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ssgA Is Essential for Sporulation of *Streptomyces coelicolor* A3(2) and Affects Hyphal Development by Stimulating Septum Formation

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The role of *ssgA* in cell division and development of streptomycetes was analyzed. An *ssgA* null mutant of *Streptomyces coelicolor* produced aerial hyphae but failed to sporulate, and *ssgA* can therefore be regarded as a novel *whi* gene. In addition to the morphological changes, antibiotic production was also disturbed, with strongly reduced actinorhodin production. These defects could be complemented by plasmid-borne *ssgA*. In the wild-type strain, transcription of *ssgA* was induced by nutritional shift-down and was shown to be linked to that of the upstream-located gene *ssgR*, which belongs to the family of *iclR*-type transcriptional regulator genes. Analysis of mycelium harvested from liquid-grown cultures by transmission electron microscopy showed that septum formation had strongly increased in *ssgA*-overexpressing strains in comparison to wild-type *S. coelicolor* and that spore-like compartments were produced at high frequency. Furthermore, the hyphae were significantly wider and contained irregular and often extremely thick septa. These data underline the important role for *ssgA* in *Streptomyces* cell division.

Streptomycetes are gram-positive, filamentous soil bacteria that have become a major focus for the study of microbial development. *Streptomyces* growth on solid media is started by the development of a complex vegetative mycelium of branching hyphae. Environmental signals such as nutrient depletion cause the development of almost aseptate aerial hyphae that partially parasitize the substrate mycelium. Elongation of the cell wall takes place at the tips of the hyphae, and occasional septation leads to multinucleoid compartments separated by cross walls. Exponential growth is achieved by branching of the vegetative hyphae, resulting in an intricate mycelial network. Eventually, the aerial hyphae become subdivided into uninucleoid cells that develop into chains of hydrophobic spores (10). One of the striking features of streptomycetes and other actinomycetes is their ability to produce a wide variety of secondary metabolites, including many antibiotics, which are produced at about the same time as the onset of morphological differentiation in surface-grown cultures (19, 31).

The process leading to sporulation on solid media has been well documented, helped by the availability of a wide variety of developmental mutants (reviewed in references 8 and 26). In principle, these mutants can be divided into two classes: the bald (*bld*) mutants, which fail to produce the fuzzy aerial mycelium, and the white (*whi*) mutants, which produce aerial hyphae but cannot form the grey-pigmented spores. The *whi* genes are further subdivided into early and late *whi* genes, depending on the developmental state of the aerial hyphae. The early *whi* genes, including *whiA*, *whiB*, *whiG*, and *whiH*, are involved in the regulatory cascade involving the early stages of sporulation and fail to produce spore compartments even after prolonged incubation (15, 36). The late *whi* genes, including *whiD* and *sigF*, are involved in the final stages of sporulation and spore maturation (10, 33).

Some *Streptomyces* species, including *S. albus* (12), *S. griseus* (27), *S. roseosporus* (21), and *S. venezuelae* (17), have the capacity to produce spores in liquid cultures. This process is often elicited by nutritional shift-down from a rich medium to a defined minimal medium (14, 27), indicating a positive control by the stringent response and suggesting a possible correlation between sporulation and secondary metabolism. Interestingly, *S. roseosporus* was also shown to sporulate when grown in rich media.

Little is known about the processes underlying submerged sporulation. One of the best-characterized proteins involved is factor C, which was identified as a 34-kDa protein that restores submerged sporulation to an *S. griseus* mutant. Although antibodies against factor C cross-react with proteins in a wide variety of prokaryotic and eukaryotic organisms, no homologue has yet been identified in any of the databases (5, 6). More recently, a mutant of *S. griseus* (designated SY1) that produced submerged spores in rich as well as in minimal liquid media was identified. Introduction of a DNA fragment harboring the *ssgA* gene into SY1 suppressed submerged sporulation (23, 24). *ssgA* encodes an approximately 15-kDa protein of unknown function. Recently, data from the *S. coelicolor* genome sequencing project (www.sanger.ac.uk/projects/S_coelicolor) revealed an open reading frame (ORF) highly homologous to *ssgA*, but analysis of genomes from many other eubacteria, including other gram-positive bacteria such as *Bacillus subtilis* and the related actinomycetes *Mycobacterium leprae* or *M. tuberculosis*, did not reveal a similar ORF. This indicates that *ssgA* might be limited to the genus *Streptomyces*. Introduction of a multicopy plasmid harboring *ssgA* into *S. griseus* resulted in fragmentation of the mycelium and suppressed submerged sporulation, while it inhibited development on agar plates. Western blot analysis with polyclonal antibodies raised against SsgA revealed that timing of *ssgA* expression in *S. griseus* correlates to the onset of sporulation in liquid cultures (25).

These data suggested a possible involvement of SsgA in cell division and sporulation, although no direct evidence has been presented. Here we show that *S. griseus* strain SY1 is not

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TABLE 1. Oligonucleotides used in PCR

Primer	Sequence ^a	Position ^b (nt)
ssg1	5' <u>GGCGAATTC</u> GAACAGCTACGTGGCGAAGTCGCCA 3' <i>EcoRI</i>	−194 to −170
ssg2	5' <u>GTGGGATCCG</u> TGCTCGCGGCGCTGGTCGTCTC 3' <i>BamHI</i>	+539 to +517
ssg3	5' <u>GGGAATTC</u> ATATGCGCGAGTCGGTTCAAGCA 3' <i>EcoRI NdeI</i>	−30 to −10
ssgN3	5' <u>GGGAATTC</u> ATATGATGACGTTCTCTCCG 3' <i>EcoRI NdeI</i>	+1 to +22
ssg4	5' <u>GTGGATCCCC</u> GGTCAGCCGGCGTTCTG 3' <i>BamHI</i>	+412 to +394
Q1	5' <u>CTGAATTC</u> TAGCATCGAGGGCAGGACATCA 3' <i>EcoRI</i>	−1450 to −1427
Q6	5' <u>CTGAAGCTT</u> AACGACCGGCCAGGTGGCGAC 3' <i>HindIII</i>	+520 to +541
Q10	5' <u>CTGAATTC</u> GGCTCAGCGGTTGACAGAACGAG 3' <i>EcoRI</i>	−196 to −175
Q11	5' <u>ATAGGGATCCC</u> GGGTCTCGTAGCGCAGC 3' <i>BamHI</i>	+75 to +48
Q14	5' <u>CTGGATCCT</u> GGTGCCGCTGGGGCAGGAGG 3' <i>BamHI</i>	+310 to +330
Q15	5' <u>CTGAAGCTT</u> GAGACGGGTTACGGCCACGATGC 3' <i>HindIII</i>	+1870 to +1850

^a Underlined nucleotides indicate nonhomologous sequences added to create restriction sites (in italics) at the ends of the PCR fragments.

^b Location with respect to the first nucleotide (+1) of the ATG translational start codon of *S. griseus* *ssgA* of *S. coelicolor* *ssgA* for oligonucleotides designated “ssg” or “Q”, respectively.

mutated in the *ssgA* gene and describe a defined knockout mutant of the *S. coelicolor* homologue, which has a Whi phenotype. We have also analyzed the cytological effect of overexpression of *ssgA* and show by electron microscopy (EM) that SsgA in fact enhances cell division by stimulating septum formation in liquid-grown cultures of *S. coelicolor*.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. *Escherichia coli* K-12 strains JM109 (30) and ET12567 (28) were used for propagating plasmids. The strains were grown and transformed by standard procedures (37); transformants were selected in L broth containing 1% (wt/vol) glucose and ampicillin at a final concentration of 200 µg ml^{−1}. L broth with 1% (wt/vol) glucose and 30 µg of chloramphenicol ml^{−1} was used to grow ET12567.

