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Function and control of the ssg genes in streptomyces

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Citation

Traag, B. A. (2008, September 24). *Function and control of the ssg genes in streptomyces*. Retrieved from <https://hdl.handle.net/1887/13114>

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Function and control of the *ssg* genes in *Streptomyces*

PROEFSCHRIFT

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. P. F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op 24 september 2008

klokke 13.45 uur

door

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Geboren te Den Haag,

11 januari 1980

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Chapter I

Introduction

Streptomycetes are Gram-positive soil-dwelling bacteria, in appearance similar to filamentous fungi. In fact, *Streptomyces* owes its name to this apparent likeness, when in 1943 Waksman and Henrici combined the original names given to the first actinomycetes (Waksman and Henrici, 1943), "Actinomyces" (Greek for "ray fungus") and "Streptothrix" (Greek for "twisted hair"). *Streptomyces* genomes are larger than those of most other bacteria; with around 7950 predicted genes (7847 encoding proteins, 18 rRNAs, 65 tRNAs and several small RNAs), the genome of *Streptomyces coelicolor* encodes almost twice as many genes as well-known bacteria such as *Escherichia coli*, *Bacillus subtilis* and *Mycobacterium tuberculosis* (all have approximately 4000 predicted genes), and carries even more genes than the lower eukaryote *Saccharomyces cerevisiae* (6203 predicted genes) (Bentley *et al.*, 2002). This large genome size reflects the complex morphogenesis and saprophytic nature of streptomycetes, which is highlighted by the presence of no less than 65 sigma factors, a wealth of polysaccharide hydrolases and over 200 ABC transporters; this allows the organism to rapidly respond to environmental changes and utilise many complex carbon sources (e.g. chitin, cellulose, lignin, mannan, xylan and agar).

During its life cycle, *Streptomyces* undergoes two apparently different events of cell division. Initially, the cell division machinery produces irregularly spaced septa ("crosswalls") in the vegetative hyphae that delimit the multi-nucleoid hyphal cells. During the reproductive phase, up to a hundred septa are simultaneously formed in the sporogenic aerial hyphae, eventually resulting in the production of mono-nucleoid spores (Chater, 2001; Flärdh and van Wezel, 2003). In contrast to septation in vegetative hyphae, developmental cell division results in physical separation of the spores. Instead of the canonical cell division control proteins, most of which were identified during studies on *E. coli* and *B. subtilis*, several unique protein families have been identified that play a role in the control of cell division in streptomycetes (Chater and Chandra, 2006). This thesis deals with one such family, the SsgA-like proteins (SALPs). Since its discovery during the mid-90s, SsgA (for sporulation of *Streptomyces griseus*) has been shown to play an important role in the activation of sporulation-specific cell division in *Streptomyces* on solid surface and in liquid medium. We now know streptomycetes generally have six to eight SALP homologues, all of which play an important and unique role in the control of sporulation (Noens *et al.*, 2005). In

Chapter II, 10 years of research on the SsgA-like proteins in actinomycetes is reviewed.

This work started at a time when further insight into the regulation of *ssgA* was necessary to better understand its role during development and to place this important morphogen in the life cycle of *Streptomyces*. The transcriptional regulation of *ssgA* in the model organism *Streptomyces coelicolor* was extensively studied in different genetic backgrounds, results of which are presented in Chapter III. These indicated that transcription of *ssgA* directly depends on the DNA-binding regulator SsgR, but is independent of other essential sporulation genes known at that time. Major differences were observed compared to the regulation of the *ssgA* orthologue in *S. griseus* (Yamazaki *et al.*, 2003), which could be correlated to the ability of *S. griseus* to produce spores in submerged culture, a property that *S. coelicolor* lacks. In Chapter IV, the survey of transcriptional regulation is expanded so as to include the analysis of the six *S. coelicolor* *ssgA*-like genes (*ssgB-G*). Results indicate that all but *ssgD* are developmentally controlled and repressed by glucose. During the course of development, *ssgB* transcript levels were found to be strongly dependent on two sporulation genes (*i.e.* *whiA* and *whiH*). We furthermore obtained and compared *ssgA* sequences from a large number of streptomycetes. In addition to the role of transcriptional regulation of *ssgA*, phylogenetic evidence suggested that there is a relationship between particular amino acid residues and the ability of some strains to produce spores in submerged culture. In order to obtain more insight into the role of individual amino acid residues in its function, SsgA was subjected to an extensive mutational study by setting up a novel method to create, maintain and screen a library of random mutants (see Chapter V).

At the start of this work, SALPs had only been identified in streptomycetes, but with the publication of a great number of full genome sequences of various actinomycetes in recent years, genes encoding SALPs have been found in a number of morphologically diverse actinomycetes. In Chapter VI, genetic evidence is presented that strongly suggests that SsgB is the archetype of the SALP family. Results indicate that SsgB plays an important role in septum-site localization during developmental cell division and that this function is conserved even in distantly-related actinomycetes.

Finally, the results presented in this thesis are summarised in Chapter VII, and the implications with respect to actinomycete morphogenesis are discussed.

Chapter II

The SsgA-like proteins in actinomycetes: small proteins up to a big task

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adapted from *Antonie van Leeuwenhoek* (2008) 94: 85-97

ABSTRACT

Several unique protein families have been identified that play a role in the control of developmental cell division in streptomycetes. The SsgA-like proteins or SALPs, of which streptomycetes typically have at least five paralogues, control specific steps of sporulation-specific cell division in streptomycetes, affecting cell wall-related events such as septum localization and synthesis, thickening of the spore wall and autolytic spore separation. The expression level of SsgA, the best studied SALP, has a rather dramatic effect on septation and on hyphal morphology, which is not only of relevance for our understanding of (developmental) cell division but has also been successfully applied in industrial fermentation, to improve growth and production of filamentous actinomycetes. Recent observations suggest that SsgB most likely is the archetypal SALP, with only SsgB orthologues occurring in all morphologically complex actinomycetes. Here we review 10 years of research on the SsgA-like proteins in actinomycetes and discuss the most interesting phylogenetic, regulatory, functional and applied aspects of this relatively unknown protein family.

INTRODUCTION

Actinomycetes have an unusually complex life cycle, many aspects of which are globally similar to those observed in some lower eukaryotes, which makes them particularly interesting for the study of bacterial development and evolution (Chater and Losick, 1997). One of the best characterized genera among the actinomycetes is *Streptomyces*. As producers of over half of the known antibiotics, the Gram-positive soil-dwelling filamentous streptomyces are a paradigm of secondary metabolite-producing microorganisms (Chater and Losick, 1997; Hopwood, 1999), with *Streptomyces coelicolor* A3(2) as the most-studied streptomycete (Hopwood, 1999). Development of streptomyces is initiated by the germination of a spore, from which typically two hyphae are produced, which continue to grow and branch to form a vegetative mycelium. Exponential growth is achieved by apical (tip) growth and branching (Flärdh, 2003), with a complex mycelial network as the result. The vegetative hyphae consist of syncytial cells separated by occasional cross-walls, laid down at 5-10 μm intervals (Wildermuth and Hopwood, 1970). When development is initiated, an aerial mycelium is produced, with hydrophobic hyphae breaking through the moist surface, erected into the air. This is the start of the reproductive phase, initiated in response to nutrient depletion and the resulting requirement of mobilization. Eventually, sporulation-programmed hyphae are formed in a process requiring a complex, spatial and temporal genetic programming scheme that is switched on upon nutrient limitation (Chater, 1998). During sporulation, long chains of unigenomic spores are formed from multigenomic aerial hyphal compartments.

Multiple cell division during sporulation of streptomyces requires an unparalleled complex coordination of septum-site localization, cell division and DNA segregation (Flärdh *et al.*, 2000; McCormick *et al.*, 1994; Schwedock *et al.*, 1997; Wildermuth and Hopwood, 1970). Penicillin-binding proteins (PBPs) are key enzymes for the synthesis of the bacterial peptidoglycan, both during growth and during cytokinesis (Errington *et al.*, 2003; Holtje, 1998; Stewart, 2005). The best-studied PBPs are PBP2, which is required specifically for lateral cell-wall synthesis in *E. coli* (Den Blaauwen *et al.*, 2003), and FtsI (PBP3), which is part of the divisome and essential for synthesis of the septal peptidoglycan (Botta and Park, 1981). Streptomyces contain many PBPs and a number of these is

developmentally controlled, suggesting a role specifically during sporulation (Hao and Kendrick, 1998; Noens *et al.*, 2005). During maturation, spores are separated in a process that most likely resembles the separation of mother and daughter cells during cell division of unicellular bacteria, involving several autolytic enzymes such as amidases, lytic transglycosylases and endopeptidases (Heidrich *et al.*, 2002). While the cell division machinery of streptomycetes strongly resembles that of other bacteria (Flärdh and van Wezel, 2003), the control of septum formation is very different in these organisms. Streptomycetes lack the MinC, MinE and Sula proteins that control septum-site localization (Autret and Errington, 2001; Marston *et al.*, 1998), the nucleoid occlusion system (NOC) that coordinates septum synthesis and DNA segregation (Wu and Errington, 2004), as well as some crucial Z-ring anchoring proteins such as FtsA and ZipA (Errington *et al.*, 2003; Lowe *et al.*, 2004). How cell division is controlled in streptomycetes is unclear. In sporulation-committed aerial hyphae, FtsZ organizes into spiral-shaped intermediates along the length of the aerial hyphal cell, eventually forming up to a hundred Z-rings per aerial hyphae (Grantcharova *et al.*, 2005). At this stage MreB localizes to the septa, suggesting this actin-like cytoskeletal protein may assist in cell division (Mazza *et al.*, 2006).

