence of this region (see below) identified the 1·02 kb EcoT14I–MluI fragment as essential for suppression. When we introduced pAFB19L containing the 1·02 kb region into strain HH1, aerial mycelium formation on R medium supplied with 1·5 μg A-factor at the time plates were spread was normal (Fig. 5). Scarce growth and almost no aerial mycelium formation around the paper disc were observed for strain HH1 harbouring the vector plasmid pKU209. The presence of a doughnut-like zone of sporulation indicated that A-factor at a lower concentration induced aerial mycelium formation. On
growth disturbance by A-factor at a high concentration was present 9 bp upstream of the initiation codon. The A-factor and we determined the nucleotide sequence of turbulance on R medium caused by high concentrations of osmolality was mediated by ArpA. Because strain HH1 harbouring pAFB18 (Fig. 2) showed wild-type behaviour, the 1-72 kb PstI-MluI region was presumed responsible for suppressing the growth disturbance of strain HH1 by high concentration under high osmolality during early growth phase (suppression of growth disturbance caused by A-factor at high osmolality). We spread mycelium of mutant KM7 on R medium and placed paper discs containing varying amounts of A-factor on the agar surface at varying periods after inoculation. Similar studies with mutant KM7 just as for strain HH1 (data not shown). No growth disturbance of mutant KM7 by A-factor disturbed the growth of this strain, because introduction of arpA into mutant KM7 gives the same phenotype for both strains (suppression of growth disturbance caused by A-factor in S. griseus). We therefore concluded that the growth disturbance of strain HH1 by large amounts of A-factor under conditions of high osmolality was mediated by ArpA.

Involvement of ArpA in the growth disturbance of strain HH1 by A-factor at high osmolality

Since the growth disturbance of strain HH1 by high concentrations of A-factor was observed only when it was supplied early in growth, we examined the possible involvement of the A-factor receptor protein, ArpA. Strain KM7, derived from strain HH1 bearing a large deletion that includes the entire 9-1 kb PstI region (Fig. 1), is deficient in ArpA; it forms aerial mycelium and spores and produces streptomycin due to relief from the repression-like behaviour of ArpA (Miyake et al., 1990). Immunoblot analysis of the lysate of mutant KM7 with anti-ArpA antibody indicated that this mutant produced no detectable ArpA (unpublished data). Strains KM7 and HH1 appear to have the same genetic background except for arpA, because introduction of arpA into mutant KM7 gives the same phenotype for both strains in all the aspects so far examined (Onaka et al., 1995). We spread mycelium of mutant KM7 on R medium and placed paper discs containing varying amounts of A-factor on the agar surface at varying periods after inoculation. Neither growth inhibition nor morphological defects were seen, even when a paper disc containing 5 µg A-factor was added immediately after inoculation. Similar studies with mutant KM7 harbouring arpA on pKU209 (plasmid pAFB19L) showed that 0.5 µg A-factor disturbed the growth of this strain, just as for strain HH1 (data not shown). No growth disturbance of mutant KM7 by 5 µg A-factor was observed on media containing high concentrations of sorbitol, mannitol, KCl or NaCl. We therefore concluded that the growth disturbance of strain HH1 by large amounts of A-factor under conditions of high osmolality was mediated by ArpA.

Nucleotide sequence of the region that suppresses growth disturbance by A-factor at a high concentration

Because strain HH1 harbouring pAFB18 (Fig. 2) showed wild-type behaviour, the 1-72 kb PstI-MluI region was presumed responsible for suppressing the growth disturbance on R medium caused by high concentrations of A-factor and we determined the nucleotide sequence of this region. FRAME analysis (Bibb et al., 1984) of the sequence predicted the presence of two complete genes and one truncated gene. As described above, pAFB19L (Fig. 1) containing one of the genes, named sgaA (suppression of growth disturbance caused by A-factor at a high concentration under high osmolality during early growth phase), suppressed the growth disturbance. A probable ribosome-binding sequence, GAGGAGA, was present 9 bp upstream of the initiation codon. The sgaA gene encoded a protein of 264 aa with a calculated molecular mass of 28 kDa. Introduction of sgaA on pKU209 (plasmid pAFB19L) into the wild-type strain caused no detectable effect on morphogenesis and streptomycin production on R or SD-R medium.

A computer-aided search indicated that the deduced amino acid sequences of Orf2 and Orf0, also present in the 1.72 kb region, showed no homology with the proteins in databases. On the other hand, the search predicted that a gene product (OrfX) encoded by a region just in front of S. griseus afsK is homologous to SgaA. The function of afsK in S. griseus has been studied in this laboratory, but is still unclear. OrfX, consisting of 270 aa, showed end-to-end similarity to SgaA (Fig. 6). We cloned a region including orfx together with 773 bp upstream of the putative initiation codon of orfx in pKU209. The resulting plasmid (pAFKIL) was introduced into strain HH1 and the ability of orfx to suppress growth disturbance by A-factor was examined (Fig. 5). The growth of strain HH1 harbouring pAFKIL on R medium was disturbed by A-factor when supplied at the time of inoculation, just as it was in strain HH1 harbouring the vector pKU209; thus orfx was ineffective.