S. griseus (ATCC 23345) was obtained from the American Type Culture Collection, and *S. griseus* mutant strain SY1 was described previously (23). *S. coelicolor* A3(2) M145 (prototrophic, SCP1[−] SCP2[−]), obtained from the John Innes Centre strain collection, was used for transformation and propagation of *Streptomyces* plasmids. Protoplast preparation and transformation were performed as described by Hopwood et al. (20). SFM (16) was used to make spore suspensions. R2YE (20) was used for regenerating protoplasts and, after addition of the appropriate antibiotic, for selecting recombinants. For liquid culturing of *Streptomyces* YEME (20), tryptone soy broth (Difco) containing 10% (wt/vol) sucrose (TSBS) or standard minimal medium (MM [20]) with 1% (wt/vol) mannitol as the carbon source was used. For nutritional shift-down, *S. coelicolor* M145 was grown in TSBS to an optical density at 550 nm (OD₅₅₀) of 0.7, washed, and transferred to MM.

Plasmids pUC18 (42), pIJ2925 (22), and pSET152 (4) were used for cloning experiments. While pSET152 is a conjugative shuttle plasmid, in the experiments described in this study the plasmid and its derivatives were introduced by standard protoplast transformation. The *E. coli*-*Streptomyces* shuttle vector pWHM3 (39) was used as a high-copy-number vector (approximately 100 copies per chromosome) in *S. coelicolor*. Plasmid pWHM3-E is a derivative of pWHM3 harboring the 300-bp *EcoRI*-*BamHI* fragment containing the *ermE* promoter (*P_{ermE}*) (3) in pWHM3. Standard procedures were used to isolate plasmid DNA from *E. coli* (37), and to isolate plasmid and total DNAs from actinomycetes (20).

PCR conditions. PCRs were performed in a minicycler (MJ Research, Watertown, Mass.) using *Pfu* polymerase (Stratagene, La Jolla, Calif.) and the buffer provided by the supplier, in the presence of 5% (vol/vol) dimethyl sulfoxide and 200 µM deoxynucleoside triphosphate (dNTP). No additional Mg²⁺ was added to the reaction mixture. The following PCR program was used for 30 cycles: 45 s of melting at 94°C, 1 min of annealing at 54°C, and 90 s of extension at 72°C. The reaction was completed by an additional 10-min incubation at 72°C. Oligonucleotides used for PCR are shown in Table 1.

Construction of the *ssgA* deletion mutant. Two DNA fragments of approximately 1.5 kb were amplified from the *S. coelicolor* M145 chromosome by PCR with oligonucleotides Q1 plus Q11 and Q14 plus Q15 (Table 1). Digestion of these PCR fragments with the appropriate enzymes resulted in an *EcoRI*-*BamHI* fragment and a *BamHI*-*HindIII* fragment, encompassing nucleotides (nt) −1450 to +75 and +310 to +1870, respectively, relative to the translational start (+1) of *ssgA*. These fragments were ligated together into *EcoRI*-*HindIII*-digested pUC18. Subsequently, the *aadA* gene, conferring resistance to spectinomycin and streptomycin (34), was inserted into the *BamHI* site in the plasmid-borne and streptomycin (34), and the *aacC4* gene, conferring apramycin resistance (7), was inserted into the *HindIII* site, resulting in the disruption construct pΔ*ssgA*. As a result, the construct has the +75–+310 region of *ssgA* replaced by *aadA*. Apra-

mycin resistance, the selectable marker for the plasmid, is present after integration of the plasmid in the chromosome but should be lost after a second mutational crossover event. Therefore, after transformation of the plasmid to *S. coelicolor* M145 and a double-crossover event between pΔ*ssgA* and the chromosome, the desired mutant is expected to be resistant to spectinomycin and streptomycin and sensitive to apramycin.

Constructs for the expression of *ssgA*. A 750-bp DNA fragment containing the *S. griseus* *ssgA* gene (accession no. D50051) was amplified from the *S. griseus* chromosome by PCR, using primers ssg1 and ssg2 (Table 1). The PCR fragment was cloned as an *EcoRI*-*BamHI* fragment in pIJ2925, giving pGWS1. The insert of pGWS1 was cloned behind *P_{ermE}* in pWHM3-E, and the *P_{ermE}*-*ssgA* cassette was transferred to pSET152, resulting in pGWS4 (Table 2). From earlier work we know that the ribosome binding site of *S. ramocissimus* *tufI* is efficiently recognized by ribosomes and hence typically results in high expression (40). We therefore replaced the upstream region of *S. griseus* *ssgA* in some of the constructs by that of *S. ramocissimus* *tufI*, to allow higher expression of the gene. To achieve this, a 560-bp fragment was amplified by PCR using oligonucleotides ssgN3 and ssg2 and cloned as an *EcoRI*-*HindIII* fragment into pIJ2925, giving pGWS5 (Table 2). The *ssgA* insert of pGWS5 was then cloned as an *NdeI*-*BglII* fragment into *EcoRI*-*BamHI*-digested pUSRT3-3 containing the *tufI* ribosome binding site (40) after filling in the 5' protruding ends of the *NdeI* and *EcoRI* sites, using the Klenow fragment of DNA polymerase I and dNTPs according to standard procedures (37). The resulting clone was designated pGWS1-SD. From this clone, the derivative pGWS4-SD was made similarly as described for pGWS1 and pGWS4 (Table 2).

For homologous expression of *ssgA* in *S. coelicolor* and for complementation of the *ssgA* null mutant, we amplified the *ssgA* gene from the *S. coelicolor* M145 chromosome by PCR with oligonucleotides Q10 and Q6, designed to encompass nt −196 to −175 and +520 to +541, respectively, relative to the start of the gene. The PCR fragment was cloned in pIJ2925 or behind *P_{ermE}* in pWHM3-E, giving pGWS6 or pGWS7, respectively.

Western analysis of SsgA. Protein extracts were prepared by ultrasonication of the mycelium on ice, at 30 W for 300 s in standard buffer (10 mM Tris-HCl [pH 7.6], 60 mM NH₄Cl, 10 mM magnesium acetate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Samples were then centrifuged at 30,000 × *g* for 30 min. The resulting S30 protein extract (supernatant) was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In all lanes, approximately 5 µg of protein was loaded. Gels were either stained with Coomassie brilliant blue or blotted onto Hybond-C Super nylon membranes (Amersham) and immunostained with antibodies raised against SsgA (25).

