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Function and control of the ssg genes in streptomyces

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Chapter IV

Control of *Streptomyces* differentiation through the regulation of SALPs

Bjørn A. Traag, Giovanni Sandrini and Gilles P. van Wezel

ABSTRACT

SsgA-like proteins (SALPs) control sporulation-specific cell division and autolytic spore separation in aerial hyphae of streptomycetes. With the exception of the vegetatively expressed *ssgD*, all *ssg* genes are repressed by glucose, which highlights a possible mechanism for carbon-source-dependent repression of development. Previous work indicated that *ssgA* is expressed in a *whi*-independent manner. Transcriptional analysis of all *ssgA*-like genes (*ssgB-G*) in early developmental mutants demonstrated that transcription of *ssgB*, itself essential for sporulation and extremely well-conserved in streptomycetes, was dependent on *whiA* and *whiH*, while the expression of other *ssg* genes was less affected in the developmental mutants. The transcriptional activation of SsgB may explain the sporulation-deficient phenotype of *whiH* mutants. In liquid-cultures SsgA has a major effect on morphogenesis of streptomycetes. While several conserved features were found in the *ssgA* promoter region from 18 *Streptomyces* species, its divergent transcriptional control could be related to their efficiency of producing submerged spores. Interestingly, besides these transcriptional aspects, phylogenetic analysis suggests a direct relationship between the SsgA amino acid sequence and liquid-culture morphology, with specific amino acid residues conserved only in SsgA orthologues from streptomycetes that sporulate in submerged cultures. The implications of these observations for the control of morphogenesis are discussed.

INTRODUCTION

Streptomycetes have an amazing potential to adapt to diverse natural habitats. This is highlighted by the presence of more than 20 clusters coding for secondary metabolites, around 65 sigma factors and an unprecedented number of sugar transporters and polysugar hydrolases on the genomes of *Streptomyces coelicolor* (Bentley *et al.*, 2002), *Streptomyces avermitilis* (Ikeda *et al.*, 2003), *Streptomyces griseus* and *Streptomyces scabies* (sequences available through the internet). The study of *Streptomyces* development is carried out primarily on solid-grown cultures, where aerial hyphae act as an intermediate between vegetative growth and spore formation (Chater, 1972). Most developmental genes that control aerial development (the so-called *whi* genes) encode transcription factors (Chater, 1972; Flärdh *et al.*, 1999; Ryding *et al.*, 1999). Aerial hyphae are by definition not produced in submerged culture. Nonetheless, several streptomycetes have the ability to produce spores in liquid cultures, such as *S. griseus* and *S. venezuelae* (Glazebrook *et al.*, 1990; Kendrick and Ensign, 1983). Some *whi* genes also play a role in submerged sporulation. For example, overexpression of the sporulation-specific σ-factor WhiG induces some submerged sporulation in liquid-grown mycelium of *S. coelicolor* (Chater *et al.*, 1989), and deletion of a number of *whi* gene orthologues in *S. venezuelae* (*i.e. whiA, whiB, whiD, whiG*, *whiH* and *whiI*) resulted in a failure to sporulate on agar plates and in liquid-grown cultures (M.J. Buttner and M.J. Bibb, pers. comm.). This suggests significant overlap between the sporulation pathways under both conditions.

SsgA was originally identified as a suppressor of a hyper-sporulating *S. griseus* mutant (designated SY1) and was shown to be essential for submerged sporulation (Kawamoto and Ensign, 1995a; Kawamoto *et al.*, 1997). Similar to σ^{WhiG}, overexpression of *S. griseus* SsgA in liquid-grown mycelium of *S. coelicolor* induced mycelial fragmentation and spore formation (van Wezel *et al.*, 2000a). On solid media *ssgA* mutants have a conditional "white" phenotype, capable of producing spores on mannitol-containing medium, but not in the presence of glucose (Jiang and Kendrick, 2000a; van Wezel *et al.*, 2000a). Although many early developmental (*bld*) mutants are carbon source dependent (Merrick, 1976; Pope *et al.*, 1996), similar dependence is rare among *whi* mutants. Transcription

of *ssgA* has been extensively studied in the model streptomycetes *S. coelicolor* and *S. griseus*, and important differences were observed. In *S. coelicolor*, transcription is *trans*-activated by and dependent on SsgR (Traag *et al.*, 2004). In contrast, in *S. griseus* expression of *ssgA* is only slightly affected by SsgR and dependent on the the A-factor pathway-controlled AdpA (Horinouchi and Beppu, 1994; Ohnishi *et al.*, 2005). There is no detectable transcription of *ssgA* in submerged cultures of *S. coelicolor*, while it is strongly expressed in *S. griseus* (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a; van Wezel *et al.*, 2000b). The difference in transcriptional control is possibly one of the main reasons why *S. griseus* is able to sporulate in submerged culture, while *S. coelicolor* is not. Of all *S. coelicolor* SALP null mutants, *ssgA* and *ssgB* mutants have a "white" phenotype, while the *ssgG* mutant produced significantly less spores than the wild-type strain (Noens *et al.*, 2005). In contrast to *ssgA* mutants, *ssgB* mutants have a non-conditional "white" phenotype, producing straight aerial hyphae and no spores on all media (Keijser *et al.*, 2003; Sevcikova and Kormanec, 2003). SsgB is most likely the archetype of the SALP family, with functional orthologues occuring in distantly related actinomycetes (Chapter VI of this thesis).

In this study, we investigated *ssg* gene expression on different carbon sources by promoter probing assays, and their transcriptional dependency on six early *whi* genes (*i.e. whiA*, *whiB*, *whiG*, *whiH*, *whiI* and *whiJ*) and *ssgB*. Furthermore, the control of transcription and translation of *ssgA* in distantly related streptomycetes was adressed, and new insights into its role during submerged sporulation are discussed.

MATERIALS AND METHODS

Bacterial strains and culturing conditions

E. coli K-12 strains JM109 (Sambrook *et al.*, 1989) and ET12567 (MacNeil *et al.*, 1992) were used for propagating plasmids, and were grown and transformed using standard procedures (Sambrook *et al.*, 1989). Transformants were selected in L broth containing 1% (w/v) glucose and the appropriate antibiotics. The *Streptomyces* strains used in this work are listed in Table 1. M145 was used for transformation and propagation of *Streptomyces* plasmids. M512 Δglk was made

by protoplast fusion of M512 (Floriano and Bibb, 1996) and J1915 (Kelemen *et al.*, 1995). Preparation of media for streptomycete growth, protoplast preparation and transformation were done according to standard procedures (Kieser *et al.,* 2000).

Table 1. *Streptomyces* strains

Strains	Description	Reference
S. coelicolor M145	Wild type *S. coelicolor* A3(2)	(Kieser *et al.*, 2000)
S. coelicolor M512	M145 Δ*actII*-ORF4 Δ*redD*	(Floriano and Bibb, 1996)
S. coelicolor J1915	M145 Δglk	(Kelemen *et al.*, 1995)
S. coelicolor M512 Δ*glkA*	M512 and J1915 protoplast fusion	This work
S. coelicolor GSA2	M145 harboring pGWS4SD	(van Wezel *et al.*, 2000a)
S. coelicolor GSA3	M145 Δ*ssgA*	(van Wezel *et al.*, 2000a)
S. coelicolor GSB1	M145 Δ*ssgB*	(Keijser *et al.*, 2003)
S. coelicolor J2401	M145 Δ*whiA*	(Flärdh *et al.*, 1999)
S. coelicolor J2402	M145 Δ*whiB*	(Flärdh *et al.*, 1999)
S. coelicolor J2400	M145 Δ*whiG*	(Flärdh *et al.*, 1999)
S. coelicolor J2210	M145 Δ*whiH*	(Ryding *et al.*, 1999)
S. coelicolor J2450	M145 Δ*whiI*	(Ainsa *et al.*, 1999)
S. coelicolor C77	*S. coelicolor* A3(2) *whiJ* locus *77*	(Ryding *et al.*, 1999)
S. griseus B2682	Wild type *S. griseus*	
S. griseus SY1	*S. griseus* mutant strain	(Kawamoto and Ensign, 1995a)
S. albus DSM40313	Wild type *S. albus*	
S. clavuligerus NRRL8165	Wild type *S. clavuligerus*	
S. collinus DSM40733	Wild type *S. collinus*	
S. diastatochromogenes Tü1062	Wild type *S. diastatochromogenes*	
S. filipinensis	Wild type *S. filipinensis*	
S. fradiae Tü1222	Wild type *S. fradiae*	
S. granaticolor	Wild type *S. granaticolor*	
S. lividans 1326	Wild type *S. lividans*	
S. ramocissimus	Wild type *S. ramocissimus*	
S. roseosporus ATCC31568	Wild type *S. roseosporus*	
S. venezuelae ATCC15439	Wild type *S. venezuelae*	
Streptomyces species Wlb19	Novel soil isolate	This work
Streptomyces species Che26	Novel soil isolate	This work
Streptomyces species Gre54	Novel soil isolate	This work

SFM medium was used to make spore suspensions; R2YE medium was used for regenerating protoplasts and, after addition of the appropriate antibiotic, for selecting recombinants; minimal medium (MM) was used to prepare total RNA samples, and for promoter-probe experiments on different carbon sources. For

standard cultivation of *Streptomyces* in liquid cultures YEME (yeast extract malt extract with 30% (w/v) sucrose); TSBS (tryptone soy broth (Difco) containing 10% (w/v) sucrose) and NMMP (normal minimal medium) were used.

