Expression analysis of the ssgA gene product, associated with sporulation and cell division in *Streptomyces griseus*

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The ssgA gene of *Streptomyces griseus* B2682, when present in high copy number, results in both suppression of sporulation and fragmented growth of mycelia. Western analysis with polyclonal antibodies against the gene product (SsgA) revealed a close correlation between SsgA accumulation and the onset of sporulation in wild-type cells. The protein was only detected in the cytoplasm. Certain developmental mutants of *S. griseus* (afs, relC and brgA) which are defective in aerial mycelium formation in solid culture and submerged spore formation in liquid culture failed to accumulate SsgA. The SsgA protein appeared shortly (1 h) after nutritional shift-down of strain B2682 cells. afs mutant cells sporulated and expressed SsgA only when A-factor was present both before and after nutritional shift-down. Introduction of the ssgA gene in a low-copy-number vector into strain B2682 resulted in fivefold overexpression of SsgA, and was accompanied by fragmented growth of mycelia and suppression of submerged spore formation (in liquid culture) and aerial mycelium formation (in solid culture). Streptomycin production was not inhibited. In a control experiment, a nonfunctional ssgA gene possessing a frameshift mutation near its N-terminus had no effect on either growth or sporulation. It is proposed that the ssgA gene product plays a role in promoting the developmental process of *S. griseus*.

**Keywords:** *Streptomyces griseus*, sporulation, cell division, ssgA gene

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**INTRODUCTION**

Streptomycetes are unique bacteria which grow vegetatively as multinucleoidal branched substrate mycelia containing occasional cross-walls. Reproduction occurs by the synchronous and regularly spaced septation of specialized aerial hyphae to form uninucleate dormant spores (Chater, 1984, 1989). Most *Streptomyces* species sporulate only during growth on solid media, presumably in response to nutrient depletion signals, i.e. a decrease in intracellular GTP pool size (Ochi, 1987a, b, 1990; Itoh et al., 1996). Sporulation in *Streptomyces* is a complicated process involving expression of genes which are both temporally and spatially regulated (Chater, 1984, 1993).

Studies of sporulation in most streptomycetes are hampered by asynchronous growth, because cultures contain a mixture of young and senescent vegetative mycelia and hyphae in various stages of the sporulation process. *Streptomycetes griseus*, however, sporulates relatively synchronously over a short time period when cultured in shaken submerged conditions (Kendrick & Ensign, 1983; Ensign, 1988). Thus, this organism provides a feasible system for the physiological and genetic study of sporulation, as demonstrated in previous physiological (Ochi, 1987a, b; Kendrick & Ensign, 1983) and molecular genetic (Babcock & Kendrick, 1988, 1990) studies.