Nuclease S1 protection assays. RNA was purified as described by Hopwood et al. (20), except that DNase I treatment was used in addition to salt precipitation to eliminate DNA from the nucleic acid preparations. The concentration and the integrity of the RNA were checked by spectrophotometry and by gel electrophoresis. For each nuclease S1 protection assay, about 0.02 pmol (approximately 10⁴ Cerenkov cpm) of labeled probe was hybridized to 30 µg of RNA in NaTCA buffer (32) at 45°C overnight after denaturation at 70°C for 15 min. All subsequent steps were carried out as described previously (38), using an excess of probe. Experiments were carried out twice on independently isolated RNA. The 330-bp *ssgA* probe (Fig. 1) for mapping *ssgA* transcripts was generated by PCR amplification using the universal primer (17-mer) and ³²P-end-labeled Q11 and with pGWS6 as the template. The probe contains an approximately 50-nt non-homologous extension at the 3' end, to allow discrimination between DNA-RNA hybrids and reannealed probe.

Phase-contrast microscopy. Cultures were examined by light microscopy using a Zeiss standard 25 phase-contrast microscope. For photography, we used a Zeiss MC80 camera.

EM. Samples of M145 and GSA2 for EM were prepared as follows. Mycelium was washed in phosphate-buffered saline, centrifuged at 2,300 rpm for 1 min, and

TABLE 2. Plasmids and strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
pUSRT3-3	Derivative of pUC18, harboring <i>S. ramocissimus tuf3</i> behind the <i>S. ramocissimus tuf1</i> ribosome binding site	40
pWHM3-E	pWHM3 with the constitutive P_{ermE}	This study
pGWS1	pIJ2925 containing the 750-bp <i>S. griseus ssgA</i> PCR (ssg1-ssg2) product	This study
pGWS1-SD	pGWS1 with the upstream region of <i>ssgA</i> replaced by nt -1 to -70 of <i>S. ramocissimus tuf1</i>	This study
pGWS4	pSET152 containing <i>ssgA</i> behind P_{ermE}	This study
pGWS4-SD	pGWS4 with the upstream region of <i>ssgA</i> replaced by nt -1 to -70 of <i>S. ramocissimus tuf1</i>	This study
pGWS5	pIJ2925 with 560-bp <i>S. griseus ssgA</i> PCR (ssgN3-ssg2) product	This study
pGWS6	pIJ2925 with 280-bp PCR (Q10-Q6) product of <i>S. coelicolor ssgA</i>	This study
pGWS7	<i>S. coelicolor ssgA</i> behind P_{ermE} in pSET152	This study
pGWS7-SD	pGWS7 with the upstream region of <i>ssgA</i> replaced by nt -1 to -70 of <i>S. ramocissimus tuf1</i>	This study
p Δ ssgA	Construct for disruption of <i>S. coelicolor ssgA</i>	This study
Streptomyces strains		
M145	Wild-type <i>S. coelicolor</i> A3(2)	20
GSA2	M145 harboring pGWS4-SD	This study
GSA3	<i>ssgA</i> knockout mutant of M145	This study
GSA4	GSA3 with pGWS7 integrated into the chromosome	This study
B2682	Wild-type <i>S. griseus</i> NRRL B2682	
SY1	Mutant of <i>S. griseus</i> B2682 that sporulates in rich liquid media	23

resuspended in a fixative containing 1.5% (wt/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4; 360 mosmol) at room temperature for 20 h. Mycelium was pelleted, rinsed twice in phosphate-buffered saline, and postfixed in 1% (wt/vol) osmium tetroxide in Millonig phosphate buffer (pH 7.3; 330 mosmol) at room temperature for 20 h. After rinsing, the samples were resuspended in 2% Bacto Agar at 60°C, centrifuged at 11,000 rpm for 2 min, cut in 1-mm³ blocks, and dehydrated in a graded series of ethanol. After incubation in a graded series of epoxy resin LX-112 (Ladd Research Industries, Burlington, Vt.) in propylene oxide, the blocks were placed in capsules filled with epoxy resin and polymerized at 60°C for 72 h. Ultrathin sections (70 nm) were cut on an ultramicrotome (Reichert OM U3), collected on copper grids, stained with uranyl acetate and lead hydroxide, and examined in a Philips EM410 transmission electron microscope.

Computer analysis. The BLAST search engines BLASTN, BLASTP, and BLASTX (1) were used to perform database searches, and the Wisconsin Package (13) was used for DNA and protein sequence alignments. Figure 2 was produced using the Boxshade program (www.ch.embnet.org/software/box_form.html).

Nucleotide sequence accession numbers. The sequences shown in Fig. 1 have been assigned GenBank accession no. Q9X9U1 and Q9X9U3.

RESULTS

Genomic organization around *S. coelicolor ssgA*. The sequence of *S. griseus ssgA* was published previously (accession no. D50051). Extensive recent searches of both the translated nucleotide and protein databases using the BLAST search engines BLASTX and BLASTP identified one *ssgA* homologue, which is located on cosmid Q11 (accession no. AL096823) of the *S. coelicolor* cosmid library (35), with the predicted gene product showing 78% amino acid identity to *S. griseus* SsgA. The organization around *S. coelicolor ssgA* is shown in Fig. 1. Upstream of *ssgA* lies a gene encoding an IclR regulator-like protein (241 amino acids), with significant end-to-end homology to several regulatory proteins, for example, 28% amino acid identity (38% similarity) to *S. coelicolor* GylR (accession no. P15360) and *Acinetobacter calcoaceticus* PobR (accession no. Q43992). Experiments described below provide evidence that transcription of *ssgA* is linked to this upstream ORF, and we therefore tentatively call the gene *ssgR*. Downstream of *ssgA* lies a gene encoding a putative membrane protein (513 amino acids), positioned in the opposite direction.

The *S. coelicolor* genome sequencing project (www.sanger.ac.uk/projects/S_coelicolor) revealed several more ORFs with relevant homology to *ssgA* (see the legend to Fig. 2 for accession numbers), with their predicted gene products showing

between 30 and 45% amino acid identity to *S. griseus* SsgA. The functions of these ORFs, which, like *ssgA* itself, have no relevant homology to any other gene in the databases, are unknown.

An alignment of the SsgA homologues of *S. griseus* and *S. coelicolor* and the four SsgA-like proteins identified on the *S. coelicolor* genome is shown in Fig. 2.

***S. griseus* SY1 is not an *ssgA* mutant.** A mutant of *S. griseus* NRRL B2682, designated SY1, is able to produce submerged spores not only in minimal media but also in rich media (23). This phenotype could be suppressed by the introduction of a DNA fragment harboring *ssgA*. While introduction of plasmid-borne *ssgA* did not restore the wild-type phenotype to this mutant, we sought to ascertain that the aberrant phenotype of SY1 was not due to a mutation in the chromosomal *ssgA*. Therefore, we cloned the *ssgA* gene from SY1 by PCR with oligonucleotides ssg1 and ssg2 (Table 1) and determined its sequence. The DNA sequence was shown to be identical to

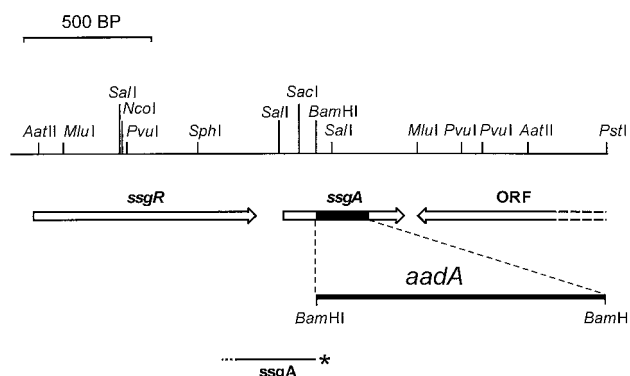


FIG. 1. Restriction map of *S. coelicolor ssgA* and flanking regions and representation of the *ssgA* disruption mutant. The dark area in the *ssgA* gene is replaced by *aadA* (conferring resistance to spectinomycin and streptomycin; presented as a thick line and not exactly to scale) in the *ssgA* knockout mutant GSA3. The probe (*ssgA*) used in nuclease S1 mapping experiments is shown; the asterisk represents the ³²P-labeled 5' end, and the dotted part represents an approximately 50-nt nonhomologous extension at the 3' end, all drawn to scale. ORF, gene encoding a putative membrane protein (accession no. Q9X9U1); *ssgR*, gene encoding an IclR-like regulatory protein (accession no. Q9X9U3).

