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

Shinichi Kawamoto,¹ Hajime Watanabe,¹ Andrew Hesketh,¹
Jerald C. Ensign² and Kozo Ochi¹

Author for correspondence: Shinichi Kawamoto. Tel: +81 298 38 8124. Fax: +81 298 38 7996.
e-mail: taishi@ss.nfri.affrc.go.jp

¹ National Food Research Institute, 2-1-2 Kannondai, Tsukuba, Ibaraki 305, Japan

² Department of Bacteriology, 1550 Linden Drive, University of Wisconsin-Madison, Madison, WI 53706, USA

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

INTRODUCTION

Streptomycetes are unique bacteria which grow vegetatively as multinucleoid 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. *Streptomyces 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

Abbreviation: MBP, maltose-binding protein.

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 sporulating in the rich medium. One of the DNA clones (named *ssgA*) was further analysed and the *ssgA* gene was found by DNA sequence analysis to encode an acidic protein of approximately 15 kDa which showed no significant sequence homology to known proteins (Kawamoto & Ensign, 1995b). The *ssgA* gene, when present in high copy number in the parent cells, not only resulted in the suppression of sporulation but also caused the cells to grow in a fragmented rather than mycelial fashion. Thus, the *ssgA* gene product was proposed to act both as a positive effector of cell division and as a negative effector of sporulation (Kawamoto & 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.

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, Sigma), 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. DMCY 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, SpMR, was described previously (Babcock & Kendrick, 1988). Soluble starch/polypeptone/yeast extract (SPY) medium contained (per litre): 30 g soluble starch, 2 g yeast extract, 2 g MgSO₄·7H₂O, 30 g polypeptone, 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). SPY medium, used for streptomycin production, contained 2% (w/v) MgSO₄·7H₂O. *Streptomyces* cultures were incubated at 30 °C, unless otherwise stated. Spores were inoculated into the appropriate media at a final concentration of 2 × 10⁷ to 5 × 10⁷ spores ml⁻¹. Cultivation was performed on a rotary shaker (200 r.p.m.) using 100 ml of the medium in a 500 ml flask containing a coiled stainless steel wire to prevent clump formation. For selection of transformants, antibiotics were added to the growth media to a final concentration of 2.5 µg ml⁻¹ (liquid media) or 10 µg ml⁻¹ (solid media) for thiostrepton and/or 10 µg ml⁻¹ (liquid media) or 25 µg ml⁻¹ (solid media) for hygromycin. *E. coli* was grown with shaking in Luria broth or on Luria agar at 37 °C. When necessary, ampicillin and IPTG were added to the media to a final concentration of 50 µg ml⁻¹ and 0.5 mM, respectively.

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 × 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 titres 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 dithiothreitol 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 10 000 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 ultracentrifuged at 75 000 g for 60 min. The supernatant served as a cytoplasmic fraction, whilst the pellet was resuspended 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 Coomassie 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 dideoxynucleotide chain-termination method, using a Sequenase version 2.0 kit (US Biochemicals), alkali-denatured supercoiled plasmid DNA and [³⁵S]dCTPαS (sp. act. 1000 Ci mmol⁻¹, 37 TBq mmol⁻¹, DuPont). 7-Deaza-dGTP was substituted for dGTP to resolve