Plasmids and constructs

1. General cloning vectors

pIJ2925 (Janssen and Bibb, 1993) is a pUC19-derived plasmid used for routine subcloning. Plasmid DNA was isolated from ET12567 prior to transformation to *Streptomyces*. For selection of pIJ2925 and pIJ2587 in *E. coli* ampicillin (100 µg/ml) was used; chloramphenicol (25 µg/ml) was added for growth of ET12567.

2. Constructs for promoter probing

pIJ2587, a derivative of pHJL401 containing a promoterless version of the undecylprodigiosin activator gene *redD* as reporter gene, was used for promoter-probe experiments (van Wezel *et al.*, 2000c). Fragments harboring the upstream regions of *ssgA-G* and *ssgR* were amplified by PCR and cloned individually into pIJ2587. The constructs are summarised in Table 2. The oligonucleotides (Table 3) were designed such that restriction sites were added allowing cloning of the fragments as *Eco*RI-*Bam*HI fragments, with the *Bam*HI site proximal to the translational start of the genes. In this way, putative promoter sequences were positioned in the desired orientation and immediately upstream of the promoterless *redD* gene in pIJ2587. Transformants were plated on MM medium with mannitol, glycerol and/or glucose, and the production of the red-pigmented antibiotic undecylprodigiosin (Red) was assessed visually.

Table 2. Constructs.

Constructs	Insert*	Reference
pGWS222	-619/+70 region relative to the start of *ssgA*	This work
pGWS221	-512/+12 region relative to the start of *ssgR*	This work
pGWS216	-592/+21 region relative to the start of *ssgB*	This work
pGWS217	-216/+35 region relative to the start of *ssgC*	This work
pGWS211	-291/+22 region relative to the start of *ssgD*	This work
pGWS110	-223/+38 region relative to the start of *ssgE*	This work
pGWS218	-401/+99 region relative to the start of *ssgF*	This work
pGWS219	-467/+62 region relative to the start of *ssgG*	This work

* All constructs are pIJ2587 derivatives

RNA isolation and semi-quantitative RT-PCR analysis

For transcriptional analysis of *ssgA*-*ssgG* in surface-grown developmental (*whi*) mutants, mycelium grown on solid MM with mannitol (0.5% w/v) on cellophane discs was harvested at three time points corresponding to vegetative growth, early aerial growth, and late aerial growth or, in the case of M145, spore formation. Harvested mycelium was immediately dispersed in 3 ml of P-buffer (Kieser *et al*., 2000) containing 1 mg/ml lysozyme. The RNA was purified from the mycelium using the Kirby-based protocol (Kieser *et al.*, 2000). RNA purification columns (RNeasy, Qiagen) and DNaseI treatment were used as well as salt precipitation (final concentration 3M NaAc pH 4.8) to purify the RNA and fully remove any traces of DNA, respectively. Phase-contrast light microscopy was used to assess the developmental stage of the surface-grown mycelium prior to harvesting and RNA isolation. For transcriptional analysis of *ssgA*, *ssgR*, *afsA* and *adpA* in *S. griseus* strains B2862 and SY1 (Table 1) 50 ml YEME cultures were grown to OD_{600} 0.3-0.4, from which a 10 ml sample was taken, designated T_0. Nutritional shift-down was achieved by subsequent washing and resuspending of the mycelium in 40 ml of NMMP. The cultures were then allowed to continue growth, and samples were taken after 30 min (T_1) and 60 min (T_2).

Semi-quantitative reverse-transcriptase PCR (RT-PCR) analysis was carried out using SuperScript III one-step RT-PCR System (Invitrogen). For each RT-PCR reaction 200 ng of RNA was used together with 0.5 μM (final concentration) of each oligonucleotide. The program used was as follows: 45 min cDNA synthesis at 50°C, followed by 25-35 cycles of: 30 sec at 94°C (denaturing), 30 sec at 58°C (annealing) and 30 sec at 68°C (elongation). The reaction was completed by 5 min incubation at 68°C. Samples were tested on a 2% agarose gel in TAE buffer containing ethidium bromide. RT-PCR experiments without prior reverse transcription were performed on all RNA samples to assure exclusion of DNA contamination. Quantification of the RT-PCR results was done by scanning the gels using the GS-800 imaging densitometer followed by analysis using Quantity One software (Bio-Rad). 16S rRNA levels were analysed and quantified as a control, and values obtained for the *ssg* genes were corrected for slight differences in the16S rRNA levels in the corresponding RNA extracts.

Western blot analysis of SsgA

Protein extracts were prepared from mycelium grown in liquid TSBS medium. Mycelium was treated by ultrasonication for 5 min at 30 W at 4ºC in standard buffer (10 mM Tris-HCl (pH 7.6), 60 mM NH_4Cl, 10 mM magnesium acetate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Samples were then centrifuged at 30,000x *g* for 30 min. The resulting S30 extracts were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 5 µg of total protein was loaded. Gels were stained with Coomassie brilliant blue or blotted onto Hybond-P (PVDF; Amersham) and immunostained with antibodies raised against *S. griseus* SsgA (Kawamoto *et al.*, 1997). Full length (145 aa; see Results section) SsgA from *S. griseus* (SsgA$_{145}$) was expressed in *E. coli* as a hexahistidine-tagged fusion protein and purified by nickel-affinity chromatography. The hexahistidine tag was removed by digestion with thrombin prior to gel electrophoresis.

DNA sequencing of ssgA orthologues

The sequences of the open reading frame (ORF) of *ssgA* and flanking regions from 14 streptomycetes (Table 1) were obtained by PCR with primers ssgA-seqF

Table 3. Oligonucleotides. Underlined sequences indicate non-homologues sequence added to create restriction sites (in italics) at the ends of the PCR fragments.

Primer name	DNA sequence (5’ to 3’)*	Location 5’ end	Relative to +1 of
ssgA-seqF	GAT*GAATTC*AGCATCTGAAAACTCACTCCTTGTGATC	-140	*ssgA*
ssgA-seqR	GATC*AAGCTT*CTGCTGCTGTTCTC(C/G)ATCGC(C/G)CAGA	+709	*ssgA*
PssgA-for	GTC*GAATTC*CACCATGGCGCGCTGGCGCGAC	-619	*ssgA*
PssgA-rev	CTG*GGATCC*CCCGGGTCTCGTAGCGCAGCTC	+70	*ssgA*
PssgR-for	GTC*GAATTC*GGACTGCCGTGGTGGGTGAAGTG	-512	*ssgR*
PssgR-rev	GTC*GGATCC*CGCCCGCTGCACGGAGCCGATC	+12	*ssgR*
PssgB-rev	GTCC*GAGCTC*GCTCTCCCGAGTGATCACTGGTC	-592	*ssgB*
PssgB-for	GTC*GGATCC*GCAGCTGACCGTGGTGTTGAT	+21	*ssgB*
PssgC-rev	GTC*GGAATTC*GTCGACGCCGGGTTCACCGAGGT	-216	*ssgC*
PssgC-for	GTC*GGATCC*TGCACGACCAGGGTCTTGTGCAC	+35	*ssgC*
PssgD-for	GTC*GAATTC*GTCCCGTGCGTCGCGTGCTTCCC	-291	*ssgD*
PssgD-rev	GTC*GGATCC*ACTGCTCGATGACGGTGGAC	+22	*ssgD*
PssgE-for	GTC*GAATTC*GAGGTCGGGGCGTTGATGAATC	-223	*ssgE*
PssgE-rev	GTC*GGATCC*AGGATGTGGGCTCGTGCGTAC	+38	*ssgE*
PssgF-for	GTC*GAATTC*CGCGTGGGCCTGACCGGACATGAC	-401	*ssgF*
PssgF-rev	GTC*GGATCC*CTCGAGAGCGCCCGTCATCTG	+99	*ssgF*
PssgG-for	GTC*GAATTC*CTCGACCGGGTCCTCGTCGAAG	-467	*ssgG*
PssgG-rev	GTC*GGATCC*AGGACGAGCCTGAGCTCCAG	+62	*ssgG*

and ssgA-seqR (Table 3). Inserts were cloned into *Sma*I digested pIJ2925 by blunt ligation. DNA sequencing was done using universal primers MF and MR, specific for plasmid sequences adjacent to the *Sma*I restriction site. Newly obtained *ssgA* gene and promoter sequences have been assigned the following GenBank accession numbers (between brackets): *S. albus ssgA* (no. AF195771), *S. clavuligerus ssgA* (no. EU475893), *S. collinus ssgA* (no. EU475888), *S. diastatochromogenes ssgA* (no. EU475890), *S. filipinensis ssgA* (no. EU475891), *S. fradiae ssgA* (no. EU475889), *S. granaticolor ssgA* (no.EU475894), *S. lividans ssgA* (no. EU475887), *S. ramocissimus ssgA* (no. EU475892), *S. roseosporus ssgA* (no. EU475886), *S. venezuelae ssgA* (no. EU475895), *Streptomyces* species Wlb19 *ssgA* (no. EU475896), *Streptomyces* species Che26 *ssgA* (no. EU475898), and *Streptomyces* species Gre54 *ssgA* (no. EU475897). The latter three strains are novel strains in our collection, and submerged sporulation was observed for all three after nutritional shift-down.