DISCUSSION

On the basis of the initial finding that the A-factor-deficient mutant S. griseus HH1, which harboured pAFB2, was incapable of forming aerial mycelium on R medium, we reached the following conclusions. The defect in aerial mycelium formation of strain HH1 harbouring afsA at high copy number results from a growth disturbance associated with overproduction of A-factor, but not the AfsA protein. The growth disturbance by A-factor occurs on media of high osmolality caused by high concentrations of sucrose, mannitol, KCl or NaCl only during the very early growth phase.
and when a large amount of A-factor is supplied exogenously or by means of a gene dosage effect of afsA. The effect of A-factor is exerted via the A-factor receptor protein. The presence of sgaA suppresses the growth disturbance by A-factor.

In wild-type S. griseus strains, A-factor produced in a growth-dependent manner triggers the onset of morphogenesis and secondary metabolism, leading to formation of morphologically differentiated mycelium, spores, streptomycin and a yellow pigment. Timing is important for the switching role of A-factor since sporulation (Barabas et al., 1994) and streptomycin biosynthesis (Neumann et al., 1996) are determined at an early stage of the life cycle, usually not later than 10–12 h after initial inoculation in liquid medium. After this time, exogenously supplemented A-factor or nutrients can no longer influence physiological and morphological differentiation. These observations support the hypothesis of Ensign (1988) that sporulation of some S. griseus strains depends on a programmed developmental cycle based on a clock-like mechanism. Neumann et al. (1996) called this A-factor-sensitive period the ‘decision phase’; the presence of a decision phase has also been suggested in Streptomyces hygroscopicus (Holt et al., 1992). The very early growth period (0–8 h after inoculation on solid medium) during which the inhibitory effect of a large amount of A-factor was observed, is presumably explained in terms of the decision phase. During this phase, A-factor is thought to initiate a sequence of metabolic events. Addition of exogenous A-factor to an A-factor-deficient mutant results in severe alterations in protein turnover, as determined by SDS-PAGE (Gräfe & Sarfert, 1985; Horinouchi & Beppu, 1990); many proteins are produced, but some disappear, in response to A-factor. According to our model for the A-factor regulatory cascade, A-factor relieves the repression due to ArpA by dissociating the ArpA–DNA complex from a still unknown DNA. Consistent with this, ArpA is produced very early in a growth-dependent manner, as determined by immunoblotting with anti-ArpA antibody (unpublished data). It is possible that a large amount of A-factor during the decision phase abruptly relieves ArpA repression and sets off an anomalous sequence of metabolic events. It is also probable that the synthesis of many proteins is induced or repressed in response to high osmolality. We assume that induction and repression of protein synthesis in response to both excess A-factor and high osmolality in the absence of the sgaA product disturbs the physiology of cells, resulting in growth disturbance. Only under conditions of high osmolality is the growth disturbance enhanced to a detectable level. The sgaA product phenotypically protects against high osmolality in the presence of large amounts of A-factor during the decision phase. Further study is needed to elucidate the protective role of SgaA and to identify the protein(s) responsible for the growth disturbance.

Since the growth disturbance does not occur in arpA mutants, we assume that ArpA plays a key role during the decision phase causing all of the genes necessary for morphogenesis and secondary metabolite formation to be expressed in an orderly sequence in response to A-factor. Examination of the time course of A-factor production by the wild-type strain and strain HH1 harbouring afsA on a low-copy-number plasmid indicates that the amount of A-factor during the decision phase is low and reaches a maximum at the end of the exponential growth phase (Fig. 3). Since arpA-null mutants, such as strains KMS, KM7 and KM12, form spores more abundantly and begin to produce 10 times more streptomycin 1 d earlier than the wild-type strain (Miyake et al., 1990), the programmed developmental cycle appears to be different in these mutants. This may explain the lack of growth disturbance of arpA mutants, since the growth disturbance caused by large amounts of A-factor under high osmolality presumably results from a disorder of the programmed developmental cycle in which ArpA plays a key role.

Streptomycin production by strain HH1 harbouring afsA on high- or low-copy-number plasmids in a medium of high osmolality did not differ significantly. This means that A-factor at the concentration produced by strain HH1 containing multiple copies of afsA does not disturb the sequence of signal transfer leading to streptomycin production to a detectable extent. In aerial mycelium formation, many enzymes and regulatory proteins are presumably required. It is probable that disturbance of the complex network by a combination of excess A-factor and high osmolality leads to a failure to grow normally and to form aerial mycelium. Streptomycin production seems to be less susceptible to the change in physiological conditions, although it may be affected to an undetectable extent.

Since the sgaA product shows no similarity in amino acid sequence to known proteins, its role is unclear. Although a large amount of A-factor added to cultures at inoculation has no effect on the growth of the wild-type strain even on medium of high osmolality, it is possible that sgaA exerts a subtle fine-tuning role ensuring healthy growth under certain environmental conditions. A better understanding of the sequence of gene expression during the decision phase and under conditions of high osmolality will be needed to elucidate the role of SgaA.

ACKNOWLEDGEMENTS

This work was supported in part by the Nissan Science Foundation, the Proposal-Based Advanced Industrial Technology R&D Program of the New Energy and Industrial Technology Development Organization (NEDO) of Japan, the ‘Research for the Future’ Program of JSPS and the Ministry of Agriculture, Forestry and Fisheries of Japan (BMP 97-V-1-(3)-2).

REFERENCES


Received 3 January 1997; revised 2 April 1997; accepted 17 April 1997.