Instead of the canonical cell division control proteins, several unique protein families have been identified that play a role in the control of cell division in streptomycetes, and notably the CrgA-like proteins and the SsgA-like proteins (Chater and Chandra 2006; Flärdh and van Wezel 2003). CrgA-like proteins comprise a family of small integral membrane proteins, thought to play a role in the inhibition of FtsZ-ring formation during *Streptomyces* development (Del Sol *et al.* 2006). In this review, we will have a closer look at the SsgA-like proteins or SALPs, which play an important role in the control of sporulation-specific cell division in sporulating actinomycetes.

The cell division activator SsgA

In the mid-90s Kawamoto and Ensign identified a genomic DNA fragment of *Streptomyces griseus* that inhibited submerged sporulation of a hyper-sporulating *S. griseus* strain at multiple copies (Kawamoto and Ensign, 1995b). The same genomic fragment induced fragmented growth of the otherwise branching mycelial filaments and the responsible gene was designated *ssgA* (for

sporulation of *Streptomyces griseus*). A direct correlation between SsgA accumulation and the onset of sporulation in wild-type cells, and the failure of some developmental mutants of *S. griseus* to accumulate SsgA, further demonstrated the sporulation-related function of SsgA (Kawamoto *et al.*, 1997). SsgA is member of a family of small acidic 14-17 kDa proteins now known as the SsgA-like proteins or SALPs (see next section).

ssgA null mutants of both *S. coelicolor* and *S. griseus* produce an aerial mycelium but fail to sporulate except on mannitol-containing media, where some spores are produced after prolonged incubation; this makes *ssgA* a rather unique example of a conditional white (*whi*) mutant (Jiang and Kendrick, 2000b; van Wezel *et al.*, 2000a). Later studies showed that SsgA directly activates sporulation-specific cell division, and over-production results in a dramatic morphological change of the vegetative hyphae of *S. coelicolor*; the vegetative hyphae become approximately twice as wide (average of 800 nm instead of 400-500 nm) and form spore-like compartments separated by massive and aberrant septa, resulting in hyper-fragmenting hyphae in submerged culture that occasionally produce submerged spores (van Wezel *et al.*, 2000a; van Wezel *et al.*, 2000b). The large impact of SsgA on morphogenesis was underlined by microarray analysis, which showed that deletion of *ssgA* affects expression of an unprecedented large number of genes, with many hundreds of genes up- or downregulated by at least two-fold, including most developmental genes (*e.g. bld*, *whi* and *ssg* genes), as well as many genes involved in DNA segregation and topology (Noens *et al.*, 2007). The remarkable upregulation of many of the known *bld* and *whi* genes, which are essential for aerial mycelium and spore formation, respectively, is best explained as a stress response to try and compensate for the absence of an important morphogen (*i.c. ssgA*). The same is probably true for the strong upregulation of *ftsI* (septum synthesis) and of *divIVA* (apical growth), whose functions relate to and maybe assisted by SsgA. The genes that are by far the most strongly upregulated in *ssgA* mutants are the chaplin and rodlin genes. These genes encode hydrophobic proteins that form the water-repellent sheath of aerial hyphae that allows them to break through the soil surface (Claessen *et al.*, 2003; Claessen *et al.*, 2004; Elliot *et al.*, 2003). Further research is required to explain the correlation between these developmental coat proteins and SsgA.

The SsgA-like proteins exclusively occur in morphologically complex actinomycetes

SALPs typically are proteins of 120-150 amino acid (aa) residues with a sequence identity between 30 and 50%. Seven SALPs were found in the fully sequenced genomes of *S. coelicolor* (designated SsgA-G; in this review we will use the *S. coelicolor* nomenclature throughout, unless stated otherwise) and *S. scabies*, six in that of *S. avermitilis*, and five in the recently sequenced genome of *S. griseus*. Alignment of the 24 known SALP proteins of streptomycetes (presented in Traag *et al.*, 2007) showed that, despite the relatively low conservation, 11% (16 residues) of all amino acids are completely conserved. Surprisingly, this number does not change much when all 53 SALPs are taken into account, including the SALPs from sometimes quite distantly related actinomycetes. In that case 11 residues are still fully conserved, and if one of the SALPs from each of the *Frankia* species (FrS2 in Figure 1 and Table 1) is left out of the comparison, 15 residues are completely conserved among 50 SALP sequences. SALPs do not carry any known protein motif and to date there is not a single protein in the databases that has significant sequence similarity to a SALP. A random mutant library was created for *S. coelicolor ssgA*, from which approximately 800 *ssgA* variants were sequenced and screened for their ability to complement the *ssgA* null mutant. The essential residues for SsgA function were predominantly found in the first two thirds of the analysed part of the protein and most of these are highly conserved among all SALPs, while the penalty for mutations in the C-terminal domain was much lower. Three aa residues (L39, D68, and S99) are essential for SsgA function but are not significantly conserved in other SALPs and these may therefore have an SsgA-specific function (Traag *et al.*, 2007). During the writing of this thesis, the crystal structure of the single SALP from *Thermobifida fusca*, which is most likely an SsgB orthologue (based on results presented in Chapter VI of this thesis), was resolved by Ashley Deacon and Qingping Xu at the Joint Center for Structural Genomics (California, USA) and was made publicly available in the protein data base (PDB ID 3cm1). The structure revealed unexpected structural similarity to a class of a so-called "whirly" ssDNA/RNA-binding proteins (Desveaux *et al.*, 2002) and is highly similar to the mitochondrial RNA-binding protein MRP2 from the parasitic protozoan *Trypanosoma brucei* (Schumacher *et al.*, 2006), which is involved in

kinetoplastid RNA editing (Simpson *et al.*, 2003). In contrast to other “whirly” ssDNA/RNA binding proteins which function as dimers or tetramers, SALPs likely function as trimers. Combining the results obtained from the mutant library of SsgA with this three-dimensional structural data will most likely provide very useful information on the role of the individual residues in SsgA (and SALP) function.

Five of the SALPs, namely SsgA, SsgB, SsgD, SsgE and SsgG, have orthologues in all streptomycetes analysed (Noens *et al.*, 2005 and our unpublished hybridization data), with the exception of SsgG, absent from the *S. avermitilis* genome. Additionally, a few species-specific SALPs are typically found in all streptomycetes. SALPs can be divided into phylogenetic subfamilies, namely the SsgA branch, the SsgBG branch, the SsgDE branch and the species-specific SALPs, which include SsgC and SsgF (Figure 1). Unexpectedly, the genome of *S. griseus* contains an additional three much larger proteins with an approximately 120 aa C-terminal SsgA-like domain, which contains the typical sequence identity to other SALPs of approximately 30-50%. Two of these (SGR7098t and SGR41t) are identical proteins of 654 aa, while the third (SGR128) is a 651 aa protein with an end-to end sequence identity of 67% to the other two (approximately 84% in the 120 aa SsgA-like domain). Apart from the SsgA-like domain the remainder of these proteins have no significant similarity to other proteins. So far, SALPs have exclusively been found in actinomycetes. The genomes of *Thermobifida fusca*, *Kineococcus radiotolerans*, *Nocardioides*, *Acidothermus cellulolyticus*, *Salinispora tropica* and *Salinispora arenicola*, all of which are either not known to form spores or produce single spores borne on the vegetative mycelium, contain a single *ssg* gene, which is most likely functionally related to *ssgB* (see below). Similar to streptomycetes, the multispore-forming actinomycetes have multiple homologues, namely two in *Saccharopolyspora erythraea*, three in *Frankia sp. CcI3* and *Frankia sp. EAN1pec* and five in *Frankia alni* (Table 2). Remarkably, there is a clear correlation between the complexity of the morphology of actinomycetes and the number of SALPs found in actinomycetes. No SALPs were identified in *Mycobacterium*, *Rhodococcus* or *Corynebacterium* species.