*S. griseus* strain NRRL B2682 sporulates after approximately 30 h of growth in a semi-defined liquid medium (Ensign, 1988; Kawamoto & Ensign, 1995a). On inclusion of casein hydrolysate (1%, w/v) and yeast extract (1%, w/v) in the medium, the organism grows profusely as branched mycelia but does not sporulate. Kawamoto & Ensign (1995a) isolated mutants that sporulate in such a nutritionally rich medium, in which sporulation is normally suppressed. Transfer of six
different genomic DNA fragments in high-copy-number plasmids from the parent to mutant cells caused the mutants to exhibit the original phenotype of not present in high copy number in the parent cells, not only no significant sequence homology to known proteins or on Luria agar at 37 °C. When necessary, ampicillin and IPTG were added to the media to a final concentration of 50 ng ml⁻¹ and 0.5 mM, respectively.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Strains and plasmids are listed in Table 1. The afs mutant BB51, defective in A-factor synthesis, was obtained from strain B2682 by incubating the cells at 36 °C as described previously (Ochi, 1987b). DM1 medium, a glucose/ammonium/mineral salts medium, contained 25 mM MOPS (pH 7.2 with KOH), 5 mM (NH₄)₂SO₄, 0.5 mM MgSO₄, 0.1% (w/v) casein hydrolysate (Hy-Case SF), 50 mM glucose, 10 mM K₂HPO₄/KH₂PO₄ buffer (pH 7.0), 67.5 μM CaCl₂, 18 μM FeSO₄, 25 μM MnSO₄, and 0.43 μM ZnSO₄. Glucose and the potassium phosphate buffer were autoclaved separately. DCMY medium contained 1% (w/v) Hy-Case SF and 1% (w/v) yeast extract (Difco) in addition to the components of DM1. GYM medium contained (per litre): 4 g glucose, 4 g yeast extract, 10 g malt extract, 1 g NZ-amine (type A, Wako), 2 g NaCl, 5 mg CuSO₄, 5H₂O, 7.5 mg FeSO₄, 7H₂O, 3.6 mg MnSO₄·5H₂O, 15 mg CaCl₂, 2H₂O, and 9 mg ZnSO₄·7H₂O (adjusted to pH 7.3 with NaOH). For preparation of protoplasts or plasmid DNA, cells were grown in Bacto-tryptic soy broth supplemented with 10% (w/v) sucrose, 5 mM MgCl₂ and 0.3% (w/v) glycine. Regeneration medium, SpFR, was described previously (Ensign, 1995b). The aim of the present study was to clarify further the possible role of the ssgA gene product (SsgA) in relation to both cell division and morphological development by investigating its expression using Western analysis with an anti-SsgA serum.

**Physiological methods.** For streptomycin production, spores or cells of B2682 (or its transformants previously grown on selective agar plates) were inoculated into SPY medium followed by incubation at 30 °C for 3 d. Then 3 ml aliquots of the culture were used to inoculate 100 ml SPY medium containing 2% MgSO₄·7H₂O, followed by incubation at 25 °C for 3 d. Streptomycin was assayed as described previously (Ochi, 1986). A-factor was prepared by extracting the culture broth of *S. griseus*IFO13189 with ethyl acetate. The concentration of A-factor in the preparation was determined by bioassay (Ochi, 1987b) with *S. griseus* mutant BB51, defective in A-factor synthesis. Authentic A-factor was a gift from K. Ueda. Nutritional shift-down was performed as described previously (Ochi, 1987a), except that cells grown to mid-exponential phase (approx. 1.5 mg dry cell weight ml⁻¹) in DMCY medium were filtered and transferred to the same volume of DM1 medium without 0.1% casein hydrolysate. In plate cultures, a 0.1 ml spore suspension (approximately 2 x 10⁸ spores) was spread on a cellophane sheet (diameter 8 cm; UV-sterilized) previously placed on the agar medium. At various times the cellophane sheet with mycelia was removed, and the cells were collected by scraping off with a spatula. Harvested cells were stored at −30 °C until use. The intracellular concentrations of ppGpp and other nucleotides were assayed by HPLC as described by Ochi (1987a). Spore titer were determined by direct counting in a Petroff–Hauser counting chamber using phase-contrast optics. Samples were sonicated for 5 min prior to counting.

**Preparation of crude extract.** Cells were suspended in TGE buffer containing 10 mM Tris/HCl (pH 7.0), 1 mM EDTA, 1 mM diethiothreitol and 10% (v/v) glycerol. The cell suspensions (0.5 g cells ml⁻¹) were sonicated for 3 min in an ice-cold bath. Cell debris was then removed by centrifugation at 10000 g for 60 min, and the resulting supernatant was used as a crude extract for Western analysis. Crude extracts thus prepared typically contained 2–10 mg protein ml⁻¹. In a cell fractionation experiment, the crude extract was ultra-centrifuged at 75 000 g for 60 min. The supernatant served as a cytoplasmic fraction, whilst the pellet was re-suspended in 0.2 vol. TGE buffer with brief sonication and used as a membrane fraction. All procedures were performed at 4 °C.