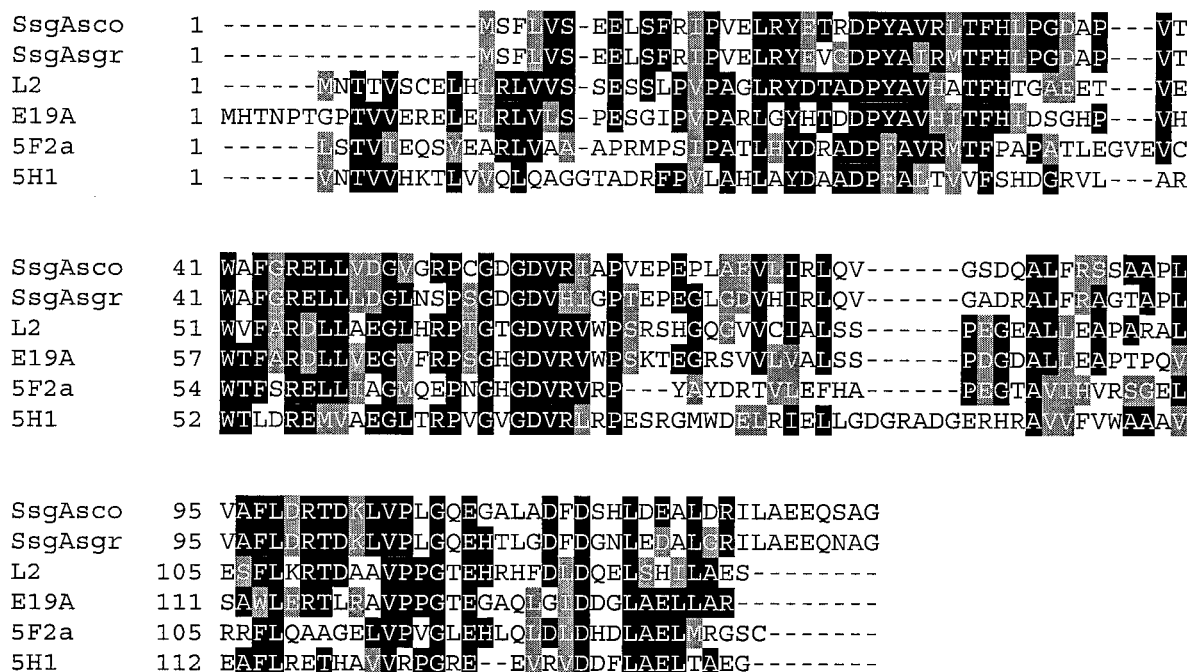


FIG. 2. Alignment of amino acid sequences of SsgA and SsgA-like proteins of *S. griseus* and *S. coelicolor*. SsgAsco and SsgAsgr refer to the SsgA homologues from *S. coelicolor* and *S. griseus*, respectively. 5F2a, 5H1, E19A, and L2 refer to the cosmids on which the four SsgA-like proteins of *S. coelicolor* were located. The protein accession numbers are CAB40672, CAB42965, CAB51005, and CAB70943, respectively. Amino acid identities/similarities to *S. coelicolor* SsgA are as follows: SsgAsgr, 78/82%; L2, 45/52%; E19a, 42/50%; 5H1, 33/39%; 5F2a, 32/38%.

that of the wild-type gene. We also analyzed the expression of *ssgA* in both wild-type *S. griseus* NRRL B2682 and its mutant SY1 by Western analysis with antibodies raised against SsgA (25). In solid-grown cultures (MM or R2YE) or in liquid minimal medium cultures, conditions that allow either strain to sporulate, SsgA protein levels increased with the culture age, reaching a peak at a time correlated to sporulation (25). In contrast to the wild-type strain, *S. griseus* SY1 also sporulates in rich liquid media. Under these conditions, SsgA was produced abundantly in SY1 but at significantly lower levels in B2682, although the two strains are similar in growth phase dependence of *ssgA* (Fig. 3A). Apparently, a certain threshold level of SsgA is required to stimulate submerged sporulation. These data show that the mutation in SY1 does not lie in the *ssgA* gene, although expression of the gene is increased in rich liquid cultures, allowing *S. griseus* to sporulate under these conditions.

These data prompted the creation of an *ssgA* null mutant. Since *S. coelicolor* is genetically by far the best-characterized

Streptomyces strain, with the genome sequencing project almost completed, we decided to analyze the role of *ssgA* in this strain.

Construction of an *ssgA* null mutant of *S. coelicolor*. To establish the role of *ssgA* in the development of *S. coelicolor*, we created an *ssgA* null mutant of this species. For this purpose, nt +75 to +310 of *ssgA* (relative to the start of the gene) were replaced by the *aadA* gene, conferring resistance to spectinomycin and streptomycin. The construct is shown in Fig. 1. *S. coelicolor* M145 was transformed with the disruption construct pΔ*ssgA* (see Materials and Methods), and colonies resistant to spectinomycin and streptomycin were selected. Three of these did not show resistance to apramycin (the marker for the plasmid), indicative of the absence of the plasmid due to a second crossover event. Since *aadA* was apparently still present, these colonies were considered to be possible *ssgA* disruption mutants. The putative *ssgA* mutants were screened by three Southern hybridizations, one with a probe recognizing the *aadA* gene, one with a probe recognizing *ssgA*, and one

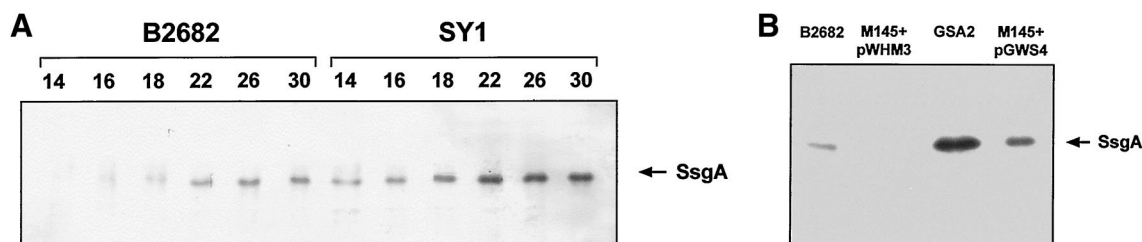


FIG. 3. Western analysis of SsgA expression. (A) Expression of *ssgA* in *S. griseus* B2682 and *S. griseus* SY1 during normal growth in rich medium (TSBS). Samples were taken 14, 16, 18, 20, 26, and 30 h after inoculation of the cultures. Under these conditions, spores emerged in SY1 cultures after 18 h; B2682 failed to produce submerged spores in TSBS. (B) Production of SsgA in *S. griseus* B2682 and *S. coelicolor* M145 harboring pWHM3 (control plasmid), GSA2 (high-level expression), and pGWS4 (low-level expression) during growth in TSBS (mycelium was harvested 30 h after inoculation).

with a probe confirming the absence of *aacC4* (hybridization data not shown). All three had the +75–+310 part of *ssg4* replaced by the *aadA* gene and lacked the *aacC4* gene, showing that they were indeed true *ssg4* disruption mutants. Since the ORF located downstream of *ssg4*, which encodes a putative membrane protein, is oriented in the opposite direction, effects of the *ssg4* disruption on its expression are unlikely. One of the *ssg4* knockout mutants was selected and was designated GSA3.