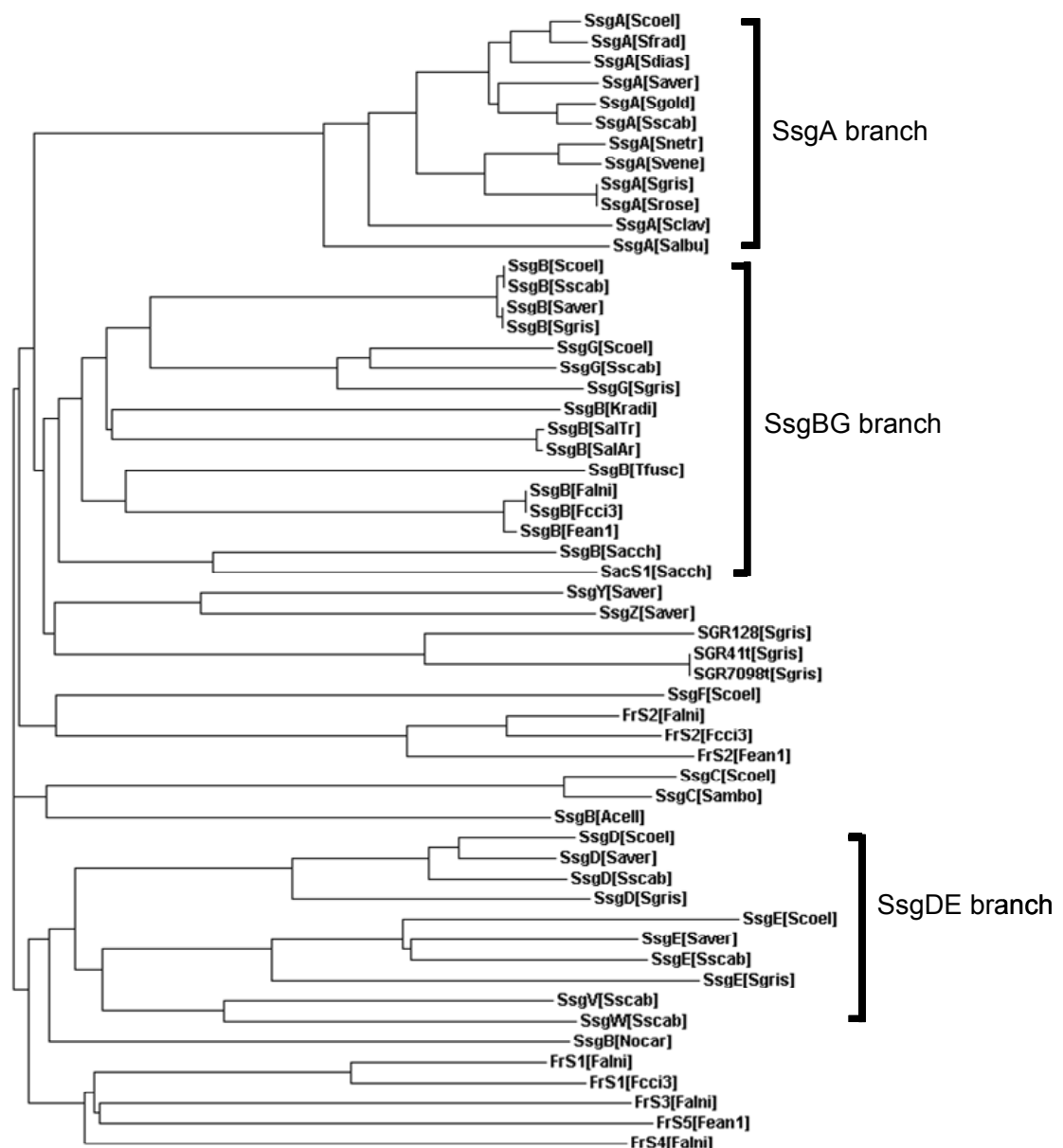


Figure 1. Phylogenetic tree of the SALP proteins listed in Table 1. Phylogenetic analysis of all SsgA-like proteins (SALPs) from different actinomycetes was performed using the CLUSTALX program (Thompson et al., 1997). Accession numbers and other information on the proteins in the tree are listed in Table 2. The three subfamily branches (SsgA, SsgBG, and SsgDE) are indicated.

Table 1. SALP proteins that were subjected to phylogenetic analysis in Figure 1. Nomenclature is based on highest sequence homology to the *S. coelicolor* SALP homologues. Database tags refer to the locus name given by the respective genome sequencing projects. Contigs refer to short sequence files extracted from the *S. scabies* database (www.sanger.ac.uk/Projects/S_scabies).

Protein name	Organism of origin	Database tag /contig number
SsgA[Scoel]	<i>S. coelicolor</i>	SCO3926
SsgB[Scoel]	<i>S. coelicolor</i>	SCO1541
SsgC[Scoel]	<i>S. coelicolor</i>	SCO7289
SsgD[Scoel]	<i>S. coelicolor</i>	SCO6722
SsgE[Scoel]	<i>S. coelicolor</i>	SCO3158
SsgF[Scoel]	<i>S. coelicolor</i>	SCO7175
SsgG[Scoel]	<i>S. coelicolor</i>	SCO2924
SsgA[Saver]	<i>S. avermitilis</i>	SAV4267
SsgB[Saver]	<i>S. avermitilis</i>	SAV6810
SsgD[Saver]	<i>S. avermitilis</i>	SAV1687
SsgE[Saver]	<i>S. avermitilis</i>	SAV3605
SsgY[Saver]	<i>S. avermitilis</i>	SAV570
SsgZ[Saver]	<i>S. avermitilis</i>	SAV580
SsgA[Sscab]	<i>S. scabies</i>	contig: scab0274d04.q1k
SsgB[Sscab]	<i>S. scabies</i>	contig: scab0975f02.p1k
SsgD[Sscab]	<i>S. scabies</i>	contig: scab0372d01.q1k
SsgE[Sscab]	<i>S. scabies</i>	contig: scab0136f09.q1k
SsgG[Sscab]	<i>S. scabies</i>	contig: scab0313c04.q1k
SsgV[Sscab]	<i>S. scabies</i>	contig: scab0162c08.q1k
SsgW[Sscab]	<i>S. scabies</i>	contig: scab0039b09.q1k
SsgA[Sgris]	<i>S. griseus</i>	SGR3655
SsgB[Sgris]	<i>S. griseus</i>	SGR5997
SsgD[Sgris]	<i>S. griseus</i>	SGR1004
SsgE[Sgris]	<i>S. griseus</i>	SGR4320
SsgG[Sgris]	<i>S. griseus</i>	SGR4615
SGR128[Sgris]	<i>S. griseus</i>	SGR128
SGR41t[Sgris]	<i>S. griseus</i>	SGR41t
SGR7098t[Sgris]	<i>S. griseus</i>	SGR7098t
SsgA[Salbu]	<i>S. albus</i>	AF195771
SsgA[Sgold]	<i>S. goldeniense</i>	AF195773
SsgA[Snetr]	<i>S. netropsis</i>	AF195772
SsgB[Tfusc]	<i>Thermobifido fusca</i>	Tfu_2111
SsgB[Kradi]	<i>Kineococcus radiotolerans</i>	Krad_3069
SsgB[Nocar]	<i>Nocardioide</i>	Noca_2368
SsgB[Acell]	<i>Acidothermus cellulolyticus</i>	Acel_1369
SsgB[SalTr]	<i>Salinispora tropica</i>	Strop_1600
SsgB[SalAr]	<i>Salinispora arenicola</i>	Sare_1560
SsgB[Falni]	<i>Frankia alni</i> ACN14a	FRAAL2127
FrS1[Falni]	<i>Frankia alni</i> ACN14a	FRAAL5533
FrS2[Falni]	<i>Frankia alni</i> ACN14a	FRAAL4594
FrS3[Falni]	<i>Frankia alni</i> ACN14a	FRAAL5373
FrS4[Falni]	<i>Frankia alni</i> ACN14a	FRAAL6494
SsgB[Fcci3]	<i>Frankia</i> sp. CcI3	Franci3_1359
FrS1[Fcci3]	<i>Frankia</i> sp. CcI3	Franci3_3418
FrS2[Fcci3]	<i>Frankia</i> sp. CcI3	Franci3_1632
SsgB[Fea1]	<i>Frankia</i> sp. EAN1pec	Franea1_5158
FrS2[Fea1]	<i>Frankia</i> sp. EAN1pec	Franea1_2058
FrS5[Fea1]	<i>Frankia</i> sp. EAN1pec	Franea1_4060
SsgB[Sacch]	<i>Saccharopolyspora erythraea</i>	SACE_1961
SacS1[Sacch]	<i>Saccharopolyspora erythraea</i>	SACE_5535

Table 2. Distribution of SALPs across actinomycetes.