Computer analysis

The program ClustalW was used for DNA and protein sequence alignments, and to make phylogenetic trees presented in Figure 7 (Higgins *et al.*, 1996). Figures 2 and 4 were made using the program Boxshade (www.ch.embnet.org/ software/BOX_form.html). Sequences in Figure 2, 4 and 7 were labeled by their strain of origin and abbreviated as follows: **(S.albu)** *S. albus*, **(S.aver)** *S. avermitilis*, **(S.clav)** *S. clavuligerus,* **(S.coel)** *S. coelicolor*, **(S.coll)** *S. collinus*, **(S.dias)** *S. diastatochromogenes*, **(S.fili)** *S. filipinensis*, **(S.frad)** *S. fradiae*, **(S.livi)** *S. lividans*, **(S.gran)** *S. granaticolor*, **(S.gris)** *S. griseus*, **(S.rose)** *S. roseosporus*, **(S.ramo)** *S. ramocissimus*, **(S.scab)** *S scabies*, **(S.vene)** *S. venezuelae*, **(S.Wlb19)** *Streptomyces* species Wlb19, **(S.Che26)** *Streptomyces* species Che26, **(S.Gre54)** *Streptomyces* species Gre54.

RESULTS

Glucose repression of developmental *ssg* genes

Sporulation of streptomycetes is delayed on glucose-containing media, but the molecular basis for this phenomenon is largely unknown. The *ssg* genes encode

SsgA-like proteins or SALPs, which control specific stages of sporulation-specific cell division in streptomycetes (Noens *et al.*, 2005). To investigate the effect of carbon sources on *ssg* gene expression, the *ssgA*-G and *ssgR* promoter regions were cloned into pIJ2587 in front of the promoterless *redD* gene and introduced into M512 and its *glkA* mutant derivative (Table 1). M512 lacks the pathway-specific activator genes *actII-4* and *redD* for the Act and Red pathways, respectively, and expression of *redD* in pIJ2587 will restore biosynthesis of the red-pigmented undecylprodigiosin, thus allowing monitoring of promoter activity. Glucose kinase (Glk, encoded by *glkA*) is essential for growth on glucose and for carbon catabolite control in *S. coelicolor* (Angell *et al.*, 1992), and M512 *glkA* can thus be used to study promoter activity insensitive to carbon catabolite control. On MM agar plates with mannitol or glycerol as the sole carbon source (glycerol data not shown), the promoters of *ssgR* and of any of the *ssg* genes but *ssgD* were active from the onset of aerial mycelium formation onwards, although the transcriptional activity of the fragments harbouring the promoters *ssgA*p, *ssgE*p or *ssgG*p was low. In contrast, *ssgD*p is a very strong promoter, with large amounts of Red visible already during very early growth (Figure 1, left column). In M512, all promoters except *ssgD*p were repressed by glucose (Figure 1, middle column). In M512 Δ*glkA*, promoter activity was not repressed by glucose, and similar red pigmentation were observed as compared to M512 transformants on mannitol (Figure 1, right column). This strongly suggests that the promoters of the developmental *ssg* genes are subjected to glucose repression, while the life-cycle independent *ssgD*p is not.

Transcription of *ssg* genes in sporulation mutants of *S. coelicolor*

We have previously shown that *ssgRA* are transcribed independently of the essential sporulation genes *whiA*, *whiB*, *whiG*, *whiH*, *whiI* and *whiJ* in *S. coelicolor* (Traag *et al.*, 2004). Here we expanded this survey so as to include the transcriptional analysis of the six *ssgA*-like genes (*ssgB-G*) in the genetic background of these 'classical' *whi* mutants as well as in *ssgB* mutants (Keijser *et al.*, 2003), using semi-quantitative RT-PCR. 16S rRNA was used as the control (Figure 2A), and the RT-PCR data were quantified and corrected for differences in the 16S rRNA levels (see "Materials and Methods" section). In accordance with the promoter-probe experiments, in M145 transcription of *ssgA*, *ssgB*, *ssgC*,

ssgE, *ssgF* and *ssgG* was life-cycle-dependent, with increased transcript levels at later time points, while *ssgD* transcript levels were equally high at all time points (Figure 2B). As reported earlier, *ssgA* transcription was not significantly (less than two-fold) altered relative to M145 in any of the *whi* mutant backgrounds (although *ssgA* levels appeared somewhat lower in the *whiH* mutant), and the same was true for *ssgD*.

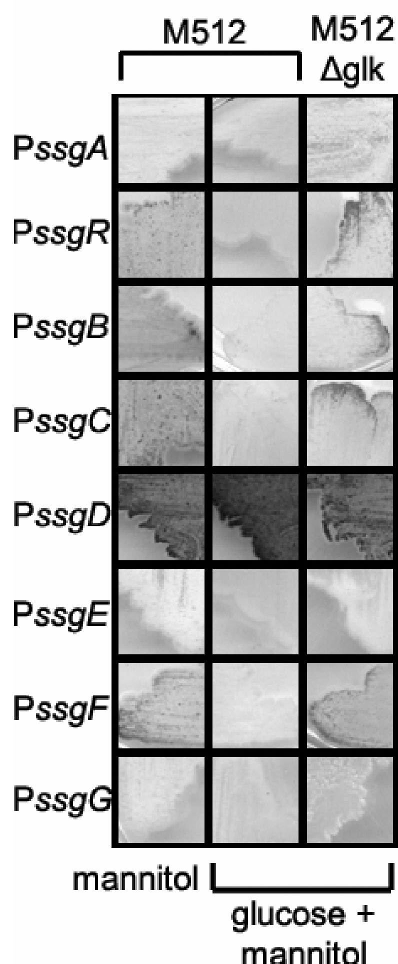
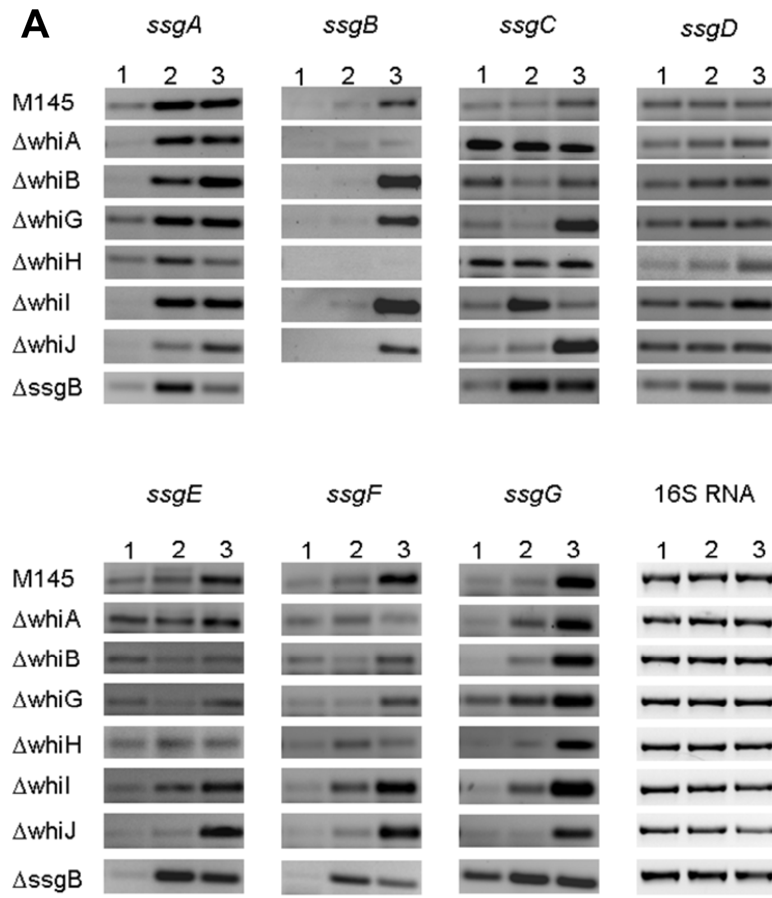


Figure 1. Dependence of the activity of *ssg* gene promoters on different carbon sources (For colour figure see Appendix A). The ability to stimulate Red production by the upstream fragments of *ssgA-G* and *ssgR* (*ssgX*p) was tested on minimal medium containing mannitol (left panel) or mannitol + glucose (middle and right panel). Red production is not evidently stimulated by the promoter fragments of *ssgA*, *ssgE* and *ssgG* on minimal medium. Promoter fragments of *ssgB*, *ssgC, ssgD*, *ssgF* and *ssgR* stimulated Red production in M512 on mannitol (left panel), however only the promoter of *ssgD* did so on mannitol + glucose (middle panel). Promoter activity of all fragments was restored on mannitol + glucose in M512 Δ*glkA* (right panel).