**PAGE and immunoblotting.** SDS-PAGE was performed using a 12% gel according to the method of Laemmli (1970). Proteins on the gel were visualized using Comassie blue. For immunoblotting, proteins separated on the gel were transferred to an Immobilon-P membrane (Millipore) using a semi-dry blotting apparatus (Atto) with blotting buffer consisting of 25 mM Tris base, 192 mM glycine, and 20% (v/v) methanol (pH 8.3). An electric field of 2 mA cm⁻² was applied for 1 h. Western blots were developed using the ECL Western blotting chemiluminescent detection system (Amersham) as specified by the manufacturer. The polyclonal anti-SsgA serum, prepared as described below, was used as the primary antibody at a dilution of 1:5000. Incubation of the luminol-saturated blot in the presence of chemical enhancers and subsequent exposure to X-ray film were performed at room temperature.

**General DNA techniques and transformation.** Restriction and modifying enzymes were used according to the manufacturer’s recommendations. Plasmid DNA was prepared by alkaline lysis (Kieser, 1984). DNA fragments were purified from the agarose gels by using a Gene Clean kit (Bio101). Nucleotide sequencing was done by the dideoxy nucleotide chain-termination method, using a Sequenase version 2.0 kit (US Biochemicals), alkali-denatured supercoiled plasmid DNA and [³²P]dCTP25 (sp. act. 1000 Ci mmol⁻¹, 37 TBq mmol⁻¹, DuPont). 7-Deaza-dGTP was substituted for dGTP to resolve the problems of deamination and dimer formation.
were screened for the presence of the larger 23.7 kb pIJ941 plasmids (Lydiate et al., 1985) with a 1.5 kb BamHI-PstI fragment containing ssgA from pUWB which contains the ssgA gene product of S. griseus. This study

E. coli plasmids

pUWB pGEM-3Zf(−) (Amp') with a 1.5 kb BamHI–PstI fragment containing ssgA Kawamoto & Ensign (1995b)
pUWASA pGEM-3Zf(−) (Amp') with same fragment as in pUWB but with a 28 bp deletion between Sac1 sites resulting in a frameshift mutation in the ssgA gene This study

pMAL-c BR322 (Amp') derived vector for overexpression of a fusion protein with a maltose binding protein (MBP) New England Biolabs

pMGC1 pMAL-c (Amp') derivative containing a MBP–SsgA fusion protein gene This study

Streptomyces plasmids

pIJ941 Low-copy-number cloning vector (Hyg' Tsr') Lydiate et al. (1985)
pV1 pIJ941 derivative (Hyg' Tsr' Amp') containing pBluescript SK(+) sequence This study

pIJ702 High-copy-number cloning vector (Tsr' Mel') Katz et al. (1983)
pLSA pV1 (Tsr' Hyg' Amp') with a 1.5 kb EcoRI–PstI fragment containing ssgA This study

pLASA pV1 (Tsr' Hyg' Amp') with a 1.5 kb EcoRI–PstI fragment containing the mutated ssgA from pUWASA This study

pMSA pIJ702 (Tsr' Mel') with a 1.5 kb BamHI–PstI fragment containing ssgA This study

Construction of recombinant plasmids. pV1 was constructed using pIJ941 (Lydiate et al., 1985) fragments produced following digestion with BamHI and XhoI. The fragments (23.7 kb and 1.3 kb) were dephosphorylated and ligated to a pBluescript SK(+) vector (Stratagene) digested with BamHI and XhoI in the polylinker region. The ligated products were introduced into E. coli DH5α and the transformants were selected for ampicillin resistance. The recombinant plasmids were screened for the presence of the larger 23.7 kb pIJ941 fragment insert by restriction analysis. The plasmid pV1 is an E. coli–Streptomyces shuttle vector which possesses the ampicillin resistance gene as a selective marker in E. coli, and the thiostrepton resistance or hygromycin resistance gene as a selective marker in Streptomyces. Like pIJ941, pV1 retains the same unique restriction sites for gene cloning (BamHI, BglII, ClaI, EcoRV, EcoRI and PstI). The copy number of the plasmid pV1 is estimated to be as low as 5–10 copies per genome in S. griseus, and 1–2 copies per genome in S. coelicolor (S. Kawamoto, unpublished results). The Strepto-

myces vector plasmid pIJ702, which is commonly used for gene cloning, is estimated to be present at 40–300 copies per genome (Katz et al., 1983).