***ssg4* is essential for sporulation of *S. coelicolor* M145.** The *S. coelicolor* *ssg4* mutant GSA3 was plated on several solid media, including R2YE, SFM, and MM with mannitol as the carbon source. On R2YE, SFM, and MM, aerial mycelium was produced normally, but the hyphae failed to produce spores within 2 weeks (Fig. 4A). After prolonged incubation (more than 2 weeks), colonies remained white on R2YE plates; some spores were produced on SFM and MM with mannitol as the carbon source, although titers were low in comparison to the parental strain M145. Interestingly, antibiotic production was disturbed in GSA3, with almost complete absence of the blue-pigmented actinorhodin.

To verify that the phenotypes described above were solely due to the deletion of *ssg4*, we complemented mutant strain GSA3 by transformation with plasmid pGWS7, an integrative vector harboring *ssg4* behind P_{ermE} . This plasmid allows constitutive expression of *ssg4* in *S. coelicolor*. One of several integrants with the same phenotype was selected and designated GSA4. As shown in Fig. 4A, sporulation is fully restored to the complemented strain, showing that the Whi phenotype of GSA3 was indeed due to the deletion of the *ssg4* knockout. In GSA4, production of actinorhodin was also restored to levels similar to those in the wild-type strain, indicating that disturbance of the regulation of antibiotic biosynthesis could also be ascribed to the *ssg4* deletion. Interestingly, GSA4 consistently produced more spores than M145, as judged by the denser layer of spores (determined by microscopy) and the increased brown-grey pigmentation of the colonies. This apparent increase in sporulation is most likely due to the constitutive and enhanced expression of *ssg4* in the complemented mutant (Fig. 3B). Finally, GSA4 shows increased fragmentation in submerged culture, which again can be ascribed to the increased expression of *ssg4* in this strain.

The sporulation mutants are categorized in various classes, depending on the stage in which they are blocked in their development; while some are blocked in an earlier phase, some have differentiated so far that coiling of their tips is normal, and chains of immature spores are produced (9, 14). To analyze the developmental state of the aerial hyphae, we made impressions of the top layer of 7-day-old surface-grown cultures of *S. coelicolor* M145 and GSA3 by gently pressing a moist coverslip onto the surface and analyzed these by phase-contrast microscopy. While the wild-type strain produced large amounts of spores after 7 days (Fig. 4B), the *ssg4* mutant GSA3 showed typical coiling of the tips of the aerial hyphae but failed to sporulate (Fig. 4C). This indicates that the arrest occurs in a later stage of aerial hyphae development, as early *whi* mutants fail to coil. We plan to compare the phenotype of the *ssg4* mutant to that of other *whi* mutants by scanning EM, to determine what mutant class it falls into.

***ssg4* and *ssgR* are transcriptionally linked.** To analyze the transcription of *ssg4* in *S. coelicolor* and establish whether *ssg4* is transcribed from a promoter directly upstream of the gene, we performed nuclease S1 mapping experiments on RNA isolated from liquid cultures of *S. coelicolor* M145, using the 330-bp *ssgA* probe resulting from PCR with 32 P-end-labeled oligonucleotide Q11 and the universal primer on pSCF6 DNA. The generated probe harbors 280 nt homologous to the *ssg4*

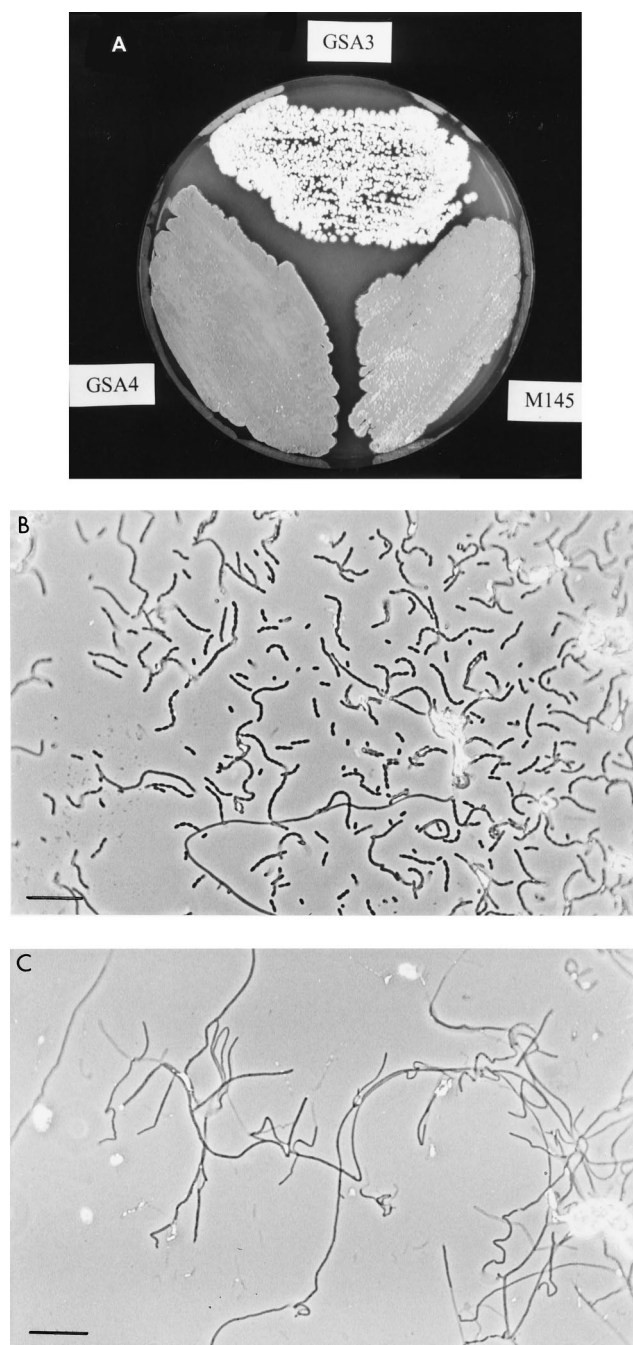


FIG. 4. (A) Phenotype of the *ssg4* null mutant of *S. coelicolor* M145 on SFM medium. GSA3 is the *ssg4* mutant of M145; GSA4 is the *ssg4* mutant complemented with pGWS7. The white appearance of GSA3 is due to the failure of the mutant to produce the grey-pigmented spores. GSA4 consistently produces more spores than M145. (B and C) Impression prints of the surfaces of solid-grown cultures of *S. coelicolor* M145 (B) and its *ssg4* null mutant GSA3 (C). While M145 sporulates abundantly, aerial hyphae of GSA3 show typical coiling, but no spores (bars = 10 μ m).

locus and 50 nt of nonhomologous extension at the 3' end to discriminate between DNA-RNA hybrids and reannealing of the probe. The location of the probe is shown in Fig. 1.