A
ssgA
ssgB
ssgC
ssgD
1 2 3
M145
ΔwhiA
ΔwhiB
ΔwhiG
ΔwhiH
ΔwhiI
ΔwhiJ
ΔssgB
ssgE
ssgF
ssgG
16S RNA

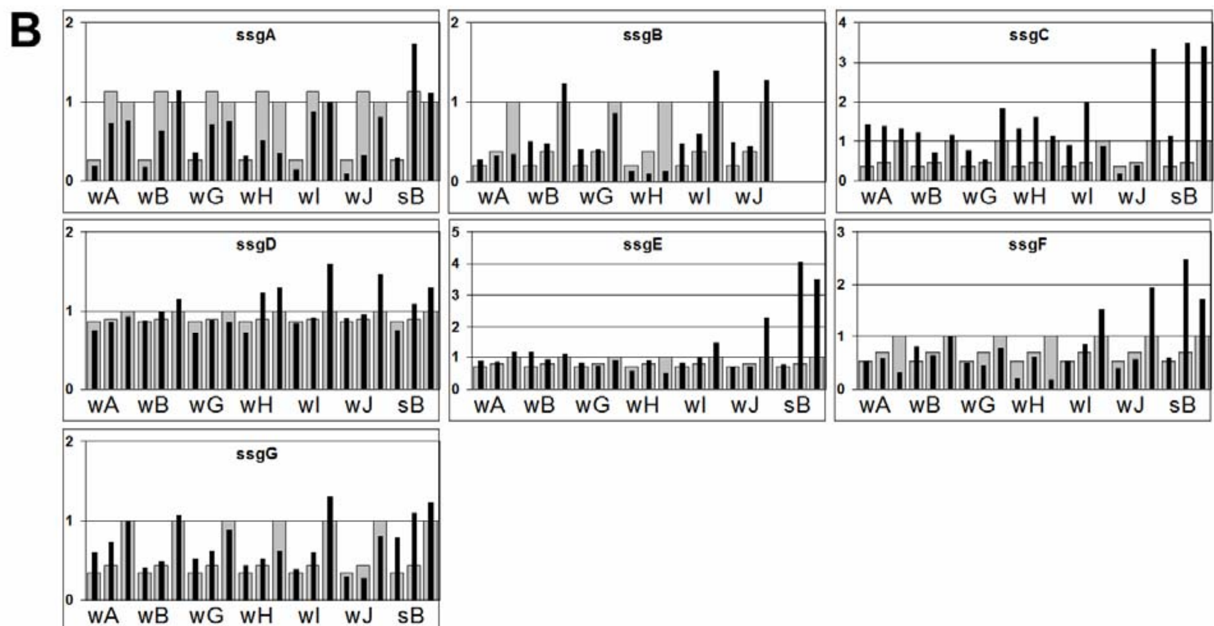

B
ssgA
ssgB
ssgC
ssgD
ssgE
ssgF
ssgG
wA wB wG wH wI wJ sB

Figure 2. Transcriptional analysis in *S. coelicolor whi* mutants. A. Representative picture of semi-quantitative RT-PCR results of *ssgA-G* and 16S rRNA (top) in RNA purified from the parental strain M145 and mutants of *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, *whiJ* and *ssgB* (left). For all strains, time points **1**, **2** and **3** correspond to vegetative growth (approx. 24h), early aerial growth (approx. 48h) and late aerial growth or, in the case of M145, sporulation (approx. 72h), respectively. **B.** Bar graphs showing quantified intensities of the bands in Figure 2A, with the background value substracted, and corrected for differences in 16S rRNA. For each gene, the bar values are relative to the value of time point 3 in M145, which was arbitrarily set to one (y-axis). Each set of three bars corresponds to the time points **1**, **2** and **3** of each strain. Bars for the wild type M145 are shown in light grey and are duplicated behind each set of bars of the mutants, in order to highlight the differences. The sets of bars are labelled (x-axis) as follows: **wA** (Δ*whiA*), **wB** (Δ*whiB*), **wG** (Δ*whiG*), **wH** (Δ*whiH*), **wI** (Δ*whiI*), **wJ** (Δ*whiJ*) and **sB** (Δ*ssgB*).

ssgG transcripts were only affected in the *ssgB* mutant, with expression already during vegetative and early aerial growth, although maximal transcript levels (during late aerial growth) were similar between M145 and the *whiH* mutant. Several differences were observed for the other *ssg* genes in particular mutants. Transcript levels of *ssgB*, *ssgE* and *ssgF* were all reduced to some extent in *whiA* and/or *whiH* mutants. Strikingly, *ssgB* levels were more than three-fold reduced in the *whiA* mutant, and almost completely absent in the *whiH* mutant. SsgA and SsgB are the only SALPs essential for sporulation, and it is interesting that where SsgA is expressed in a *whi* independent manner, SsgB is subjected to sporulation control and its gene is transcriptionally dependent on WhiA and WhiH. This may finally explain the sporulation-deficient phenotype of *whiH* mutants. *ssgC* transcription was enhanced at one or more time points of several mutants, and significant up-regulation was observed in mutants of *whiG*, *whiI*, *whiJ* and *ssgB*. Finally, the spore maturation genes *ssgE* and *ssgF* were somewhat upregulated in *whiJ* and *ssgB* mutants.

SY1: a hyper-sporulating *S.griseus* strain

SY1 is a mutant derivative of *S. griseus* B2682 that sporulates profusely in submerged cultures and not only in minimal media (required for submerged sporulation of its parent B2682 (Kendrick and Ensign, 1983), but also in rich media (Kawamoto and Ensign, 1995a). Significantly more SsgA protein is produced from an earlier time point in SY1 when compared to the wild-type strain in complex liquid media (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a),

although the *ssgA* genes and promoters of both strains are identical (van Wezel *et al.*, 2000a). Hence, a mutation has occurred that should shed more light on the regulation of SsgA and its relationship to submerged sporulation. We therefore analysed the genomic sequences of all genes relating to the control of *ssgA* transcription, namely the specific regulator *ssfR* (ortholog of *ssgR* in *S. griseus*), the A-factor dependent regulatory gene *adpA* (activator of *ssgA*) and the known genes of the A-factor pathway, namely *afsA* (the A-factor synthetic gene) and *arpA* (encoding the A-factor responsive protein that represses *adpA*) (Horinouchi and Beppu, 1994; Ohnishi *et al.*, 2005). No mutations were found in the DNA sequences of any of these genes or in their promoter sequences. Since the known regulatory pathways of *ssgA* transcription are seemingly unaffected, we investigated the transcription of *ssgA*, *ssfR*, *afsA* and *adpA* in B2682 and SY1 (Figure 3). SY1 and its parent *S. griseus* B2682 were grown in YEME which allows submerged sporulation of SY1 but not B2682. When the cultures reached an OD_{600} of 0.3-0.4, they were subjected to a nutritional shift-down (see "Materials and Methods"), under which conditions B2682 also produced submerged spores. Total RNA was purified from samples before and after nutritional shift-down and transcript levels of *ssgA*, *ssfR*, *afsA* and *adpA* were analysed by semi-quantitative RT-PCR, again with 16S rRNA as the control (Figure 3). In the rich pre-culture (T_0), *ssgA* levels were only slightly higher in SY1 (approximately 1.5-fold), while – surprisingly - *afsA* transcription was approximately seven-fold upregulated (Figure 3b). Transcript levels of *ssfR* and *adpA* were similar in pre-cultures of either strain. After nutritional shift-down (T_1 and T_2) *afsA* transcription was induced in B2682, but remained higher in SY1, while no differences were observed for *adpA* transcription. In B2682, *ssfR* transcription was slightly induced after nutritional shift-down (approximately 1.5-fold; Figure 3b), but this was not observed for SY1. As expected, in B2682 *ssgA* transcription increased about five-fold after nutritional shift-down. In contrast, *ssgA* transcript levels were not induced by nutritional shift-down in SY1, again suggesting that the control of *ssgA* is different in B2682 and its SY1 mutant.