Strain	Genome accession number	Genome size (Mbp)	Number of SALPs	Cell morphology; development
<i>Streptomyces avermitilis</i>	BA000030	9	6	Filamentous growth, spore chains on aerial hyphae
<i>Streptomyces coelicolor</i>	AL645882	8.7	7	
<i>Streptomyces griseus</i>	(project ID: 20085)	8.5	5	
<i>Streptomyces scabies</i>	(project ID:12985)	10.1	7	
<i>Frankia alni</i>	CT573213	7.5	5	Filamentous growth, multilocular sporangia either terminally or intercalary
<i>Frankia sp. CcI3</i>	CP000249	5.4	3	
<i>Frankia sp. EAN1pec</i>	AAII00000000	9	3	
<i>Saccharopolyspora erythraea</i>	AM420293	8.2	2	Filamentous growth, short spore chains on fragmented aerial hyphae
<i>Acidothermus cellulolyticus</i>	CP000481	2.4	1	Slender floccules, non-spore forming
<i>Kineococcus radiotolerans</i>	AAEF00000000	4.9	1	Cocci with polar flagella or symmetrical multi-cell clusters, non-spore forming. Ageing colonies form an extracellular polymer shell around individual colonies.
<i>Nocardioides sp. JS614</i>	CP000509	4.9	1	Single rods/cocci, non-spore forming.
<i>Salinispora arenicola</i>	AAWA00000000	5.7	1	Filamentous growth, single spores borne on substrate mycelium.
<i>Salinispora tropica</i>	AATJ00000000	5.2	1	
<i>Thermobifida fusca</i>	CP000088	3.6	1	Filamentous growth, single spores borne on dichotomously branched sporophores.

SsgB is most likely the SALP archetype

Analysis of the genetic locus of the single SALP-encoding genes of *Thermobifida*, *Kineococcus*, *Nocardioides*, *Acidothermus* and *Salinispora* showed that all resembled the gene organization around *ssgB* in *Streptomyces*. In fact, all SALP-containing actinomycetes have one *ssg* gene with a similar genetic locus to *Streptomyces ssgB* (Bjørn Traag and Gilles van Wezel, unpublished data), which is preceded by a homologue of SCO1540 (the gene directly upstream of *S. coelicolor ssgB*) in most actinomycetes and in all genera a gene for tRNA^{val}

somewhat further upstream. A number of other genes are conserved in several genera, for example at least three additional tRNA genes are found in all except *Salinispora*, and a threonine-tRNA synthetase (SCO1531) is present in *Streptomyces*, *Acidothermus*, *Kineococcus*, *Nocardioides* and *Thermobifida*. Hence, gene synteny evidence strongly suggests that all SALP-encoding genes have been derived from spread and/or gene duplication of *ssgB* in actinomycetes and that perhaps this gene has a universally conserved function in actinomycete morphogenesis. In a phylogenetic tree nearly all of the putative SsgB orthologues group together in the above mentioned SsgBG branch (Figure 1), with the exception of the orthologues from the non-sporulating *Acidothermus* and *Nocardioides*. Whether these two SALP orthologues are still functional in these actinomycetes remains to be elucidated. More phylogenetic evidence for the importance of SsgB is provided by the fact that SsgB orthologues found in different species within a specific genus are almost completely conserved. This is true for the SsgBs from *Streptomyces*, from *Frankia* and from the salt-water actinomycete *Salinispora*. The orthologues from *Streptomyces* are identical except for aa position 150 (Gln or Thr), those from *Salinispora* differ only at aa position 137 (Asn or Ser), and in *Frankia* two orthologues are identical while the third has three aa changes (two conserved Ile/Val changes, and more importantly Ser or Ala at aa position 105). Furthermore, many nucleotide changes occur that do not lead to changes in the predicted proteins. This extraordinary conservation within genera perhaps reflects an inflexible co-evolution with an interaction partner.

ssgB mutants produce long aseptate aerial hyphae in seemingly 'immortal' white colonies (Keijser *et al.*, 2003), indicating a possible role for SsgB in the cessation of aerial growth prior to the onset of sporulation-specific cell division. Interestingly, many electron-dense granules were seen in hyphae of both *ssgB* mutants and PBP2 mutants, which perhaps reflect the accumulation of peptidoglycan subunits in both mutants (Noens *et al.*, 2005). Importantly, the *ssgB* genes from *Salinispora tropica* and *Saccharopolyspora erythraea* restored sporulation to the otherwise non-sporulating *ssgB* mutant of *S. coelicolor*, even though end-to-end sequence homology to *S. coelicolor ssgB* for both is only around 50% (Bjørn Traag and Gilles van Wezel, unpublished data). This strongly suggests that the SsgB proteins of *Salinispora* and *Saccharopolyspora* are indeed

functional orthologues of SsgB. However, defects in septum placement, DNA segregation and spore size indicate that control of septum-site localization depends on (the amino acid sequence of) SsgB. Indeed, localization of SsgB to both tips of growing aerial hyphae and to immature sporulation septa suggests the protein functions in both tip growth and cell division (E.E. Noens, J. Willemse and Gilles van Wezel, unpublished data).

Phylogenetically the closest relative of SsgB is SsgG (see Figure 1), and this SALP plays a role specifically in the control of septum-site localization. *ssgG* mutants have a light grey phenotype, resulting from the production of significantly fewer spores than wild-type *S. coelicolor*. Closer inspection revealed that sporulation septa are regularly 'skipped', resulting in many spores of exactly two, three or even four times the normal size. This showed that SsgG is required to ensure that the divisome is localised to all division sites (Noens *et al.*, 2005). Interestingly, despite the lack of cell division in the multiple-sized spores of the *ssgG* mutant the chromosomes were segregated normally, which clearly demonstrates that septum synthesis is not a prerequisite for DNA segregation in streptomycetes.

S. coelicolor ssgC-F null mutants produced an abundance of grey-pigmented spores after a few days of incubation, and *ssgC* mutants even hypersporulate. Microscopic analysis showed that aerial hyphae of the *ssgC* mutant produce very long ladders of septa, resulting in seemingly endless spore chains, while at the same time chromosome segregation in aerial hyphae was disturbed. In fact, *ssgC* mutants in many ways resemble strains over-producing SsgA and *vice versa*, and it was therefore proposed that SsgC may function as an antagonist of SsgA (Noens *et al.*, 2005). Orthologues of *ssgC* are so far only found in streptomycetes that have a low expression of *ssgA* under conditions of normal growth and as a result produce large clumps in liquid-grown cultures, namely in *S. coelicolor*, in *S. ambofaciens* and, as determined by hybridization studies, in *S. lividans* (Gilles van Wezel, unpublished data). Disruption of *ssgD* pleiotropically affected integrity of the cell wall in aerial hyphae and spores, with many spores lacking the typical thick peptidoglycan layer, which rather resembles the wall of aerial hyphae. Finally, correct autolytic spore separation depends on SsgE and SsgF. *ssgE* mutants produce predominantly single spores indicating an enhanced or accelerated autolytic activity, while mutation of *ssgF*

leads to incomplete detachment of spores, which remain attached by a thin peptidoglycan linkage, suggesting reduced or incorrect function of the secreted lytic transglycosylase SLT (Noens *et al.*, 2005).

Transcriptional regulation of the *ssg* genes in streptomycetes

Microarray analysis of *S. coelicolor* M145 and MT1110 and promoter-probe experiments in *S. coelicolor* indicated that all *ssg* genes except *ssgD* are developmentally regulated (Noens *et al.*, 2005; Noens *et al.*, 2007). In contrast to the other *ssg* genes, *ssgD* is transcribed at a much higher level and its transcription is life cycle-independent (Traag *et al.*, 2004), suggesting a role for SsgD at different stages of the life cycle. Furthermore, all *ssg* genes but *ssgD* are catabolically repressed by glucose. The transcription of *ssgA* has been studied extensively (see below). The only other *ssg* gene whose transcription has been studied in more detail is *ssgB*. Transcription of *ssgB* is directed from a single promoter 52 nt upstream of the most likely ATG start codon, resulting in a protein of 137 aa. Expression occurs in a life-cycle dependent manner and is strongly activated towards sporulation (Kormanec and Sevcikova, 2002a). In an *E. coli* two-plasmid system the *ssgB* promoter was found to be active in the presence of several members of the SigB-like sigma factor family (*i.e.* σ^B , σ^F and σ^H). SigB-like σ factors, of which the *S. coelicolor* genome encodes nine paralogues, are implicated in morphological differentiation and/or responses to different stresses (Kelemen *et al.*, 2001; Lee *et al.*, 2004). Expression of *ssgB* was unaffected in an *sigF* mutant of *S. coelicolor*, while in a *sigH* mutant no *ssgB* transcripts were detected. Furthermore, His-tagged σ^H initiated transcription from the *ssgB* promoter in *in vitro* run-off transcription assays (Kormanec and Sevcikova, 2002a). However, since a *sigH* mutant of *S. coelicolor* still produced some spores (Sevcikova *et al.*, 2001) and a *ssgB* mutant does not, *sigH* cannot be solely responsible for transcription of *ssgB*.