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
<i>S. griseus</i>		
B2682	Wild-type	Kendrick & Ensign (1983)
IFO13189	Wild-type	Ochi (1987a)
SY1	Spontaneous mutant of B2682 that sporulates in rich media	Kawamoto & Ensign (1995a)
BB51	<i>afs</i> mutant of B2682	This study
No. 14	<i>afs</i> mutant of IFO13189	Ochi (1987b)
3-3	<i>relC</i> mutant of IFO13189	Ochi (1990)
No. 4	<i>brgA1</i> (formerly <i>aba-1</i>) of IFO13189	Ochi <i>et al.</i> (1992)
<i>E. coli</i>		
DH5 α	Laboratory K-12 strain	
<i>E. coli</i> plasmids		
pUWB	pGEM-3Zf(–) (Amp ^r) with a 1.5 kb <i>Bam</i> HI– <i>Pst</i> I fragment containing <i>ssgA</i>	Kawamoto & Ensign (1995b)
pUWASA	pGEM-3Zf(–) (Amp ^r) with same fragment as in pUWB but with a 28 bp deletion between <i>Sac</i> I sites resulting in a frameshift mutation in the <i>ssgA</i> gene	This study
pMAL-c	pBR322 (Amp ^r) derived vector for overexpression of a fusion protein with a maltose binding protein (MBP)	New England Biolabs
pMGC1	pMAL-c (Amp ^r) derivative containing a MBP–SsgA fusion protein gene	This study
<i>Streptomyces</i> plasmids		
pIJ941	Low-copy-number cloning vector (Hyg ^r Tsr ^r)	Lydiat <i>et al.</i> (1985)
pV1	pIJ941 derivative (Hyg ^r Tsr ^r Amp ^r) containing pBluescript SK(+) sequence	This study
pIJ702	High-copy-number cloning vector (Tsr ^r Mel ^r)	Katz <i>et al.</i> (1983)
pLSA	pV1 (Tsr ^r Hyg ^s Amp ^r) with a 1.5 kb <i>Eco</i> RI– <i>Pst</i> I fragment containing <i>ssgA</i>	This study
pLASA	pV1 (Tsr ^r Hyg ^s Amp ^r) with a 1.5 kb <i>Eco</i> RI– <i>Pst</i> I fragment containing the mutated <i>ssgA</i> from pUWASA	This study
pMSA	pIJ702 (Tsr ^r Mel ^r) with a 1.5 kb <i>Bam</i> HI– <i>Pst</i> I fragment containing <i>ssgA</i>	This study

ambiguities and compressions. Transformation of *S. griseus* was essentially the same as described previously (Hopwood *et al.*, 1985; Kawamoto & Ensign, 1995a), except that the recombinant plasmid DNA prepared from *E. coli* was alkali-denatured prior to use. Typically, 5–10 μ g plasmid DNA in 100 μ l TE buffer (10 mM Tris/HCl pH 7.5 and 1 mM EDTA) was treated with 10 μ l of an alkaline solution consisting of 2 M NaOH and 2 mM EDTA. After incubation at 37 °C for 30 min the DNA solution was neutralized by addition of 10 μ l 3 M sodium acetate (pH 5.2) and then precipitated with ethanol. The resulting DNA pellet was dissolved in 10 μ l TE buffer and then used for transformation. This procedure was found to enhance the transformation efficiency of the plasmid DNA more than 10-fold. Transformation of *E. coli* was performed by standard procedures (Sambrook *et al.*, 1989).

Construction of recombinant plasmids. pV1 was constructed using pIJ941 (Lydiat *et al.*, 1985) fragments produced following digestion with *Bam*HI and *Xho*I. The fragments (23.7 kb and 1.3 kb) were dephosphorylated and ligated to a pBluescript SK(+) vector (Stratagene) digested with *Bam*HI and *Xho*I in the polylinker region. The ligated products were introduced into *E. coli* DH5 α 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 (*Bam*HI, *Bgl*II, *Cl*aI, *Eco*RV, *Eco*RI and *Pst*I). 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 *Streptomyces* 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 pLSA were constructed as follows. For pMSA, the 1.5 kb *Bam*HI–*Pst*I 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 *Bgl*II and *Pst*I. 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 pLSA, a 1.5 kb fragment containing *ssgA* was produced by digestion of pUWB

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 pLΔSA, 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 & Ensign, 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 nucleotide 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 lies within the hygromycin resistance gene of pV1. The ligated products were introduced into *E. coli* DH5α and the transformants were selected for ampicillin resistance. pLSA was examined for the presence of the correct insert by restriction analysis.

For construction of pLΔSA, pUWΔSA was first prepared as follows. pUWB was digested with *SacI*, cutting at sites in the polylinker region of the vector sequence, and also at positions between the 9th and 10th codons and between the 17th and 18th codons of the *ssgA* polypeptide sequence (see Fig. 1). The products were blunt-ended by removal of the 3' overhangs using T4 DNA polymerase, and then digested with *BamHI* and *PstI*. The resulting 416 bp and 1069 bp fragments, containing the N- and C-terminal portions of the *ssgA* gene respectively, were gel purified and ligated into the vector pGEM-3Zf(−) that had been digested with *BamHI* and *PstI*. The ligated products were introduced into *E. coli* DH5α and the transformants were selected for ampicillin resistance and for white colony colour. pUWΔSA was examined for the presence of the correct insert by restriction analysis. This plasmid is expected to have a 28 bp deletion between the *SacI*

sites, resulting in a frameshift mutation in the *ssgA* gene (see Fig. 1). The mutation in pUWΔSA was verified by nucleotide sequencing with a custom-made primer, 5'-ATGCGCGAGTCGGTTCAA-3' (see Fig. 1). pUWΔSA was digested with *EcoRI* and *PstI* in the polylinker region of the vector sequence and the 1.5 kb fragment containing the mutated *ssgA* gene was gel-purified. This fragment was ligated to the dephosphorylated 26.1 kb pV1 fragment obtained following digestion with *EcoRI* and *PstI*, yielding pLΔSA.