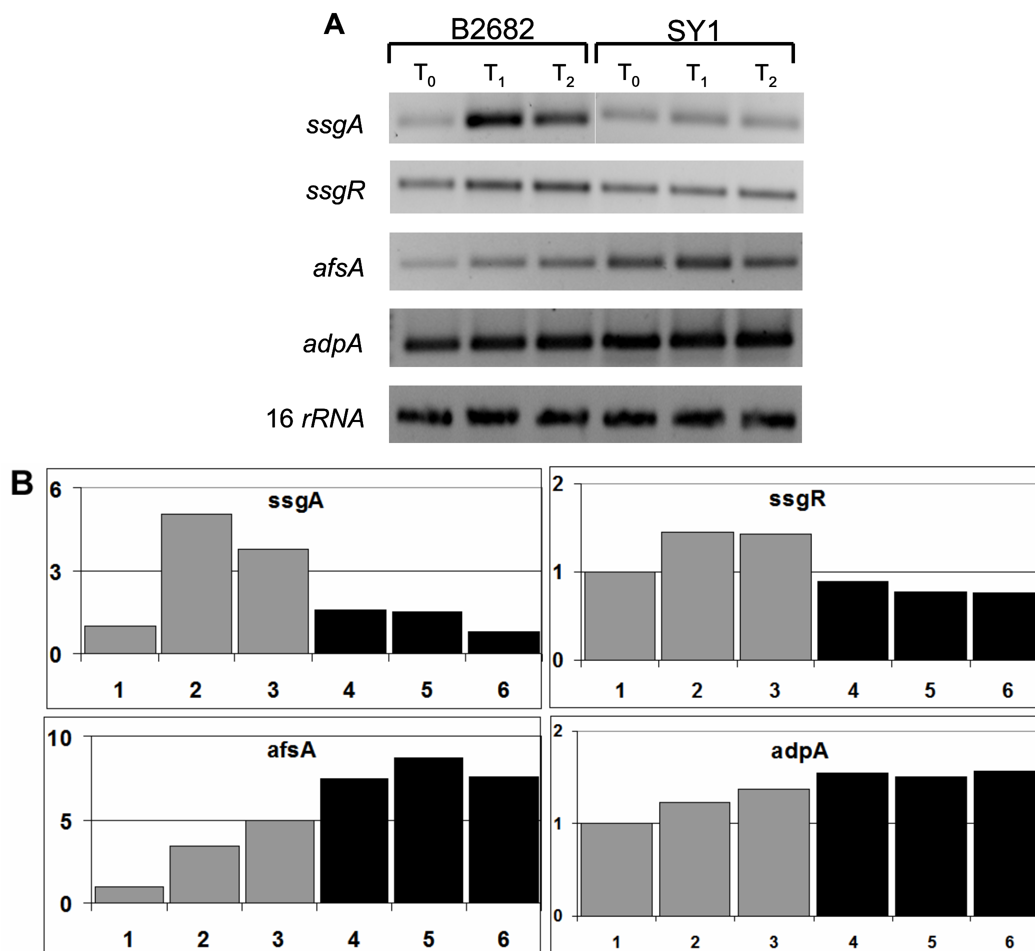


Figure 3. Transcriptional analysis in *S. griseus* B2682 and SY1. A. Semi-quantitative RT-PCR on *ssgA*, *ssfR*, *afsA, adpA* and 16S rRNA (control) in RNA purified from liquid cultures of *S. griseus* B2682 and SY1. RNA extracts were purified from rich liquid (pre-)cultures (T_0), and after nutritional shift-down after 30 (T_1) and 60 min (T_2). **B.** Bar graphs showing quantified intensities of the bands, with the background value substracted. For each gene, bar values are relative to the T_0 value, which was set to one. On the X-axis of each graph, bar numbers 1-3 correspond to T_0, T_1 and T_2 of B2682 (grey bars), and bar numbers 4-6 correspond to T_0, T_1 and T_2 of SY1 (black bars).

Transcriptional control of *ssgA*

The control of transcription of *ssgA* is strikingly different in the morphologically distinct species *S. coelicolor* and *S. griseus*, which perhaps relates to the morphological differences between the two strains, especially in respect to the ability of *S. griseus* to produce submerged spores (Traag *et al.*, 2004; Yamazaki *et al.*, 2003). To establish if a relationship could exist between the sequence of *ssgA* and *Streptomyces* morphogenesis, *ssgA* genes and their promoter sequences were compared for 18 different streptomycetes. These included the

sequenced genomes of *S. avermitilis*, *S. coelicolor*, *S. griseus*, and *S. scabies* (Figure 4), while the other 14 were cloned and sequenced. For this purpose, primers ssgA-seqF and ssgA-seqR (Table 3) were used to amplify the *ssgA* orthologues and their promoter region (up to and including the *ssgR* stop codon) from genomic DNA of the remaining 14 streptomycetes (see Table 1).

The length of the *ssgRA* intergenic regions of *S. granaticolor* and *S. venezuelae* (138 bp), and to a lesser extent of *S. clavuligerus* (117 bp) are significantly longer than those of other streptomycetes (around 100 bp). Additionally, the longer promoter regions contain an unusually long A/T-rich stretch (17/26 nt in *S. clavuligerus*, 21/38 nt in *S. granaticolor* and 20/38 nt in *S. venezuelae*). A/T-rich DNA sequences have been implemented in modulation of the efficiency of transcription initiation and promoter clearance (Tang *et al.*, 2005). Alternatively, in the G/C-rich genomes of streptomycetes such sequences can provide sequence specificity for a DNA-binding transcription factor. The region around the stop codon of *ssgR* – which lies at the centre of a DNA fragment bound by SsgR *in vitro* and involved in the *trans*-activation of *ssgA* - is highly conserved in all species. In *S. coelicolor* and *S. griseus* transcription of *ssgA* is directed from two start sites (Traag *et al.*, 2004; Yamazaki *et al.*, 2003), one of which is essentially the same in both species (p1 in *S. coelicolor* (^{Sc}) and p2 in *S. griseus* (^{Sg}), indicated as "B" in Figure 4), while the second transcripts detected in both species (indicated as "A" and "C" in Figure 4) originated from markedly different sequences and positions relative to the *ssgA* translational start site. The common promoter (corresponding to B) is in fact highly conserved in all 18 promoter sequences (100% conservation of the -35 sequence and 4/6 nt of the -10 sequence), while the second *S. coelicolor* promoter sequence (corresponding to C) is well conserved in half of the aligned sequences (from *S. coelicolor* to *S. scabies* in Figure 4), but shares little similarity with sequences upstream of *ssgA* orthologues of species in the bottom half of the figure (from *S. venezuelae* to *S. griseus*). The second *S. griseus* promoter sequence (corresponding to A) is located in the highly conserved region around the translational stop codon of *ssgR*, and is therefore highly conserved (Figure 4). However, remarkably, at least under the conditions tested no transcripts were initiated from this sequence in *S. coelicolor* (Traag *et al.*, 2004).