Transcriptional and translational control of *ssgA*

Transcription of *ssgA* is strongly induced towards sporulation in both *S. griseus* and *S. coelicolor* (Traag *et al.*, 2004; Yamazaki *et al.*, 2003). In both strains transcription is directed from two transcriptional start sites, one of which is essentially the same in both species (p1 in *S. coelicolor* and p2 in *S. griseus*;

indicated as "B" in Figure 2) while the other is different. Transcriptional analysis in six early *whi* mutants of *S. coelicolor* (*whiA*, *whiB*, *whiG*, *whiH*, *whiI* and *whiJ*) revealed that both *ssgA* and *ssgR* are transcribed independently from these crucial sporulation genes (Traag *et al.*, 2004). Directly upstream of *ssgA* lies a gene called *ssgR*, encoding an IclR-type DNA binding transcriptional regulator. In-frame deletion mutants of *ssgR* orthologues in *S. griseus* and *S. coelicolor* resulted in a sporulation deficient phenotype very similar to that of the *ssgA* mutants, and transcription of both *ssgA* and *ssgR* is strongly induced towards the onset of sporulation (Traag *et al.*, 2004; Yamazaki *et al.*, 2003), suggesting that *ssgR* regulated *ssgA* transcription. Indeed, transcriptional and DNA binding studies showed that *ssgA* is *trans*-activated by and completely dependent on SsgR in *S. coelicolor* (Traag *et al.*, 2004). Suggestively, the A/T-rich region around the stop codon of *ssgR* lies at the centre of a DNA fragment that was bound by SsgR *in vitro* and is strongly conserved among different streptomycetes, while the sequences surrounding it are far less well conserved (Figure 2). The conserved region around the stop codon is quite possibly the binding site for SsgR (Traag *et al.*, 2004). Comparison of the global expression profiles of the *ssgA* and *ssgR* mutants of *S. coelicolor* by microarray analysis revealed extraordinary similar expression profiles, providing further evidence that *ssgA* may well be the only target of SsgR (Noens *et al.*, 2007). In contrast, in *S. griseus* expression of *ssgA* is only slightly affected by SsfR (the SsgR ortholog in this species) and instead fully dependent on AdpA, a transcriptional activator in the A-factor regulatory cascade essential for differentiation and antibiotic production of *S. griseus* (Horinouchi and Beppu, 1994; Ohnishi *et al.*, 2005). AdpA binds to three adjacent sites upstream of *ssgA* (Yamazaki *et al.*, 2003). Conversely, in *adpA* (*bldH*) mutants of *S. coelicolor* transcription of *ssgA* was readily detected, though deregulated in earlier stages of the life cycle (Traag *et al.*, 2004). Different functions for the SsgR orthologues from both species are also suggested by the observation that *ssfR* from *S. griseus* failed to complement the *S. coelicolor* *ssgR* mutant (Traag *et al.*, 2004). The discrepancy in the transcriptional control of *ssgA* and the resulting enhanced expression in *S. griseus* is at least one of the main reasons for the major morphological differences between *S. coelicolor* (clumps) and *S. griseus* (submerged spores) in submerged cultures.

There has long been a controversy about the position of the *ssgA* translational start site. While initially *S. griseus ssgA* was considered to encode a 145 aa protein (Kawamoto and Ensign, 1995b), the presence of a more likely ribosome binding site (RBS) further downstream led to reassignment of the translational start to the third of three in-frame ATG codons, that lies 30 nucleotides (10 codons) downstream of the originally predicted start (Kawamoto *et al.*, 1997).

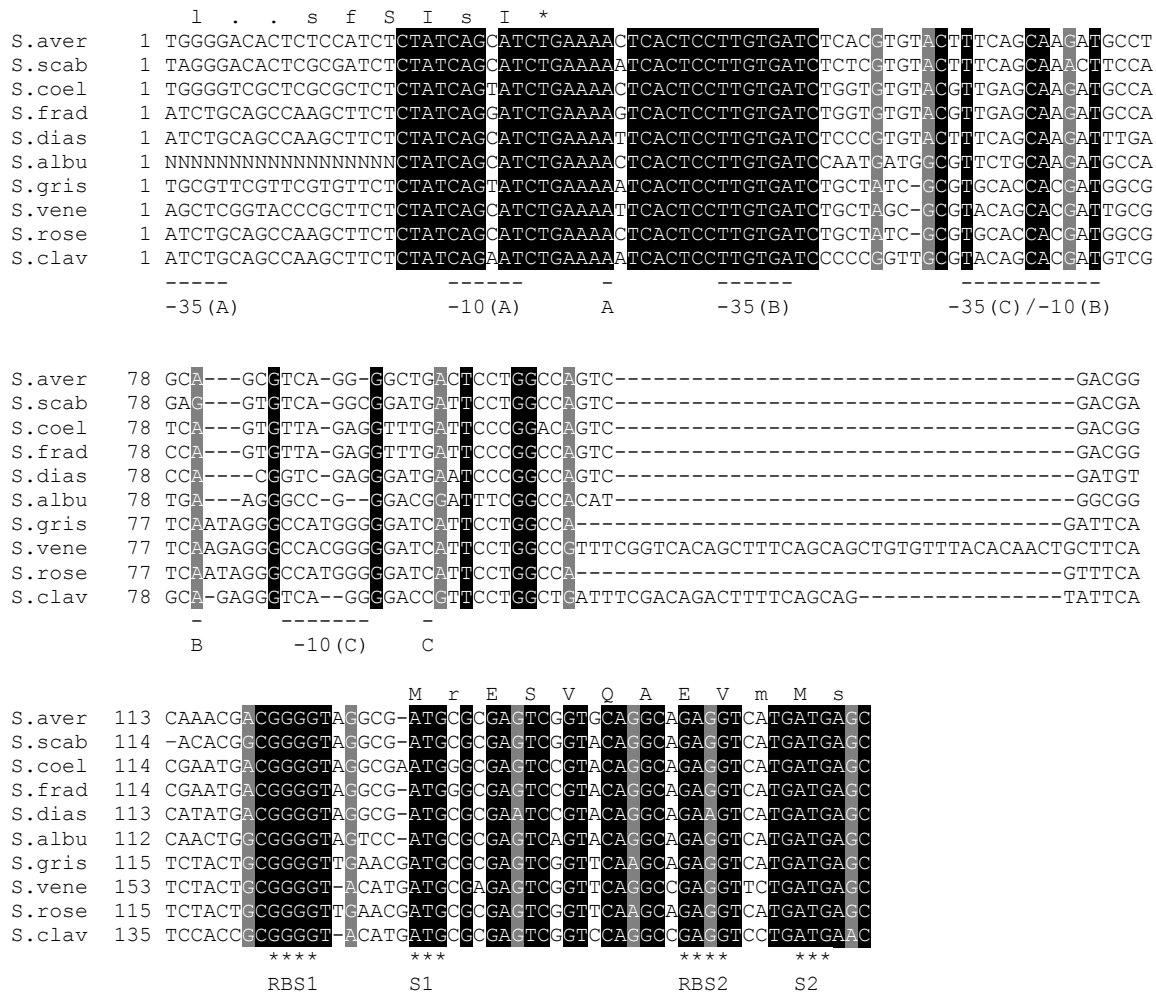


Figure 2. Alignment of *ssgA* promoter regions. Alignment was produced using the Boxshade program (www.ch.embnet.org/software/BOX_form.html). Only completely conserved nucleotides are shaded; nucleotides shaded in light grey refer to conserved purines. The two alternative start codons (S) for *ssgA* and their respective ribosome binding sites (RBS) 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 indicated by broken lines, 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*. Consensus amino acid sequences of the C-terminus of SsgR proteins and the N-terminus of SsgA proteins are given above the aligned DNA sequences. Residues conserved in all species are in capital letters and highly conserved residues in lower-case letters. The TGA stop codon for *ssgR* is indicated with an asterisk.

Alignment of ten *ssgA* ORFs and flanking regions from the sequenced genomes of *S. avermitilis*, *S. coelicolor*, *S. griseus*, and *S. scabies* and from sequenced *ssgA* clones in our laboratory (namely from *S. albus*, *S. clavuligerus*, *S. diastatochromogenes*, *S. fradiae*, *S. roseosporus*, and *S. venezuelae*), showed that two out of three ATG start codons and the putative RBS sequences preceding them are fully conserved (Figure 2), with precious few differences in the sequences between these possible start sites among the different *ssgA* orthologues. This suggests that perhaps both ATG codons could function as start codons. Indeed, we recently obtained evidence from Western blot analysis that both start codons may be used *in vivo*. Interestingly, only expression of the longer version resulted in soluble protein in *E. coli* (our unpublished data). This surprising new regulatory aspect of *ssgA* requires further analysis.