pMSA and pLASA were constructed as follows. For pMSA, the 1.5 kb BamHI–PstI insert from pUWB which contains the ssgA gene sequence (Kawamoto & Ensign, 1995b; see Fig. 1) was gel-purified and ligated to a gel-purified 5.1 kb fragment resulting from the digestion of pIJ702 with BglII and PstI. The ligated products were introduced into S. griseus B2682 and the transformants were selected for thiostrepton resistance and for non-production of melanin (by insertional inactivation). pMSA was examined for the presence of the correct insert by restriction analysis. For construction of pLASA, a 1.5 kb fragment containing ssgA was produced by digestion of pUWB...
S. KAWAMOTO and OTHERS

Fig. 1. Restriction map and structure of the S. griseus ssgA gene region. Partial nucleotide and amino acid sequences of the ssgA gene relevant in this study, and the structure of plasmid pLASA, which has a frameshift mutation (28 bp deletion between SacI sites) within the ssgA gene, are also shown. The initiation codon ATG shown in this study differs from that previously reported (Kawamoto & Ensing, 1995b) and is preferred because it is preceded by a more probable ribosome-binding site (RBS, underlined below the nucleotide sequence). The oligonucleotide primer used for DNA sequencing to confirm the mutated ssgA gene is shown by the thin arrow above the fusion sequence. The bold arrow below the amino acid sequence represents the portion of the SsgA ORF included in the fusion protein used for the preparation of the anti-SsgA serum (see Methods). The DDJB/EMBL/GenBank accession number of the ssgA gene is D50051.

with EcoRI and PstI in the polylinker region of the vector sequence and gel-purified. This fragment was ligated to the dephosphorylated pV1 fragments (26.1 and 0.6 kb) obtained following digestion with EcoRI and PstI. The single EcoRI site containing the N-terminal truncated ssgA gene was then gel-purified. The protein fusion vector, pMAL-c (New England Biolabs), was digested with EcoRI, blunt-ended by filling the 3’ overhangs using T4 DNA polymerase, and then digested with HindIII (cutting at the polylinker region of the vector sequence). The resulting 1073 bp fragment containing the N-terminal truncated ssgA gene was then gel-purified. The protein fusion point, vector pMAL-c (New England Biolabs), was digested with EcoRI, blunt-ended by filling the 3’ overhangs using T4 DNA polymerase, and then digested with HindIII in the polylinker region. The two DNA fragments were ligated, introduced into E. coli DH5α, and the transformants were selected for ampicillin resistance. pMGCl is expected to direct the synthesis of a fusion protein of 57 kDa which consists of the N-terminal 42 kDa portion of MBP (deleting its signal sequence) and the C-terminal 119 amino acid residues of the SsgA protein. The protein gene fusion point between the insert and the vector plasmid was verified by nucleotide sequencing with a commercial malE primer (New England Biolabs). In addition, overexpression of the fusion protein (expected size 57 kDa) in the E. coli pMGCl transformant in the presence of IPTG was confirmed by SDS-PAGE analysis (see below).