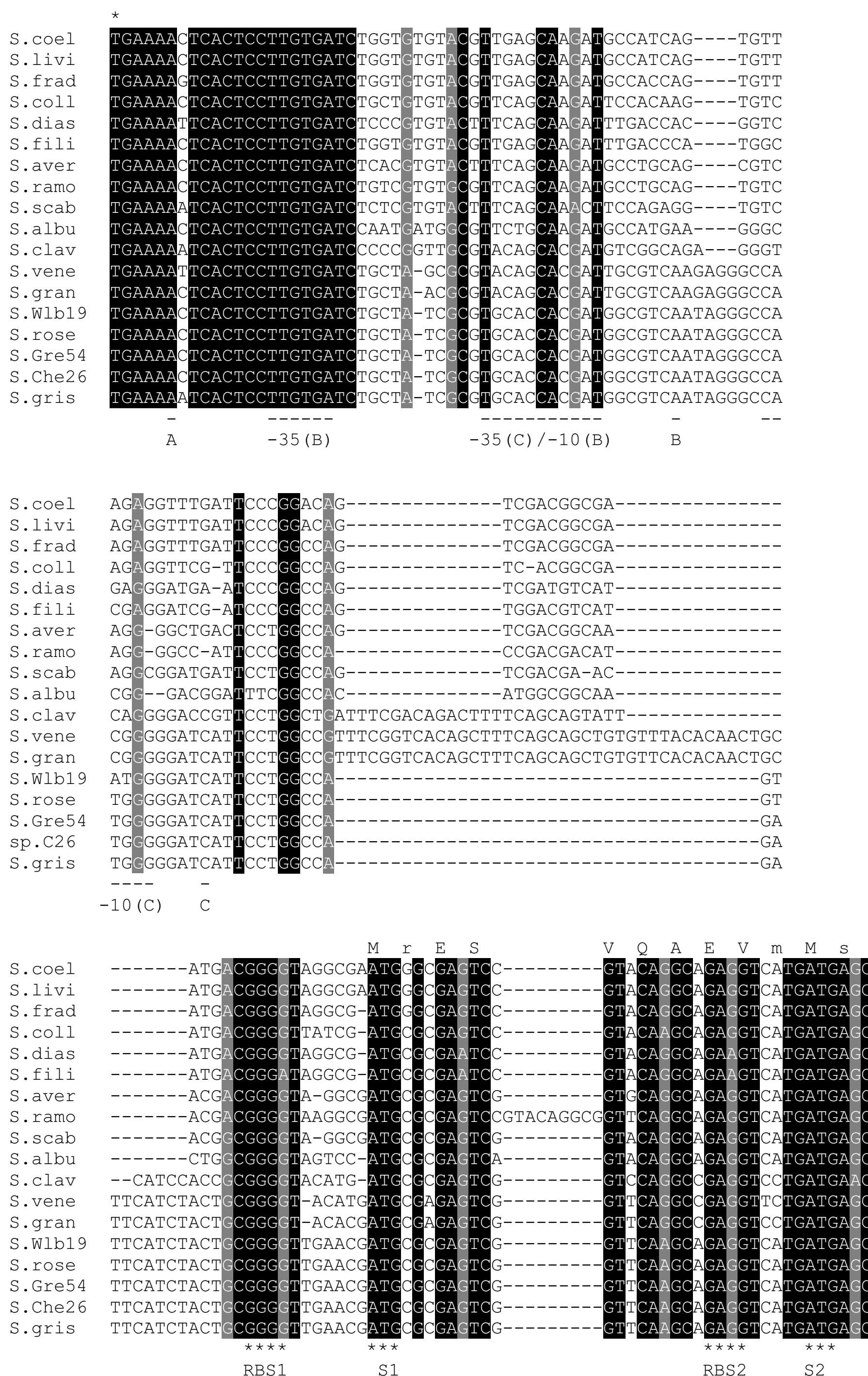


Figure 4. Alignment of *ssgA* promoter regions. Only completely conserved nucleotides are shaded; nucleotides shaded in light grey refer to conserved purines. The two alternative start codons (S1 and S2) for *ssgA* and their respective ribosome binding sites (RBS1 and RBS2) are indicated below the aligned sequence. The two transcriptional start sites and their respective -35 and -10 recognition sequences from *S. griseus* (Yamazaki et al. 2003) and *S. coelicolor* (Traag et al. 2004) are underlined, where "A" refers to p1 from *S. griseus*, "B" to p1 from *S. coelicolor* or p2 from *S. griseus*, and "C" to p2 from *S. coelicolor*. The stop codon for *ssgR* is indicated with an asterisk. The consensus amino acid sequence of the N-terminus of SsgA proteins is given above the aligned DNA sequences, where residues conserved in all species are in capital letters. For sequence labels see "Materials and Methods".

Alternative translational start sites for SsgA?

Alignment of the promoter regions of the 18 *ssgA* orthologues identified three alternative translational start sites, two of which are conserved in all species, including the putative RBS sequences upstream of them (Figure 4). These are therefore considered as possible alternative translational start sites. In all streptomycetes but *S. ramocissimus*, which contains duplication of the codons for VQA, the two possible start sites are separated by precisely 30 nucleotides, or ten possible codons. Western analysis was used to analyse which of the start codons was preferred in *S. griseus in vivo*. *S. coelicolor* GSA2 overproduces the longer version of SsgA (van Wezel *et al.*, 2000a), using the first start codon and resulting in a 145 aa protein (designated $SsgA_{145}$). Western analysis indeed revealed a protein band of the same length as $SsgA_{145}$ purified from *E. coli* (Figure 5A). In *S. griseus* B2682 and its hyper-sporulating mutant SY1 (Kawamoto and Ensign, 1995a) a band was readily detected at the same position as the band in GSA2. As expected, cultures of GSA2 and SY1 (lane 2 and 4) produced more intense $SsgA_{145}$ bands than the wild-type strain (lane 3). Hence, the first out of three possible ATG start codons is preferred in *S. griseus* over the third ATG codon.

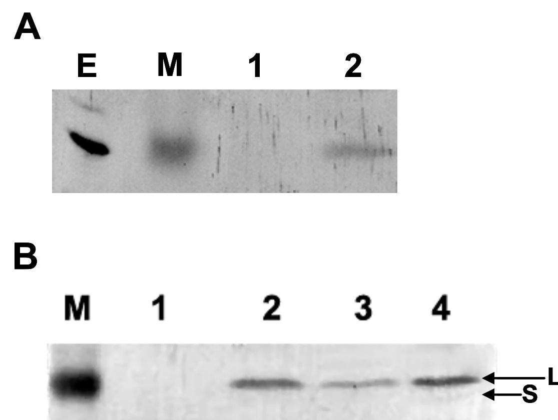


Figure 5. Western blot analysis of *S. griseus* SsgA. Western blots stained with antibodies raised against *S. griseus* SsgA. Lanes (M) pre-stained marker band (approx. 16 kda; Bio-Rad), (1) GSA3, (2) GSA2, (3) B2682, (4) SY1, (E) *E.coli purified* $SsgA_{145}$. **A.** SsgA detected in GSA2 (lane 2) runs at the same height as the SsgA protein of 145 amino acids ($SsgA_{145}$) expressed in and purified from *E. coli* (lane E). **B.** A band is readily detected at the same height in lanes 2,3 and 4 (arrow with "L"), indicating that $SsgA_{145}$ is produced abundantly. A second faint band is detected in extracts of SY1 (lane 4), which is perhaps $SsgA_{135}$ (arrow with "S").

Interestingly though, an additional fainter band was observed in SY1 below the band corresponding to $SsgA_{145}$. Perhaps this band is the result of modification or proteolysis of $SsgA_{145}$. Another possibility is that this band may reflect the production of $SsgA_{135}$, which may therefore be a functional protein with perhaps a specific function in *Streptomyces* morphogenesis (Figure 4 and 5B).

Relationship between morphology and the SsgA protein sequence

SsgA proteins from different streptomycetes generally share between 80 to 90% end-to-end sequence identity, with usually few differences occuring in the N-termini, and regions with higher variability in the core (approximately residue 63-102) and the C-termini (approximately beyond residue 120) of the proteins (Figure 6). SsgA from *S. clavuligerus* is the most distinct of all sequenced orthologues, with a sequence identity to other orthologues varying from 63% (compared to *S. ramocissimus* and *S. collinus* SsgA) to 73% (compared to *S. venezuelae* SsgA). SsgA proteins from the closely related species *S. coelicolor* and *S. lividans* are identical, while their genes contain a single nucleotide difference (His42 encoded by CAT in *S. coelicolor* and by CAC in *S. lividans*). More notably, the predicted SsgA orthologues from *S. griseus* and *S. roseosporus* are also identical, while 17 “silent” nucleotide differences occur between their respective DNA sequences, suggesting evolutionary pressure to maintain the protein sequences.

It was described previously that several species could produce spores in submerged cultures, including *S. granaticolor* (Stastna *et al.*, 1991), *S. griseus* (Kendrick and Ensign, 1983), *S. roseosporus* (Huber *et al.*, 1987) and *S. venezuelae* (Glazebrook *et al.*, 1990). In a recent survey of species in our own strain collection we discovered many others, suggesting that submerged sporulation is much more common than anticipated; this includes the novel *Streptomyces* sp. Wlb19, Che26, and Gre54 described here. Interestingly, in the phylogenetic tree SsgA proteins from strains that can produce submerged spores cluster together in a branch designated LSp (Liquid-culture Sporulation; Figure 7A). In a second branch, designated NLSp (No Liquid-culture Sporulation), only SsgA proteins are represented that were derived from strains that fail to sporulate in submerged culture and often produce mycelial clumps. *S. albus* and *S. clavuligerus* produce large, open mycelial structures and no submerged

spores. Phylogenetic analysis indicates that these species do not belong to either of the two branches, and several clear differences between their amino acid sequences and those from the other orthologues are apparent (Figure 6 and 7A).