How do SALPs function?

Considering that all *ssg* mutants invariably had defects in peptidoglycan synthesis or autolytic peptidoglycan breakdown, a direct link between the fate of peptidoglycan and SALP functions was proposed (Noens *et al.*, 2005). Detailed microscopic analysis of the respective mutants as well as localization studies resulted in a model for the function of the various SALPs in the control of sporulation of *S. coelicolor* (Noens *et al.*, 2005 and Figure 3). As discussed above, SsgA activates sporulation-specific cell division with perhaps SsgC as antagonist, SsgB localises to the growing septa and is important for the cessation of aerial tip growth, *ssgD* mutants have a defective cell wall, SsgE and SsgF ensure the correct autolysis of the peptidoglycan between spores during maturation and SsgG ensures that all sporulation septa are formed at the same time. The SALP protein sequences do not contain any motifs to indicate that they themselves possess enzymatic activity. This suggests that SALPs may control the activity and/or localization of cell-wall enzymes such as PBPs and autolysins. Interestingly, analysis of a functional SsgA-GFP fusion revealed that SsgA localizes dynamically during development and invariably to sites where future restructuring of the peptidoglycan takes place (Noens *et al.*, 2007 and Figure 4).

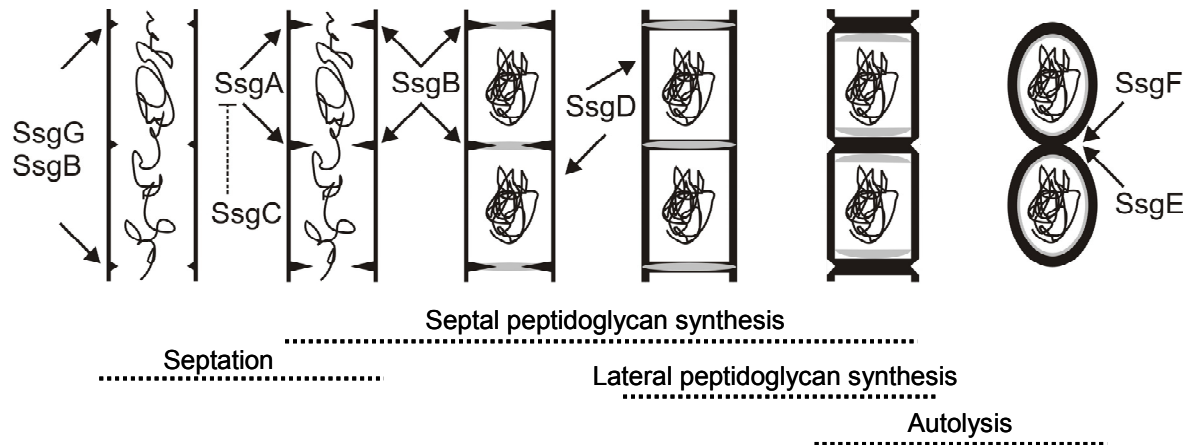


Figure 3. Model for the proposed functions of the SALPs in the control of morphogenesis of *S. coelicolor*. Arrows indicate the approximate time and place of action for the SALPs, namely septum-site localization (SsgABG), septum growth (SsgB), spore wall synthesis (SsgD) and autolytic spore separation (SsgEF). Considering its opposite effect on cell division, SsgC is proposed to act as an antagonist of SsgA. Adapted from Noens *et al* (2005).

SsgA-GFP localises to germination sites, to the tips of growing aerial hyphae, to septum sites and to branching sites, in line with the idea that SALPs functionally relate to the initiation of *de novo* peptidoglycan synthesis. In young aerial hyphae foci are found at regular intervals and relatively distant spacing, while intense foci are found at the growing tips. At the onset of sporulation these foci fade as new foci appear at alternating sides of the hyphae at positions corresponding to the septum sites. After completion of septum formation spores are produced with two foci, one at either 'pole'. These foci correspond to the future sites where germ tubes emerge; germinating wild-type spores on average produce two germ tubes, and these emerge precisely at the position where SsgA-GFP foci are found. The role of SsgA in the control of germination was further shown by the fact that strains overproducing SsgA regularly have six or seven germ tubes protruding from a single spore, while *ssgA* mutants produce fewer germ tubes. In strains expressing SsgA-GFP, signals were lost after several weeks of storage, but even after decades of storage in the freezer wild-type spores still germinate in a highly reproducible fashion, indicating that the germination sites are permanently labelled. This suggests that SsgA does not directly assist in germ tube formation, but rather marks the cell wall to identify future sites. Furthermore, overexpression of *ssgA* in *S. coelicolor* makes the mycelium very sensitive to heat, high sucrose concentrations, and SDS-treatment, all indicating a weakened cell wall. For example, such SsgA-

overproducing strains fail to grow in YEME medium (30% sucrose), We speculate that SsgA, and perhaps all SALPs, functions by recruiting (a) cell wall modifying enzyme(s) to specific locations at the cell wall.

As for how the SALPs themselves are recruited, we have strong evidence that SsgA and SsgB are recruited prior to Z-ring formation, and hence their localization is not dependent on the divisome (Joost Willemse, Bjørn Traag and Gilles van Wezel, unpublished data). We recently identified mutants in hypothetical ORFs that have an almost identical phenotype as that of SsgG, producing large spores with multiple well-segregated chromosomes. One of the genes encodes a coiled coil protein, and hence may have a cytoskeletal function, tentatively pointing at a functional relationship between SsgG and the *Streptomyces* cytoskeleton. Live-cell imaging and biochemical methods such as FRET-FLIM microscopy and two-hybrid screening should elucidate the interaction partners for the SALPs.

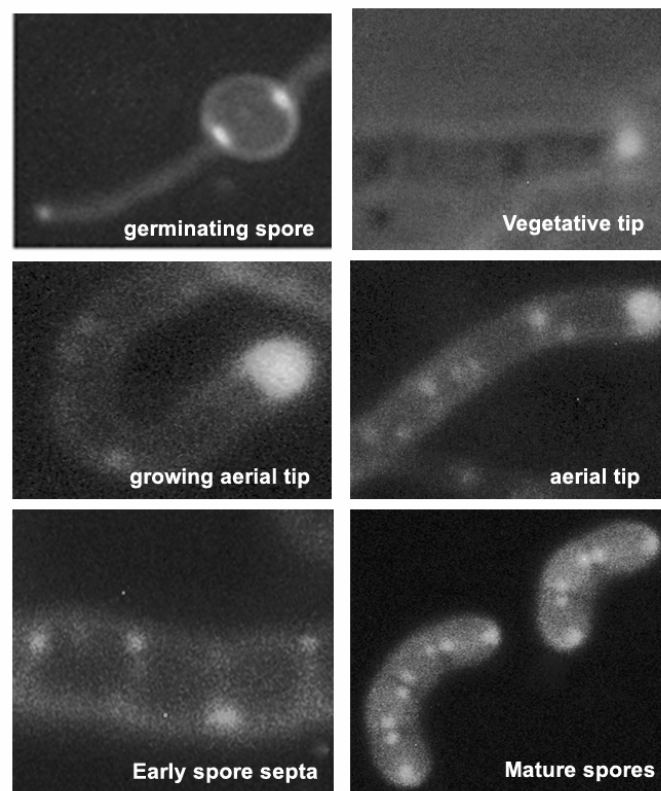


Figure 4. Dynamic localization of SsgA-GFP (For colour figure see Appendix A). Fluorescence micrographs of SsgA-GFP localization during the several stages of development of *S. coelicolor*. The width of the vegetative hyphae is around 400 nm (A, B), that of aerial hyphae and spores is around 800 nm (C-F). For further details see Noens *et al* (2007).