Purification of MBP–SsgA fusion protein. An overnight culture of E. coli DH5α (pMGCl) was diluted 50-fold into 100 ml Luria broth containing 0.2% (w/v) glucose and ampicillin. The diluted culture was shaken at 37 °C. When the OD₆₀₀ reached approximately 0.5, IPTG was added and the incubation continued for 3 h. Cells were collected by centrifugation at 5000 g for 10 min, washed once with 10 mM Tris/HCl pH 7.5 containing 150 mM NaCl, and then suspended in a column buffer (20 mM Tris/HCl pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol, 1 mM sodium azide). Cells were disrupted by sonication as described above and then debris was removed by centrifugation at 12,000 g for 30 min. The supernatant was loaded onto an amylose resin (New England Biolabs). The bound MBP–SsgA fusion protein was eluted using the column buffer containing 10 mM maltose. The eluted fusion protein was dialysed extensively against the column buffer without maltose, and then re-subjected to the affinity chromatography procedure as described above. The resulting purified fusion protein (> 95% pure) was dialysed against 20 mM potassium phosphate-buffered saline (PBS) pH 7.2. All the above procedures were carried out at 0–4 °C.

Preparation of antiserum. A white rabbit was immunized with approximately 0.5 mg purified MBP–SsgA fusion protein in complete Freund’s adjuvant. Subsequent intradermal injections with adjuvant were performed after 3, 5 and 7 weeks. The rabbit was bled 10 d after the last injection. Control preimmune serum was obtained before the first
SsgA gene product of *S. griseus*

Injection. The resultant antiserum contained polyclonal antibodies against both MBP and SsgA since it reacted strongly to the mature MBP of 43 kDa present in *E. coli* crude extract, and to a protein of 15 kDa corresponding to the expected size of SsgA in a crude extract derived from *S. griseus*. Weak reactions with several protein bands were also observed in *S. griseus*. Control preimmune serum reacted with a few protein bands but not at all with the 15 kDa protein band. The antiserum was used without removing the anti-MBP antibodies since SsgA was fully separated from MBP following SDS-PAGE.

**General reproducibility.** The results shown in each figure were all repeated two or more times to confirm the reproducibility.

**RESULTS**

**Expression and localization of SsgA protein**

The ssgA gene encodes a small acidic polypeptide (calculated isoelectric point 4.3) composed of 136 amino acid residues (Fig. 1). In initial studies the expression of the SsgA protein was determined using Western blotting with polyclonal antibodies against the protein. In submerged cultures in DM1 medium, the expression of SsgA (Fig. 2a, right) was associated with massive sporulation (5 x 10⁸ spores ml⁻¹ at 48 h as determined microscopically). In contrast, the increase in SsgA protein was much less pronounced in DMCY medium (Fig. 2a, left), in which sporulation was severely suppressed (< 1 x 10⁸ spores ml⁻¹). Likewise, the level of SsgA protein increased along with cell ageing on DM1 but not DMCY agar (Fig. 2b). Formation of aerial mycelia commenced at 24 h and coincided with accumulation of the SsgA protein. Expression of the SsgA protein therefore appears to be linked with morphological development of *S. griseus* in both liquid and solid culture. In order to study the cellular localization of the SsgA protein, crude extract obtained from cells of B2682 grown for 24 h in DM1 medium was separated into cytoplasmic and membrane fractions and subjected to Western blotting analysis. SsgA protein was found to be present exclusively (more than 95%) in the cytoplasm (Fig. 3).

**SsgA protein expression in mutant cells**

The mutant SY1 is characterized by the ability to sporulate in nutritionally rich media, such as DMCY, in which sporulation of the wild-type strain is normally suppressed (Kawamoto & Ensign, 1995a). Another mutant, BB51 (afs), is defective in both sporulation and streptomycin production, but the impaired ability is restored completely by the addition of A-factor. The expression profile of the SsgA protein in these developmental mutants was examined (Fig. 2c). The mutant SY1 exhibited a high level of SsgA protein expression in DMCY medium as well as in DM1 medium. The timing of SsgA expression of mutant SY1 in DMCY was similar to that of its parental strain B2682 in DM1 (data not shown). In contrast, the SsgA protein expression of the afs mutant BB51 remained very low throughout growth in DM1. Addition of A-factor to the growth medium, however, restored expression to the wild-type levels and was accompanied by abundant spore formation.