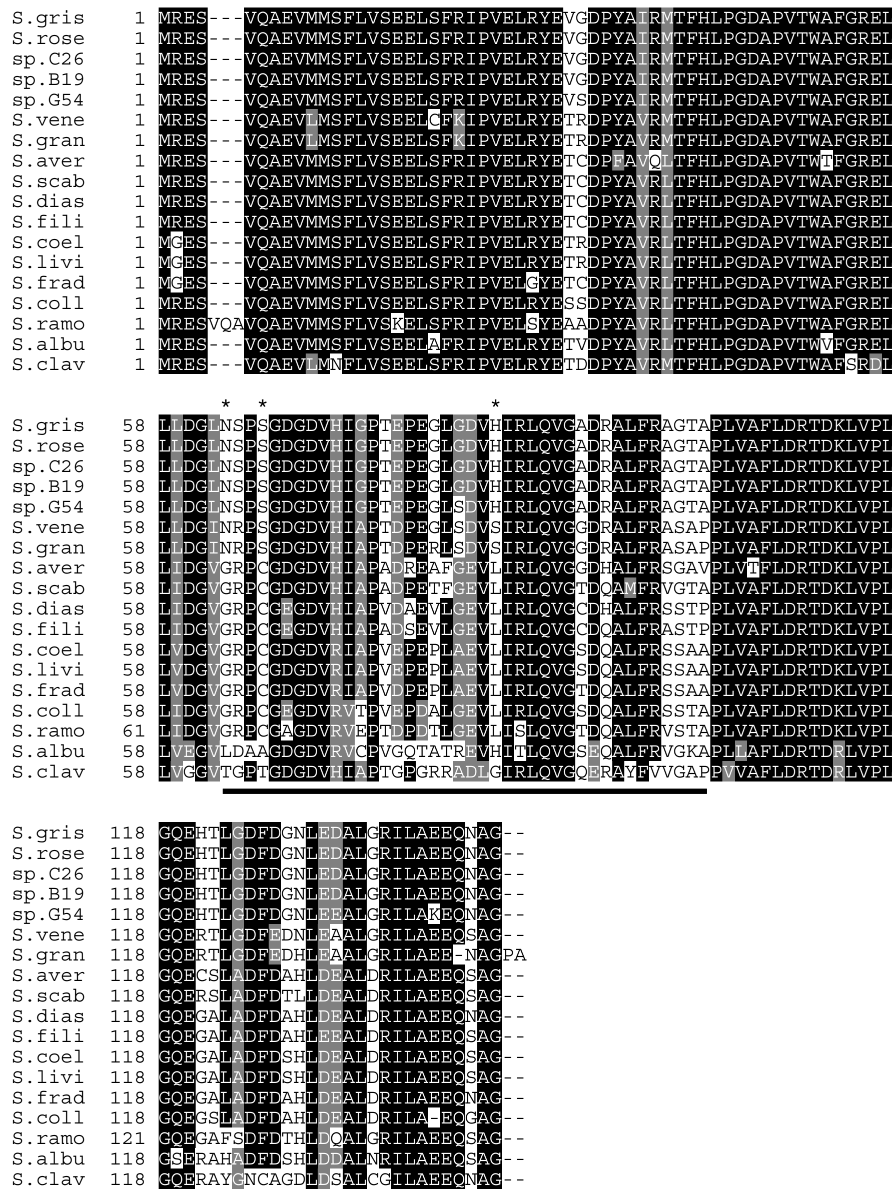


Figure 6. Alignment of SsgA orthologues. Residues conserved in at least 80% of the sequences are shaded; identical residues are shaded in black, residues with similar properties are shaded in light grey. Regions with higher variability (residues 63-102 and residues 121-stop) are underlined. Residues in position 63, 66 and 85, highlighted with an asterisk above the alignment, are conserved within- but different between the "LSp" and "NLSp" branches in Figure 7 (see also "Results" section). Sequences were labelled by their strain of origin, for see sequence labels see "Computer analysis" in "Materials and Methods".

To test if strains that sporulate in submerged cultures are evolutionary more strongly related to each other than to those that only sporulate on surface-grown cultures, we performed a phylogenetic comparison of the 16S rRNA sequences of all 18 species analysed here. In the 16S rRNA phylogenetic tree, similar branches as seen for SsgA proteins are far less apparent for the same strains (Figure 7B). These data suggest that changes in the SsgA amino acid sequence link to distinct morphological characteristics of streptomycetes, rather than highlight recent evolutionary divergence. There are several differences in the amino acid sequence of SsgA orthologues from the LSp or the NLSp branches.

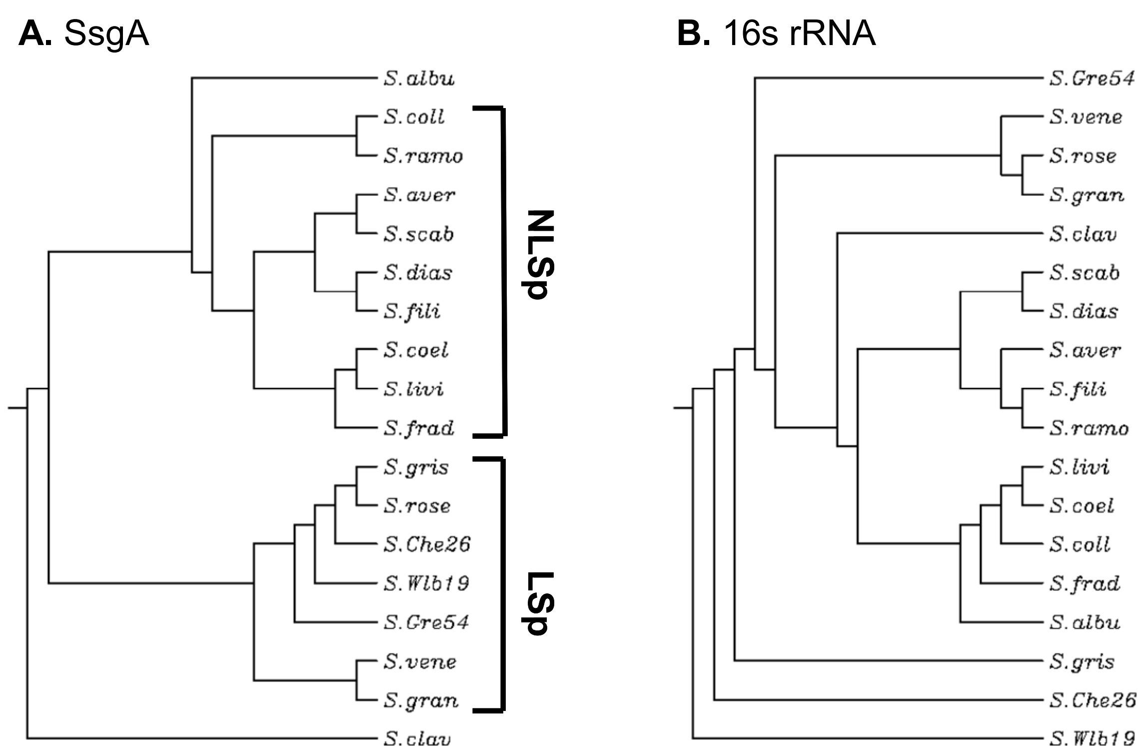


Figure 7. Phylogenetic tree of SsgA proteins and 16S rRNA sequences. Phylogenetic trees are shown for SsgA (**A**) and 16S rRNA (**B**) from 18 *Streptomyces* strains (see Results section). Two major branches of SsgA proteins are indicated, namely: SsgA orthologues from strains that produce spores in liquid-culture (LSp branch), SsgA orthologues from strains that produce dense mycelium clumps and no spores in liquid-culture (NLSp branch). SsgA and 16S rRNA were labelled by their strain of origin, for sequence labels see "Computer analysis" in "Materials and Methods"

Three residues are particularly interesting, namely those in position 63, 66 and 85 (66, 69 and 88 of *S. ramocissimus* SsgA). The relevant residues are conserved Gly63, Cys66 and Leu85 residues in orthologues from the NLSp branch, while orthologues from the LSp branch carry Asn, Ser and His/Ser residues in the corresponding positions (Figure 6).

DISCUSSION

Control of *ssg* gene expression during development

In the presence of sufficient nutrients, the soil-bound streptomycetes grow by tip extension and branching, producing an intricate network of vegetative hyphae to optimally profit from the available nutrients (Flärdh and van Wezel, 2003). When the circumstances in the habitat become less favourable, *e.g.* when cells become deprived of sufficient nutrients, the production of stress-resistant spores is essential for survival and dissemination. The decision to enter development is a critical and irreversible one, and is therefore tightly controlled in bacteria (Chater and Losick, 1997). The formation of aerial hyphae and spores by filamentous microorganisms (such as streptomycetes) is an energy-consuming process, producing a new mycelium at the expense of an existing substrate mycelium. Many genes are involved in the control of this major developmental check point, including the *bld* genes (Nodwell *et al.*, 1999), and genes involved in nutrient sensing and transport (*e.g. dasRABC* and *pts*; (Rigali *et al.*, 2006)).