We failed to identify transcripts when RNA isolated from cultures grown as a so-called normal growth curve in liquid MM was used (Fig. 5A). Since the Whi phenotype of GSA3 strongly suggests that *ssg4* is involved in sporulation (Fig. 4),

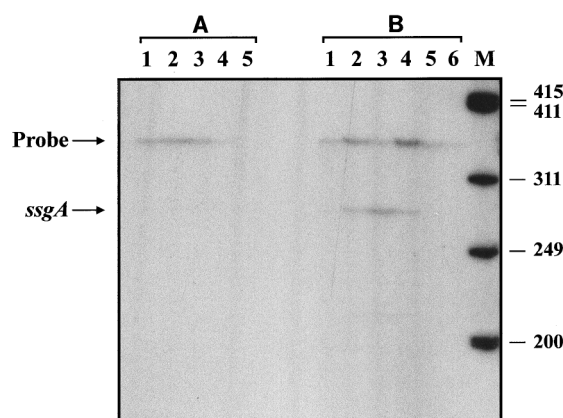


FIG. 5. Transcriptional analysis of *ssgA*. (A) Analysis of samples taken from normal growth curve in MM. Lanes 1 to 5 refer to samples taken at an OD₅₅₀ of 0.3 (early log), 0.6 (mid-log), 0.9 (late log), 1.2 (transition phase), and 1.5 (stationary phase), respectively. (B) Analysis of samples taken after nutritional shift-down. Lanes 1 to 6 refer to samples taken 0, 15, 30, 60, 120, and 240 min after shift-down, respectively. Lane M, ³²P-end-labeled *Hae*III-digested ϕ X174 size markers; numbers on the right refer to sizes of the marker bands (in nucleotides). Positions of the *ssgA* transcript and reannealed probe are shown on the left.

and the protein appears shortly after nutrient depletion in liquid cultures of *S. griseus* (25), we subjected *S. coelicolor* to nutritional shift-down conditions. The burst of ppGpp production following shift-down is generally regarded as an important signal for the onset of morphological differentiation (2) and elicits submerged sporulation in *S. griseus* (27). Cultures of *S. coelicolor* M145 were allowed to grow in TSBS to an OD₅₅₀ of 0.7, washed in MM, and transferred to MM with mannitol as the carbon source. Cultures were incubated at 30°C, and RNA was isolated after 0, 15, 30, 60, 120, and 240 min. The RNA was analyzed by nuclease S1 mapping, again using the *ssgA* probe.

While at the time just before shift-down no transcript could be identified, within 15 min afterward a band of approximately 280 nt appeared, corresponding to full-length protection of the probe (Fig. 5B). This strongly suggests that transcription of *ssgA* is initiated from a promoter either inside or upstream of *ssgR*. A linkage of transcription of *ssgA* and *ssgR* indicates that the regulatory gene *ssgR* may also play a role in the *ssgA*-mediated regulation of cell division. The *ssgRA* transcript levels reached a peak at around 30 min after shift-down; 2 h after shift-down, no transcripts could be detected.

High-level overexpression of *ssgA* in *S. coelicolor*. The phenotype of the *ssgA* null mutant (Fig. 4) and the enhanced fragmentation of *Streptomyces* strains harboring multiple copies of *ssgA* (24) both suggest possible involvement of SsgA in cell division, prompting analysis of the cytological effects of high-level overexpression of *ssgA* in *S. coelicolor*.

To optimize expression of *ssgA*, we fused the coding region to the ribosome binding site of *S. ramocissimus tufI* and placed it under the control of P_{ermE}. Cloning into the integrative vector pSET152 resulted in pGWS4-SD. Since *S. coelicolor ssgA* was not discovered until recently, we performed most of our expression studies with the *S. griseus* homologue, which has been available for several years. We recently also created plasmid pGWS7-SD, which contains *S. coelicolor ssgA* but is otherwise identical to pGWS4-SD, and confirmed that results obtained with either homologue were indistinguishable. The expression construct pGWS4-SD was introduced into *S. coelicolor* M145, and integrants were checked for the presence of *ssgA* by PCR. All transformants were correct integrants and showed the same phenotype. One was selected and designated GSA2.

SsgA protein levels in GSA2 were compared to those in the wild-type strain by Western analysis, using polyclonal antibodies raised against SsgA (see Materials and Methods). Both strains were grown at 30°C in liquid TSBS medium, and S30 extracts were prepared from mycelium grown until early exponential, mid-exponential, and late exponential phases (OD₅₅₀ 0.4, 0.8, and 1.2, respectively). We failed to detect a band corresponding to SsgA in extracts from *S. coelicolor* M145, while a strong band with an apparent molecular mass of approximately 16 kDa was observed for all growth phases of liquid-grown cultures of GSA2 integrants, indicative of significant overproduction of SsgA in this strain (Fig. 3B).

SsgA inhibits branching and induces sporulation in liquid cultures of *S. coelicolor*. The morphologies of the *S. coelicolor ssgA* null mutant and its parental strain M145 were compared by phase-contrast microscopy to those of *S. coelicolor* transformants overexpressing *ssgA*. Wild-type (i.e., harboring a single copy of *ssgA*) *S. coelicolor* produced large mycelial lumps in rich and minimal liquid cultures. The *ssgA* knockout mutant GSA3 formed even denser mycelial clumps, indicative of the formation of highly branched mycelial networks in the absence of *ssgA*. In contrast, hyphae of *S. coelicolor* containing a plasmid expressing *S. coelicolor ssgA* from P_{ermE} (pGWS4, giving increased expression of *ssgA* [Fig. 3B]) showed strongly reduced branching in complex and minimal medium cultures. This resulted in clearly less dense mycelial lumps, in which the (often long) individual hyphae can easily be discerned. Furthermore, small fragments appeared approximately 16 h after inoculation, and fragmentation increased over time. Not only did liquid-grown cultures of GSA2, expressing *ssgA* at a high level (Fig. 3B), show fragmented growth and strongly reduced branching, but the hyphae displayed a strangely swollen appearance, comparable to the tips of *S. griseus* hyphae at the time of sporulation in submerged culture (Fig. 6B). Surprisingly, chains of spore-like bodies were often observed in GSA2 cultures, most likely as the result of the SsgA-induced increase in the frequency of septation (see below and Fig. 7C). While these compartments normally did not show physical separation, we sometimes observed hyphae that had developed into chains of immature spores. These were highly irregular, and many of the spore-like compartments appeared to be empty (Fig. 6C). The abnormal length of these submerged spore chains, comprising sometimes more than 20 compartments, suggests that separation of the spores had not been completed.