Application of SsgA for improved industrial fermentations

Biotechnological relevance of actinomycetes in general, and streptomycetes in particular, is underlined by the fact that approximately 60% of all known antibiotics are produced by these organisms, as well as a large number of other biotechnologically interesting compounds and enzymes (Bennett, 1998; Demain, 1991; Hopwood *et al.*, 1995). Productivity and hence the fermentation costs are strongly affected by the morphology of filamentous microorganisms. Models for mycelial growth have been worked out for filamentous fungi, and particularly for *Penicillium chrysogenum* (Krabben, 1997; Nielsen *et al.*, 1995; Trinchi, 1971). Morphology is determined by the efficiency of germination, the tip extension rate, the degree of branching, and especially by fragmentation of the hyphae. When one considers that filamentous microorganisms can produce clumps of millimeters in diameter, the necessity to improve growth by reducing pellet formation becomes obvious. In mycelial pellets the bulk of the biomass is hidden at the inside of the clump, resulting in strongly reduced growth rates and inefficient transfer of nutrients and oxygen. Mycelial mats (large open structures) result in highly viscous broths, which is again undesirable from the production perspective. For streptomycetes the degree of hyphal fragmentation (the major determinant of mycelial clump size) is directly proportional to the frequency of septation (cross-wall formation) of the vegetative hyphae. In turn this directly depends on the *ssgA* expression level, and as a consequence the morphology of liquid-grown mycelia is dictated by SsgA (van Wezel *et al.*, 2000a; van Wezel *et al.*, 2004). The enhanced expression of SsgA was employed successfully to obtain fragmented and fast growth of pellet-forming species such as *S. coelicolor*, *S. lividans* and *S. roseosporus* in shake flasks as well as in small-scale fermentations (5-50 liter scale) (van Wezel *et al.*, 2006). Using the secreted enzyme tyrosinase as a test enzyme, an increase in the yield of around 3-fold was achieved in significantly shorter fermentation time, underlining the promise of this technology (van Wezel and Vijgenboom, 2003; van Wezel *et al.*, 2006).

Acknowledgements

We are grateful to Elke Noens and Joost Willemse for sharing unpublished data and for discussions, and to Keith Chater, Barend Kraal and Erik Vijgenboom for

discussions. We would like to thank the participants at ISBA14 in August 2007 in Newcastle (UK) for stimulating discussions. This work was supported by grants from the Netherlands Organisation for Scientific Research (NWO) and from the Royal Netherlands Academy of Arts and Sciences (KNAW) to GPvW.

Chapter III

**Transcription of the sporulation gene *ssgA* is
activated by the IclR-type regulator SsgR in a
whi-independent manner in *Streptomyces*
coelicolor A3(2)**

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Mol Microbiol (2004) 53: 985-1000

ABSTRACT

SsgA plays an important role in the control of sporulation-specific cell division and morphogenesis of streptomycetes, and *ssgA* null mutants have a rare conditionally non-sporulating phenotype. In this paper we show that transcription of *ssgA* and of the upstream-located *ssgR*, an *iclR*-type regulatory gene, is developmentally regulated in *S. coelicolor*, and activated towards the onset of sporulation. A constructed *ssgR* null mutant was phenotypically very similar to the *ssgA* mutant. The absence of *ssgA* transcription in this mutant is probably the sole cause of its sporulation deficiency, as wild-type levels of sporulation could be restored by the SsgR-independent expression of *ssgA* from the *ermE* promoter. Binding of a truncated version of SsgR to the *ssgA* promoter region showed that *ssgA* transcription is directly activated by SsgR; such a dependence of *ssgA* on SsgR in *S. coelicolor* is in clear contrast to the situation in *S. griseus*, where *ssgA* transcription is activated by A-factor, and its control by the SsgR orthologue, SsfR, is far less important. Our failure to complement the *ssgR* mutant with *S. griseus ssfR* suggests functional differences between the genes. These observations may explain some of the major differences in developmental control between the phylogenetically divergent species *S. coelicolor* and *S. griseus*, highlighted in a recent microreview (Chater and Horinouchi, 2003). Surprisingly, transcription of *ssgA* and *ssgR* is not dependent on the early *whi* genes (*whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ*)

INTRODUCTION

Streptomycetes are soil-dwelling Gram-positive bacteria that have an unusually complex life cycle, which makes them particularly interesting for the study of bacterial development and evolution (Chater and Losick, 1997). During its life cycle, *Streptomyces* undergoes two apparently different events of cell division (reviewed in Flärdh and van Wezel 2003). Initially, cell division results in the formation of semi-permeable septa in the vegetative hyphae ('crosswalls') that delimit the multi-nucleoid hyphal cells. In solid-grown cultures, the reproductive phase is initiated by the formation of an aerial mycelium, with initially aseptate hyphae; in a later sporulation-programmed stage many septa are simultaneously formed, eventually resulting in mono-nucleoid spores (Chater, 2001). Recently, the genome sequences of *S. coelicolor* and *S. avermitilis* were elucidated, taking *Streptomyces* research into the genomics era (Bentley *et al.*, 2002; Ikeda *et al.*, 2003).

Genes involved in the transition from vegetative to aerial mycelium are called bald (*bld*) genes, characterised by the bald appearance of mutants due to their failure to produce aerial hyphae, and those involved in the subsequent processes leading to sporulation, are white (*whi*) genes, characterized by the white appearance of mutants due to a failure to complete sporulation. Six *whi* loci, designated *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ*, identified by Chater (1972), are essential for the sporulation process (Flärdh *et al.*, 1999). The best characterised of these sporulation genes are *whiG*, encoding an RNA polymerase sigma factor (Chater, 1989); *whiB*, encoding a transcription factor with many homologues in streptomycetes and mycobacteria (Soliveri *et al.*, 2000), and *whiH*, encoding a GntR-family transcription factor (Ryding *et al.*, 1998). The *whiH* mutant is phenotypically similar to a mutant in which the developmental *ftsZ* promoter had been inactivated (Flärdh *et al.*, 2000).

Recently, Chater and Horinouchi compared the developmental regulatory cascades in *Streptomyces coelicolor* and *Streptomyces griseus* (Chater and Horinouchi, 2003). These two organisms probably diverged from a common ancestor around 200 million years ago (Embley and Stackebrand, 1994), and an important difference between them is that *S. griseus* sporulates in submerged

culture. The signal for the onset of this still poorly understood process is the production of the γ -butyrolactone A-factor. In *S. griseus*, A-factor plays a more direct role in developmental control than the highly similar γ -butyrolactones (called SCBs) found in *S. coelicolor*: While A-factor non-producing mutants of *S. griseus* are defective in development and antibiotic production (Chater and Horinouchi, 2003; Horinouchi, 2002), these processes seem barely affected in γ -butyrolactone-deficient mutants of *S. coelicolor* (Takano *et al.*, 2001).

One of the key targets of A-factor in *S. griseus* is *ssgA* (Yamazaki *et al.*, 2003), a gene encoding a protein unique to sporulating actinomycetes that was originally identified as an effector of cell division in *S. griseus* (Kawamoto and Ensign, 1995b). SsgA plays an activating role in the production of sporulation septa, as its enhanced expression in *S. coelicolor* resulted in fragmentation of the mycelia in submerged cultures, producing spore-like compartments at high frequency (van Wezel *et al.*, 2000a). *ssgA* mutants of *S. coelicolor* and *S. griseus* are defective in sporulation, but form apparently normal vegetative septa (Jiang and Kendrick, 2000b; van Wezel *et al.*, 2000a). In total seven *ssgA*-like genes (*ssgA-G*) occur in *S. coelicolor*, and six in *S. avermitilis* (Flärdh and van Wezel, 2003). The *ssgB* gene was recently identified as a novel *whi* gene, and a null mutant produced large non-sporulating colonies (Keijser *et al.*, 2003).

Upstream of *ssgA* lies *ssgR*, a member of the family of *iclR*-type regulatory genes. This family includes IclR itself, the repressor for the isocitrate lyase gene (Sunnarborg *et al.*, 1990) and the acetate utilization operon (Galinier *et al.*, 1990) in *E. coli*, and the glycerol regulon repressor GylR in *S. coelicolor* (Hindle and Smith, 1994). The IclR-type proteins, most often repressors, are characterised by an N-terminally situated helix-turn-helix (DNA binding) domain, and a C-terminal substrate binding domain, which is also important for oligomerization of the protein (Zhang *et al.*, 2002). None of the other *ssgA*-like genes is situated near an *iclR*-type regulatory gene. The SsgR homologues of *S. coelicolor*, *S. avermitilis*, and *S. griseus* (called SsfR) are highly similar, although the *S. griseus* homologue has a predicted N-terminal extension of 84 residues. Insertional inactivation of *ssfR* in *S. griseus* is phenotypically similar to the *ssgA* mutant, although *ssgA* transcription does not depend on SsfR *S. griseus* (Yamazaki *et al.*, 2003).

In this work, we study the transcriptional regulation of *ssgA* and *ssgR* in *S. coelicolor* A3(2) strain M145 and its early sporulation mutants *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ*, together with the transcriptional dependence of *ssgA* on *ssgR*. We identified significant differences in the regulation of *ssgA* between *S. coelicolor* and *S. griseus*, and propose that this is one of the determinants of the morphological and developmental divergence between the two microorganisms.