Sporulation is negatively affected by glucose and other type I carbon sources (Kwakman and Postma, 1994). Conversely, streptomycetes, sporulate profusely on mannitol-containing media, and this includes many *bld* mutants, which produce spores or at least aerial hyphae under these conditions (Merrick, 1976; Pope *et al.*, 1996). In this work we demonstrate that the six sporulation-specific SALPs of *S. coelicolor* (SsgABCEFG) are all subjected to carbon catabolite repression (CCR), linking nutrient availability to the later stages of development (*i.e.* sporulation). In contrast, *ssgD* was transcribed already during the earliest stages of growth, in agreement with earlier S1-nuclease protection assays (Traag *et al.*, 2004). In fact, SsgD is the only SALP that is actively expressed at different stages of the life cycle and - suggestively - the only SALP that is transcribed

independently of CCR. Mutation of *ssgD* pleiotropically affected integrity of the cell wall in aerial hyphae and spores, with many spores lacking the typical thick peptidoglycan layer. However, no clear defects were observed in vegetative hyphae (Noens *et al.*, 2005) and its role at this stage of the life cycle remains a mystery. In a *glkA* mutant background, all *ssg* genes were expressed in a CCR-independent manner, indicating that the transcriptional repression by glucose was indeed due to CCR. This provides novel insight into how nutrient availability controls the later steps in the developmental program.

Similar to *ssgRA* (Traag *et al.*, 2004), transcription of *ssgD* was not significantly affected in the six early *whi* mutants (*i.e. whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ*) or in an *ssgB* mutant, while *ssgG* transcription was only affected significantly by the absence of *ssgB*. Several differences were observed for transcription of other *ssg* genes. Transcript levels of *ssgE* and *ssgF* were reduced during late aerial growth in a *whiH* mutant, and *ssgF* in a *whiA* mutant. Since these mutants completely lack sporulation, this is in line with the proposed function of these SALPs during the autolytic separation of spores (Noens *et al.*, 2005). In contrast, transcription of *ssgC*, *ssgE* and *ssgF* was upregulated in *whiJ* and in *ssgB* mutants. It is known that *ssgA* mutants are blocked at a time coinciding with the onset of sporulation and are capable of producing some spores on particular media (Jiang and Kendrick, 2000b; van Wezel *et al.*, 2000a), while transcriptome analysis of the *ssgA* mutant revealed significant up-regulation of many known sporulation genes, most likely in an attempt to compensate for the lack of *ssgA* (Noens *et al.*, 2007). However, mutants of *ssgB* and *whiJ* are arrested during early aerial growth and prior to sporulation-specific cell division and we have as yet no explanation why *ssgEF* should be enhanced in these genetic backgrounds.

The most striking observation is that *ssgB* transcripts are strongly down-regulated in *whiA* mutants (more than three-fold), while transcription is abolished in *whiH* mutants. These results are in contrast with previous work by Kormanec and Sevcikova, who reported that *ssgB* was transcribed normally in a *whiH* mutant (Kormanec and Sevcikova, 2002b). In support of the data presented here, microarray analysis of *whiA* and *whiH* mutants also revealed strong down-regulation of *ssgB* (Klas Flärdh, pers. comm.). Mutants of *whiA*, *whiH* and *ssgB* all have white (non-sporulating) phenotypes, producing aseptate

aerial hyphae and no spores (Flärdh *et al.*, 1999; Keijser *et al.*, 2003; Ryding *et al.*, 1999). Interestingly, *whiA* and *ssgB* mutants both appear to lack the signal for aerial growth arrest that precedes the onset of sporulation (Chater, 2001), with *whiA* mutants producing very long aerial hyphae, while colonies of the *ssgB* mutant have a large colony phenotype (Flärdh *et al.*, 1999; Keijser *et al.*, 2003). FtsZ-GFP localization studies revealed occasional Z-ring formation in aerial hyphae of several *whi* mutants (*e.g. whiG*) at a frequency similar to vegetative septum formation (Grantcharova *et al.*, 2005). However, in *whiH* mutants Z-ring formation occurred at a higher frequency than in other *whi* mutants (Grantcharova *et al.*, 2005). Under some conditions enhanced Z-ring formation was also observed in the *ssgB* mutant (Elke Noens and Gilles van Wezel, unpublished data). Therefore, the sporulation-deficient phenotypes of *whiA* and *whiH* may at least in part be explained by the lack of *ssgB* expression*.* The precise relationship between the genes requires further investigation.

Regulation of *ssgA* and submerged sporulation

Comparison of the *ssgA* promoter sequences from 18 distinct streptomycetes highlighted several conserved features. An A/T rich sequence of 33 nucleotides surrounds the stop codon of *ssgR*, which lies at the heart of a fragment shown to be bound by SsgR *in vitro* (Traag *et al.*, 2004), and is nearly completely conserved in *S. avermitilis*, *S. coelicolor*, *S, griseus* and *S. scabies* (and most likely in the other 14 species described in this work - our unpublished data). Another conserved feature is the common *ssgA* promoter which is shared by *S. coelicolor* and *S. griseus* ($p1^{sc}$ and $p2^{gr}$ (Traag *et al.*, 2004; Yamazaki *et al.*, 2003), indicated as "B" in Figure 3). The putative -35 recognition sequence of this promoter is located in the highly conserved region downstream of the *ssgR* stop codon and is fully conserved (5'-TTGTGA). Separated by an ideal spacer of 17-18 nucleotides (Russel and Bennett, 1982), the -10 consensus sequence is also significantly conserved, with as consensus sequence 5'-CA(A/C)PuAT. All streptomycetes likely share at least this common *ssgA* promoter ("B" in Figure 3). Transcription from this common promoter in *S. coelicolor* depends on SsgR, while it is strongly dependent on SsfR in *S. griseus* (Traag *et al.*, 2004; Yamazaki *et al.*, 2003). On the basis of the observed sequence homologies, we anticipate that this promoter may be controlled by SsgR orthologues in perhaps all

Streptomyces species. The A/T-rich extended sequences upstream of *ssgA* in *S. granaticolor*, *S. venezuelae* and *S. clavuligerus* are perhaps involved in additional transcriptional control. Interestingly, in liquid culture *S. granaticolor* and *S. venezuelae* grow highly fragmented and produce spores (Glazebrook *et al.*, 1990; Stastna *et al.*, 1977; Stastna *et al.*, 1991), characteristics of enhanced SsgA expression. Perhaps these sequences facilitate transcriptional stimulation of *ssgA*.

It remains unclear how SsgA is upregulated in rich liquid cultures of the hyper-sporulating *S. griseus* strain SY1. We did not find any mutations in the genes known to be involved in the regulation of *ssgA*, namely *ssfR*, *adpA, afsA* and *arpA*). In *S. griseus* B2682, transcription of *ssgA* was five-fold upregulated after nutritional shift-down, a condition known to promote submerged sporulation. In contrast, *adpA* and *ssfR* did not respond significantly to the change in nutritional conditions, and were transcribed at similar levels before and after nutritional shift-down. Thus, transcription of *ssgA* is suppressed in rich cultures, while nutritional depletion triggers *ssgA* transcription, presumably in an AdpA-dependent manner. Surprisingly, transcript levels of *ssgA* in cultures of SY1 were no longer induced by nutritional shift-down. Conceivably, since SY1 sporulates in rich liquid medium, the need to induce *ssgA* is eliminated.

Phylogenetic evidence strongly suggests that a particular SsgA protein sequence is linked to the ability of streptomycetes to produce spores in liquid culture. Interestingly, while overexpression of *S. griseus* SsgA in *S. coelicolor* M145 strongly stimulates cell division and fragmentation of liquid-grown mycelium, and even the formation of spore-like bodies (van Wezel *et al.*, 2000a), overexpression of *S. coelicolor ssgA* has a far less dramatic effect, with some fragmented growth but no sporulation (Gilles van Wezel unpublished data). These observations are in line with the position of these SsgA orthologues in the different branches of the phylogenetic tree, and suggest that not only the expression level of SsgA but certainly also specific amino acids of the gene products are a major determinant of liquid culture morphology. The exciting possibility of a direct relationship between specific amino acid residues of SsgA and submerged sporulation is currently under investigation and may offer new insights into the function of SsgA in the control of *Streptomyces* development.

Acknowledgments

We are grateful to Elke Noens for sharing unpublished data and the gift of total RNA extracts from the *ssgB* mutant GSB1.