Transformation of *S. griseus* B2682 with the pLSA or pLΔSA plasmid DNA from *E. coli* transformants was performed as described above, and transformants were selected for thio-strepton resistance and for hygromycin sensitivity (by insertional inactivation). The presence of pLSA or pLΔSA was confirmed by restriction analysis.

pMGC1, expressing the maltose-binding protein (MBP)-SsgA fusion protein, was constructed as follows. pUWB was digested with *SacI* (see above and Fig. 1), blunt-ended by removing 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 vector, pMAL-c (New England Biolabs), was digested with *EcoRI*, blunt-ended by filling the 5' 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. pMGC 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* pMGC1 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(pMGC1) 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 12000 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

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 (5×10^8 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 ($< 1 \times 10^6$ 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

Fig. 2. SsgA protein expression in *S. griseus* parent and mutant strains in submerged or plate cultures. (a) Submerged cultures of strain B2682. (b) Plate cultures of strain B2682. (c) Submerged cultures of strain B2682 and its mutants. (d) Submerged cultures of strain IFO13189 and its mutants. Crude extracts were prepared from the cells grown for the indicated times (for 24 h in c and d) in the indicated media and subjected to SDS-PAGE with a 12% gel. After electrophoresis, the proteins were transferred electrophoretically to an Immobilon-P membrane and the SsgA protein on the membrane was detected using the anti-SsgA serum as the primary antibody as described in Methods. In (a) and (d) each lane contained 5 μg protein, in (b) each contained 20 μg protein, and in (c) each contained 10 μg of protein except for B2682 grown in DMCY, which contained 25 μg . Where indicated, A-factor was added to the medium at a final concentration of 40 ng ml^{-1} in (c). Sporulation was determined after 2 d cultivation. +, 5×10^8 spores ml^{-1} or more; –, less than 1×10^6 spores ml^{-1} .

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.

Fig. 3. Cellular localization of the SsgA protein. Crude extract was prepared from cells of B2682 grown for 24 h in DM1. The crude extract was further fractionated into cytoplasmic (C) and membrane (M) fractions by ultracentrifugation as described in Methods. Samples equivalent to 25 μ l of the original culture were employed for Western blotting, which was performed as described in Fig. 2. The arrow on the left indicates the position of SsgA protein. Positions of molecular mass standards are shown on the right.

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 ($< 1 \times 10^6$ spores ml^{-1} at 48 h). In contrast, the parent showed massive sporulation (2×10^9 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 &

Fig. 4. SsgA protein expression and changes in the intracellular pools of ATP, GTP, and ppGpp in wild-type strain B2682 cells subjected to a nutritional shift-down. (a) Wild-type B2682 was grown in DMCY for 18 h. The cells were harvested by filtration, and quickly transferred into DM1 medium without 0.1% casein hydrolysate. At the times indicated, the cells were filtered and the crude extracts were prepared for Western blotting as described in Fig. 2. Each lane contained 10 μ g protein. (b) Cells grown for 18 h were subjected to nutritional shift-down as described above and ATP ($\square$), GTP ($\circ$) and ppGpp ($\bullet$) were determined as described in Methods. Immediately prior to transfer, nucleotide pools of ATP and GTP were 0.7 and 0.22 nmol (mg dry wt)⁻¹, respectively.

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

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 $5 \mu\text{g}$ 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 (5×10^8 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 ($< 1 \times 10^6$ 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-

Fig. 6. Cell morphology and SsgA protein expression of B2682 transformants harbouring functional or nonfunctional *ssgA* genes in low copy number. (a) Cell morphology of transformants harbouring: the vector pV1 (top); pLSA, containing functional *ssgA* (centre); or pLSA Δ , containing mutated *ssgA* (bottom). Cells were grown for 36 h in DM1 and DMCY media containing thiostrepton. (b) SsgA protein expression. The cells were grown in the indicated media containing thiostrepton for 24 h. Crude extracts were prepared and used for Western blotting as in Fig. 2. Each lane contained $2.5 \mu\text{g}$ protein.

strepton 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* strain IFO13189 (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.*,

1994). In *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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