Analysis of *S. coelicolor* GSA2 by TEM reveals increased septation and ectopic deposits of cell wall material. To assess the effect of *ssgA* overexpression on mycelial morphology in more detail, we analyzed the hyphae of liquid-grown cultures of GSA2 by transmission EM (TEM), with M145 harboring pSET152 as the control. The wild-type strain showed normal morphology, with few thin cross walls (Fig. 7A and B). Conversely, as was also observed by phase-contrast microscopy (Fig. 6B and C), GSA2 displayed an extremely high frequency of septation, forming small compartments separated by cross-wall-like structures sometimes more than 10 times thicker than wild-type cross walls (Fig. 7C to F). Furthermore, the compartments are oddly misshapen, resulting in hyphae that resemble a string of spores rather than the typical filaments with occasional cross walls. While in *S. coelicolor* M145 hyphae cross walls appear with a frequency of approximately one per 8 μ m (as determined by TEM), hyphae of the *ssgA*-overexpressing strain GSA2 produce small compartments that are misshapen, with many having a size comparable to that of spores (approximately 1 μ m). Lumps of cell wall material are often deposited at opposite sides of the hyphal walls, perhaps indicative of

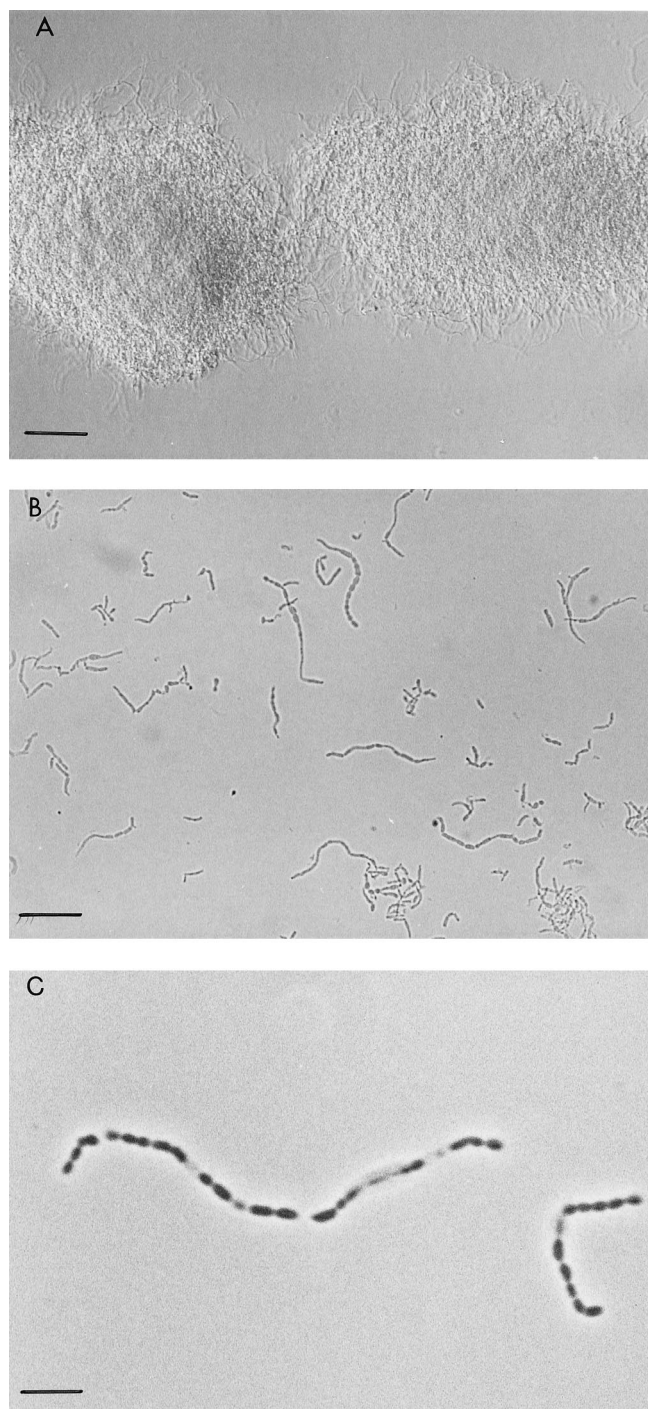


FIG. 6. Phase-contrast micrographs of liquid-grown cultures of *S. coelicolor* M145 (wild type) and GSA2 (overexpressing *ssgA*). Cultures were grown for 24 h in TSBS. (A) *S. coelicolor* M145 forming a typical lump-like mycelial network (bar = 50 μ m). (B) *S. coelicolor* GSA2. The mycelium is strongly fragmented, with hyphae that are significantly wider than in the parental *S. coelicolor* M145. Clearly visible are the spore-like bodies that are produced by this strain (bar = 25 μ m). (C) Closeup of immature spore chains of *S. coelicolor* GSA2. Note that the spores have irregular shapes, and several appear to be empty (bar = 5 μ m).

unfinished septum formation. Another striking feature is the clearly increased thickness of the hyphae, which are 1.5 to 3 times wider than wild-type hyphae, as was confirmed by light microscopy.

DISCUSSION

The data presented here show that the putative cell division gene *ssgA* is a novel *whi* gene and is essential for the proper sporulation of *S. coelicolor* A3(2). On solid media, the white, fluffy aerial mycelium is formed abundantly by the *ssgA* null mutant, as in the wild-type strain. However, spores were produced only on particular media after prolonged incubation, and at a low level. Such a conditional phenotype is typical of several *bld* mutants, several of which sporulate on minimal media containing mannitol as the carbon source, but is to our knowledge the first example of a conditional *whi* mutant. The coiling of the aerial hyphae shows that the *ssgA* mutant does not fall in the category of the early *whi* mutants. However, a more thorough analysis of the mutant, and particularly comparison to well-characterized mutants (9, 33), is required to further determine its developmental state.

Another interesting phenotype of the *ssgA* mutant is that actinorhodin production is strongly reduced, while undecylprodigiosin production is at least as strong as in the wild-type strain. Since actinorhodin is produced later in development than undecylprodigiosin (2), this phenotype may be the result of an arrest in development at a time where actinorhodin production has not yet been initiated.

Transcription of *ssgA* could not be detected during normal growth in liquid MM or TSBS but was induced by nutritional shift-down, which is known to elicit the so-called stringent response and antibiotic production in streptomycetes, and in particular submerged sporulation in *S. griseus*. Interestingly, *ssgA* was not transcribed from a promoter directly upstream of the gene under the chosen conditions but linked to that of *ssgR*, a member of the family of *iclR*-type transcriptional regulator genes. We therefore speculate that *ssgR* itself, like *ssgA*, may also be involved in the regulation of cell division. We are currently working on the creation of a knockout mutant of *ssgR* to provide more insight into its role in the *Streptomyces* life cycle.

The gene dosage of *ssgA* plays a decisive role in determining the hyphal morphology in submerged cultures of *S. coelicolor*. While the *ssgA* null mutant GSA3 produces extremely dense disk-like mycelia, increased expression of *ssgA* results in restricted branching of the hyphae, with the branches often reduced to tiny bulges, similar to the so-called sporulation branches previously described for *S. griseus* (18). Interestingly, *S. coelicolor* GSA2, which is optimized for the stable overexpression of *ssgA*, produces very small mycelial fragments and even chains of immature spores. Stimulation of sporulation was also observed when the developmental sigma factor *WhiG* was overproduced in *S. coelicolor*, although the level and frequency of spore formation were lower in that case (11). This suggests that the machinery required for submerged sporulation is present in *S. coelicolor*. However, the occurrence of many empty compartments and the impaired spore separation indicate that other conditions need to be met to produce mature submerged spores. The observation that SsgA triggers submerged sporulation seems in conflict with earlier experiments in *S. griseus*, which showed that an increased gene dosage of *ssgA* inhibits submerged sporulation in *S. griseus* SY1, a strain which normally sporulates in rich and minimal liquid media. However, since the genotype of the latter mutation could not be ascertained, it is difficult to speculate on a fitting explanation.