Another strain of *S. griseus*, IFO13189, displayed an ssgA gene expression profile similar to that of strain B2682 (data not shown). Several developmental mutants of this strain have been isolated and characterized.
Mutant strain no. 14 (afs) is defective in both sporulation and streptomycin production due to the impaired ability to synthesize A-factor (Ochi, 1987b), and mutant no. 3-3 (relC) exhibits a grossly impaired ability in sporulation and streptomycin production due to a mutation in the rplK (=relC) gene (Ochi, 1990, and unpublished results), and is defective in the ability to accumulate ppGpp. A recently isolated mutant, no. 4 (brgA; formerly aba), is also defective in both sporulation and streptomycin production, possibly due to a failure to ADP-ribosylate certain protein(s) (Ochi et al., 1992; Shima et al., 1996). As Fig. 2(d) shows, these developmental mutants all exhibited no or only slight expression of SsgA protein when cultured in GYM medium and submerged sporulation was severely suppressed (< 1 x 10⁹ spores ml⁻¹ at 48 h). In contrast, the parent showed massive sporulation (2 x 10⁹ spores ml⁻¹ at 48 h) when cultured under the same conditions. In the afs mutant, addition of A-factor to the growth medium restored SsgA expression to the parental level and was accompanied by abundant spore formation (data not shown). These results, together with those from strain B2682, further indicate the significance of SsgA protein expression for the sporulation process in *S. griseus*.

**SsgA expression following nutritional shift-down**

Using different *S. griseus* strains (B2682 and IFO13189), nutritional shift-down has been shown to be effective in producing extensive submerged spores (Kendrick & Ensign, 1983; Ochi, 1987a). This sporulation induction technique is particularly useful because at the time of shift-down, defined as time 0, metabolic events leading to developmental processes are initiated, and can be studied. The effect of nutritional shift-down on SsgA protein expression was therefore examined (Fig. 4). B2682 cells grown to mid-exponential growth phase (for 18 h) in DMCY were transferred to DM1 containing no casein hydrolysate. Significant expression of SsgA was detected 1 h after shift-down. Similar profiles of SsgA expression were observed when cells grown either for 14 h (early exponential growth phase; approx. 0.8 mg dry cell weight ml⁻¹) or for 28 h (early stationary growth phase; approx. 8.5 mg dry cell weight ml⁻¹) were employed for the shift-down experiments (data not shown). Under the shift-down conditions used, the intracellular ppGpp level increased markedly (to 360 pmol per mg dry cell wt) 15 min after shift-down whilst the GTP level did not decrease, but increased to over 300% compared to time 0 (Fig. 4b). Similar experiments were performed using the afs mutant BB51.
ssgA gene product of *S. griseus*

**Fig. 5.** Effect of A-factor addition on the SsgA protein expression of the afs mutant BB51 after a nutritional shift-down. The mutant BB51 was grown for 18 h in DMCY with or without A-factor (40 ng ml⁻¹). The cells were quickly transferred into DM1 without casein hydrolysate in the presence or absence of A-factor and the crude extracts for Western blotting were prepared as in Fig. 2. Each lane contained 5 µg protein. The figures below each panel indicate the time (h) after shift-down. (a) No A-factor. (b) A-factor present only in DMCY. (c) A-factor present only in DM1. (d) A-factor present in both DM1 and DMCY.

(Fig. 5), with cells grown in the presence or absence of A-factor. Significant expression of SsgA protein following shift-down was detected only when cells were incubated in the presence of A-factor both before and after shift-down (Fig. 5d), eventually leading to massive sporulation (5 x 10⁸ spores ml⁻¹) by 48 h. In contrast, the level of SsgA protein expression was low when A-factor was present only either before or after shift-down (Fig. 5b, c), and low spore titres were produced (< 1 x 10⁶ spores ml⁻¹).