Cross walls are often believed to provide extra stability to the hyphae, like the steps of a ladder. However, the opposite may be true. For example, the *ftsZ* null mutant produces no cross walls at all, but long hyphae are still formed in liquid culture, without any obvious sign of excessive breakage of the hyphae

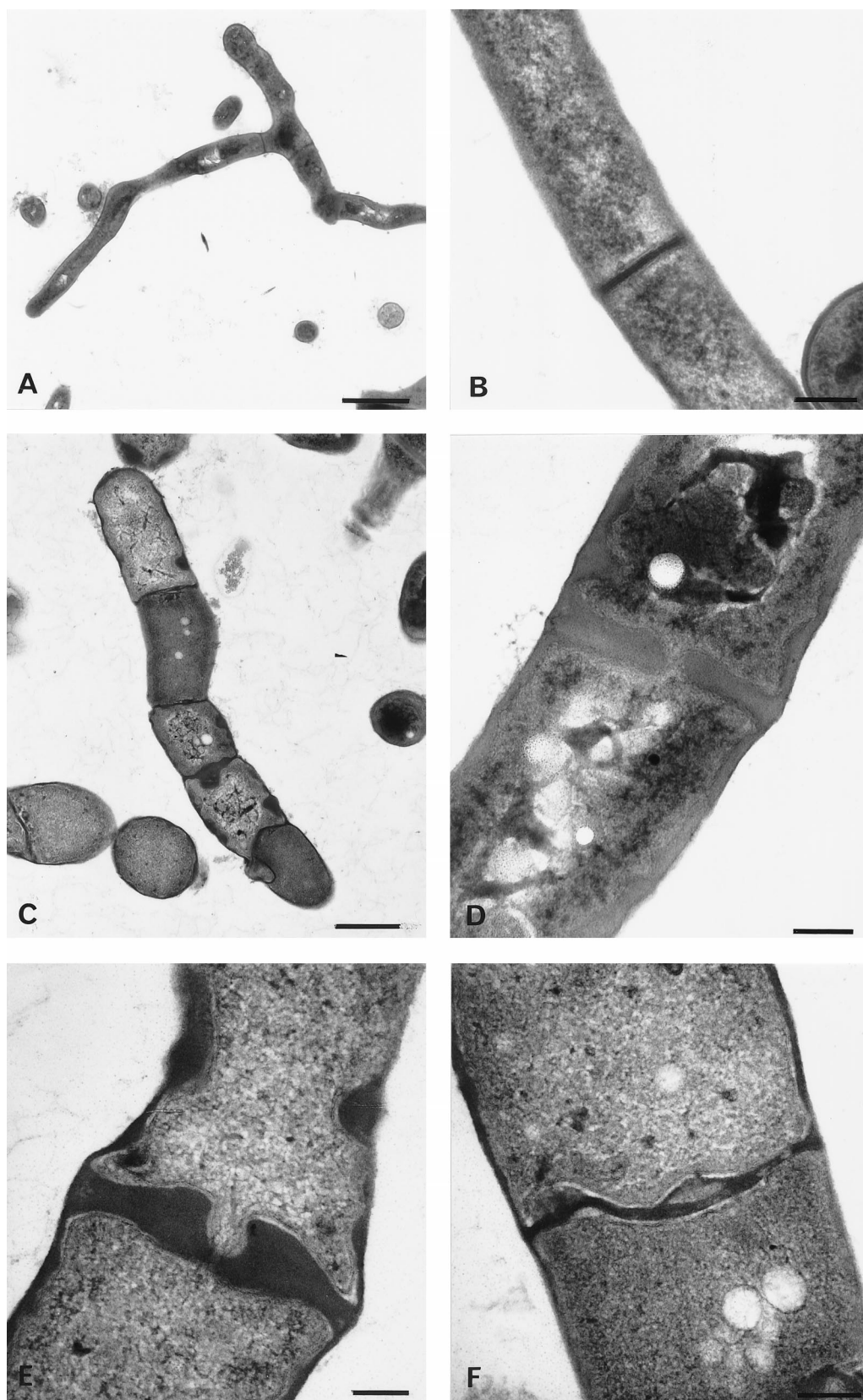


FIG. 7. Transmission electron micrographs of *S. coelicolor* M145 (harboring control plasmid pSET152) and of *S. coelicolor* GSA2. Cultures were grown for 40 h in liquid TSBS medium. (A) Image showing wild-type vegetative hyphae with cross walls (bar = 1.0 μm). (B) High magnification of wild-type cross wall (bar = 0.2 μm). (C) Image of GSA2 showing the effect of SsgA overexpression on the frequency of septum formation and hyphal morphology (bar = 1.0 μm). (D to F) High magnification of abnormally shaped septa in GSA2 hyphae (bar = 0.2 μm).

(29). Furthermore, strains that produce cross walls at higher frequency lyse rapidly in the presence of lysozyme and show increased sensitivity to high sucrose and/or glycine concentrations (e.g., several strains that sporulate in liquid culture but also *S. coelicolor* GSA2 [G. P. van Wezel, unpublished results]). Thus, the increased frequency of septation might explain the enhanced fragmentation of strains overexpressing *ssgA*.

Analysis of GSA2 by TEM shows how dramatic the effect of SsgA overproduction is on the frequency and morphology of the septa. While in *S. coelicolor* M145 hyphae cross walls appear at low frequency (one per 8 μm), hyphae of GSA2 produce compartments with a size comparable to that of spores (approximately 1 μm). There is a distinct difference between vegetative cross walls and sporulation septa; cross walls form semipermeable boundaries between different compartments, while sporulation septa lead to actual cell division (41). Considering the hyperfragmenting phenotype of strains overexpressing *ssgA* and the inhibition of sporulation on solid media in the *ssgA* null mutant, it is likely that SsgA is involved in the formation of sporulation septa. Furthermore, overproduction of SsgA (in GSA2) stimulates the formation of lumps of cell wall material at opposite sides of the hyphal walls, which might reflect the initiation of spore septation. Other observations linking *ssgA* to sporulation are the significant widening of the hyphae in this strain, resulting in a diameter similar to that of spores, and the emergence of immature spore chains in liquid-grown cultures of *S. coelicolor* GSA2.

The molecular mode of action of SsgA is still unclear. The absence of an apparent DNA binding motif in the protein suggests that SsgA does not function as a transcriptional regulator. An intriguing possibility is that SsgA might function by directly interacting with one or more proteins belonging to the cell division machinery. The availability of purified SsgA protein will allow the search for putative interaction partners for SsgA; these experiments are in progress.

In conclusion, the gene dosage of *ssgA* has a dramatic effect on *Streptomyces* hyphal morphology and particularly on sporulation. While an *ssgA* knockout mutant fails to sporulate on solid media, overexpression of the gene results in strongly increased septum formation and production of spore-like bodies by clearly widened hyphae in submerged culture, indicative of pleiotropic changes in the mycelial buildup. The regulation of *ssgA* expression as a tool for modifying the hyphal morphology may be of great value for applications in biotechnological fermentations.

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