**Effect of ssgA introduced by plasmid vectors**

We previously reported that the ssgA gene, when introduced in high copy number into B2682, resulted in suppression of sporulation and fragmentation of mycelia (Kawamoto & Ensign, 1995b). In the present study, we investigated the effect of ssgA gene introduction in both low and high copy number on sporulation, cell morphology, streptomycin production, and SsgA protein expression. The recombinant plasmids pMSA and pLSA carrying the ssgA gene were constructed as described in Methods using the high-copy-number vector pIJ702 and low-copy-number vector pV1, respectively, and used to transform B2682. Cell morphology of a transformant with the low-copy-number plasmid pLSA when grown in liquid DM1 and DMCY media (containing thio-

*streptomycin to prevent the growth of plasmid-free cells) is shown in Fig. 6(a). No significant difference in growth rate between the vector pV1 transformant and the pLSA transformant was detected. The pLSA transformant showed fragmented growth of pleomorphic rod-shaped cells and no sporulation, not only in DMCY but also in DM1 medium, whereas the transformant harbouring the vector plasmid pV1 exhibited abundant sporulation in DM1 medium (Fig. 6a). The pMSA transformant also exhibited fragmented growth indistinguishable from
that of the pLSA transformant (data not shown), as reported previously (Kawamoto & Ensign, 1995b). When cultured on agar media such as DM1, DMCY and SPY, pLSA and pMSA transformants both formed soft colonies of fragmented non-mycelial cells. These transformants overexpressed SsgA protein throughout the whole of the growth phase examined (10-36 h); results for 24 h cultures are presented in Fig. 6(b) as an example. The pLSA and pMSA transformants exhibited approximately 5- and 10-fold overexpression of SsgA, respectively, compared with the pV1 transformant when grown in DM1 medium.

Strain B2682, like S. griseus strainIFO13189 (Ochi, 1987b), produced 15 μg streptomycin ml⁻¹ after 3 d cultivation in SPY medium. Introduction of the low-copy-number plasmid pLSA (but not vector plasmid pV1) resulted in an increased ability to produce the antibiotic (45 μg ml⁻¹). These results indicate that, in contrast to sporulation, the overexpression of SsgA does not suppress streptomycin production.

A mutated ssgA gene introduced by a plasmid vector has no effect

In order to exclude the possibility that DNA sites on the low-copy-number plasmid pLSA, rather than the ssgA gene product itself, were responsible for the observed fragmented growth and non-sporulating phenotypes, pLSA, containing a nonfunctional ssgA gene, was constructed as described in Methods (also see Fig. 1) and introduced into strain B2682. The nonfunctional gene possesses a small deletion (28 bp) near its N-terminal end resulting in a frameshift mutation. Transformant cells containing pLSA exhibited sporulation in liquid DM1 medium comparable to that of the parent strain containing only the pV1 vector (Fig. 6a). The timing of aerial mycelium formation and sporulation of the pLSA transformant on DM1 agar was also similar to that of the parent strain, and on DMCY agar it formed hard, smooth, non-sporulating colonies, like the parent. These results indicate unambiguously that the ssgA gene product encoded by pLSA is responsible for the observed phenotypes, i.e. suppression of sporulation and fragmented growth of mycelia in the transformant cells.

DISCUSSION

The ssgA gene had originally been cloned on the basis of its ability to suppress, when introduced in a multicopy vector, the depressed sporulation phenotype of mutant SY1 on nutritionally rich media (Kawamoto & Ensign, 1995a). Although the nature of the mutation in the SY1 mutant has not been comprehensively characterized, in the present study we have demonstrated the significant role of the ssgA gene product in both sporulation and cell division. This conclusion has been reached from two different observations: (1) a close correlation between SsgA protein accumulation and the onset of sporulation; (2) lack of SsgA accumulation in certain developmental mutants. Although overexpression of SsgA inhibited sporulation without blocking streptomycin production, it remains unknown whether in wild-type cells SsgA regulates not only morphological differentiation but also antibiotic production. Conceivably, accurate adjustment of SsgA content within the cells is required for the normal sporulation process. Further analysis of the ssgA gene using gene disruption techniques is needed in order to elucidate the role of the SsgA protein more fully. The bldA gene encodes a leucine-tRNA which corresponds to an extremely rare TTA codon in streptomycetes (Lawlor et al., 1987; McCue et al., 1992). This gene is reported to be involved in the regulation of sporulation in S. coelicolor A3(2) (Lawlor et al., 1987). The ssgA gene, however, contains no TTA codon, indicating that expression of ssgA is not controlled by the bldA gene at the translational level.

S. griseus IFO13189 accumulates 400–500 pmol ppGpp per mg dry cell weight following nutritional shift-down (Ochi, 1987a). Strain B2682, used in the present study, accumulated a comparable amount (360 pmol mg⁻¹) of ppGpp under similar shift-down conditions (Fig. 4b). It is therefore possible that SsgA protein expression of strain B2682 induced by nutritional shift-down is elicited by the stringent response in terms of ppGpp accumulation. This is supported by the observation that a relC mutant of strain IFO13189 defective in ppGpp synthesis failed to accumulate SsgA protein during its growth cycle. It is rather surprising that unlike strain IFO13189, B2682 did not show a decrease in GTP pool size even under the physiological conditions accompanying a marked ppGpp accumulation. This fact contrasts with the results from previous studies in a variety of Streptomyces species (briefly reviewed by Ochi, 1987a), and could conceivably be due to the fact that strain B2682 (but not IFO13189) has a low GTP content throughout the growth cycle regardless of the culture media used (Ochi et al., 1994; Ochi & Inatsu, 1995). Indeed, the GTP pool size of B2682 cells growing in DMCY was only one-fourth of the levels detected in strain 13189 when grown under similar conditions (unpublished results). Another intriguing point in the present study is the fact that the afs mutant sporulated and expressed the SsgA protein only when A-factor was present both before and after nutritional shift-down. Although it is premature at present to argue for a role for A-factor in relation to sporulation induced by the shift-down, the results described above imply that A-factor is multi-functional with respect to promoting the developmental processes in this organism, as previously pointed out by Ochi (1987b) working with strain IFO13189.

The fragmented growth caused by the introduction of ssgA raises the possibility that this gene may be involved in septum formation. The ftsZ gene has been reported to play a key role as a positive effector in septum formation in bacteria (Beall et al., 1988; Beall & Lutkenhaus, 1991; Bi & Lutkenhaus, 1991; Dai & Lutkenhaus, 1991; Lutkenhaus, 1993). ftsZ homologues have recently been cloned and analysed in S. griseus (Dharmatilake & Kendrick, 1994) and S. coelicolor (McCormick et al.,...
translational features of a sporulation gene of during sporulation.

required for vegetative septation and for asymmetric septation in sporulation of Hokkaido University, for his helpful advice in the preparation of anti-SsgA serum, and to Dr M. J. Bibb for advice on the logical differentiation.

of S. coelicolor, a null mutant of ftsZ is viable and is able to produce aerial hyphae but is unable to sporulate, indicating that FtsZ is required only for sporulation septation. The ssgA gene product, a small highly acidic protein localized in the cytoplasm, is not likely to be a component of the cell division machinery in the cell membrane and shows no sequence homology to FtsZ. It is possible that ssgA is functionally analogous to the minE gene of E. coli, which acts as a topological specificity factor in septum formation during cell division (de Boer et al., 1989, 1990), and that SsgA could interact with a division inhibitor present in Streptomyces; thus overexpression would result in the observed fragmented growth. Further investigations into the functioning of the SsgA protein may provide important information on the mechanistic relationship between sporulation and cell division in Streptomyces, about which very little is currently known.

NOTE ADDED IN PROOF

The recent discovery of a GTP-binding protein, Obg, in S. griseus (Okamoto et al., 1997) provides further support for the role of GTP as a signal molecule in triggering morphological differentiation.

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