MATERIALS AND METHODS

Bacterial strains and culturing conditions

The bacterial strains used in this work are listed in Table 1. *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). *Streptomyces coelicolor* A3(2) and its derivative M145, as well as the developmental (*whi*) mutants (Table 1), were obtained from the John Innes Centre strain collection, and *adpA* mutant M851 from E. Takano (Tübingen, Germany). M145 was used for transformation and propagation of *Streptomyces* plasmids. Preparation of media, protoplast preparation and transformation were performed according to Kieser *et al.* (2000). SFM medium was used to make spore suspensions. Minimal Medium (MM) agar plates containing 0.5% (w/v) mannitol, were used for RNA isolation; R2YE agar plates were used for regenerating protoplasts and, after addition of the appropriate antibiotic, for selecting recombinants. For standard cultivation of *Streptomyces*, and for plasmid isolation, YEME or TSBS (tryptone soy broth (Difco) containing 10% (w/v) sucrose), were used.

Plasmids and constructs

The plasmids and constructs described in this paper are summarised in Table 2, and a map of the *ssgRA* gene cluster is shown in Figure 2A.

1. General cloning vectors

pIJ2925 (Janssen and Bibb, 1993) is a pUC19-derived plasmid used for routine subcloning. For cloning in *Streptomyces* we used the shuttle vectors pHJL401 (Larson and Hershberger, 1986), pWHM3 (Vara *et al.*, 1989) and pSET152

(Bierman *et al.*, 1992). All three vectors have the *E. coli* pUC19 origin of replication; maintenance in streptomycetes occurs via the SCP2* *ori* (Lydiate *et al.*, 1985) (5 copies per chromosome) on pHJL401, the pIJ101 *ori* (50-100 copies per chromosome) on pWHM3, and the *attP* sequence (allowing integration at the attachment site of bacteriophage ϕ C31) on pSET152. Plasmid DNA was isolated from ET12567 prior to transformation to *Streptomyces*. For selection of plasmids in *E. coli* ampicillin was used, except for pSET152 (apramycin); chloramphenicol was added for ET12567 transformants. For selection in *S. coelicolor* we used thiostrepton for pHJL401 and pWHM3, and apramycin for pSET152.

2. Construction of pGWR2 for in-frame deletion of *ssgR*

To create a construct for in-frame deletion of *S. coelicolor ssgR*, a 1400 bp *Bgl*II-*Bam*HI fragment containing *ssgR* and part of *ssgA* was inserted into pIJ2925 digested with *Bam*HI, and the approximately 280 bp *Nco*I-*Sph*I segment of *ssgR*

Table 1. Bacterial strains.

Bacterial strain	Genotype	Reference
<i>S. coelicolor</i> A3(2)	SCP1 ⁺ SCP2 ⁺	(Kieser <i>et al.</i> , 2000)
<i>S. coelicolor</i> M145	SCP1 ⁻ , SCP2 ⁻	(Kieser <i>et al.</i> , 2000)
M851	<i>adpA</i> (<i>bldH</i>) mutant of M145	(Takano <i>et al.</i> , 2003)
C72	<i>whiA</i> 71 <i>pgl</i> ⁺ SCP1 ⁺ SCP2 ⁺	(Chater, 1972)
C70	<i>whiB</i> 70 <i>pgl</i> ⁺ SCP1 ⁺ SCP2 ⁺	(Chater, 1972)
C71	<i>whiG</i> 71 <i>pgl</i> ⁺ SCP1 ⁺ SCP2 ⁺	(Chater, 1972)
C119	<i>whiH</i> 119 <i>pgl</i> ⁺ SCP1 ⁺ SCP2 ⁺	(Chater, 1972)
C17	<i>whiI</i> 17 <i>pgl</i> ⁺ SCP1 ⁺ SCP2 ⁺	(Chater, 1972)
C77	<i>whiJ</i> 77 <i>pgl</i> ⁺ SCP1 ⁺ SCP2 ⁺	(Ryding <i>et al.</i> , 1999)
GSA3	M145 Δ <i>ssgA</i> (::: <i>aadA</i>)	(van Wezel <i>et al.</i> , 2000a)
GSA4	M145 Δ <i>ssgA</i> + pGWS7	(van Wezel <i>et al.</i> , 2000a)
GSA5	M145 Δ <i>ssgA</i> + pGWR1	(van Wezel <i>et al.</i> , 2000a)
GSR1	M145 Δ <i>ssgR</i>	This paper
GSR2	M145 Δ <i>ssgR</i> + pGWR1	This paper
GSR3	M145 Δ <i>ssgR</i> + pGWS10	This paper
GSR4	M145 Δ <i>ssgR</i> + pGWS7	This paper
<i>E. coli</i> JM109	See reference	(Sambrook <i>et al.</i> , 1989)
<i>E. coli</i> ET12567	See reference	(MacNeil <i>et al.</i> , 1992)

(Figure 2A) was removed to create an in-frame deletion in the *ssgR* gene on the plasmid. For this purpose, the DNA was digested with *Nco*I and *Sph*I, and the protruding ends were filled in (*Nco*I) or removed (*Sph*I) using T4 DNA polymerase and dNTPs, followed by ligation and transformation. To ascertain we had created an in-frame deletion, the DNA sequence was determined and a 279 bp deletion was confirmed. Subsequently, the apramycin resistance cassette *aac(C)IV* (Kieser *et al.*, 2000) was inserted into the *Eco*RI site of the construct (outside the *ssgRA* insert), producing pGWR2. After transformation of the non-replicating construct to *S. coelicolor* M145, initial integrants (apramycin resistant) were selected, allowed to sporulate on SFM plates without antibiotics, and replicated non-selectively to allow a second recombination event to take place, and plated for single colonies. The latter were replicated to SFM containing apramycin, to screen for double recombinants, which should have lost the plasmid

Table 2. Plasmids and constructs.

Plasmid	Description	Reference
pHJL401	<i>Streptomyces/E. coli</i> shuttle vector (5-10 and around 100 copies per genome, respectively)	(Larson and Hersherberger, 1986)
pSET152	<i>Streptomyces/E. coli</i> shuttle vector (integrative in <i>Streptomyces</i> , high copy number in <i>E. coli</i>)	(Bierman <i>et al.</i> , 1992)
pIJ2925	Derivative of pUC19 (high copy number) with <i>Bg</i> /II sites flanking its multiple cloning site	(Bibb <i>et al.</i> , 1985)
Q11	Cosmid clone containing the <i>ssgRA</i> gene cluster	(Bentley <i>et al.</i> , 2002)
pGWR1	pHJL401 + 1.3 kb <i>ssgR</i> fragment (-300/+1000, relative to the translational start site of <i>ssgR</i>)	This paper
pGWR2	Construct used for creating <i>ssgR</i> deletion mutant	This paper
pGWR3	pWHM3 + 1.3 kb <i>ssgR</i> fragment (-350/+950, relative to the translational start site of <i>ssgR</i>)	This paper
pGWR5	pHJL401 + 1.3 kb fragment harbouring <i>ssfR</i> of <i>S. griseus</i> (-300/+1000, relative to <i>ssfR</i> translational start)	This paper
pGWS6	pIJ2925 harbouring PCR product of oligonucleotides Q10 and Q11, inserted as a 237 bp <i>Eco</i> RI- <i>Hind</i> III fragment.	(van Wezel <i>et al.</i> , 2000a)
pGWS7	pSET152 harbouring <i>S. coelicolor ssgA</i> expressed from the <i>ermE</i> promoter	(van Wezel <i>et al.</i> , 2000a)
pGWS10	pSET152 harbouring 1.2 kb <i>ssgA</i> fragment (-625/+540 relative to the translational start site of <i>ssgA</i>)	This paper
pGWR11	pET15b-based construct for the expression and purification of full-sized SsgR-241	This paper
pGWR12	pET15b-based construct for the expression and purification of truncated SsgR-155	This paper

and hence have become sensitive to apramycin. About 30% of all apramycin sensitive colonies were sporulation mutants. A check of four sporulating and four non-sporulating double recombinants by PCR revealed that all sporulation mutants carried the expected 300 bp in-frame deletion, while sporulating colonies had a wild-type *ssgR* gene. One of the mutant colonies was selected, and designated GSR1. The location of the deletion is shown in Figure 2A.

3. Constructs for complementation experiments

For complementation of the *ssgR* mutant, plasmid pGWR1 was designed. This low-copy-number pHJL401-based vector harbours the PCR-generated -350/+950 region (relative to the *ssgR* translational start), including *ssgR* itself and approximately 300 bp promoter sequences; the oligonucleotides used for PCR were Q16 and Q11 (Table 3), designed such as to add *EcoRI* and *BamHI* restriction sites at the *ssgR* upstream and downstream end, respectively. A multi-copy derivative of pGWR1, designated pGWR3, contains the same insert in pWHM3. Plasmid pGWR5 is essentially the same construct as pGWR1, only with the corresponding region of *S. griseus ssfR* instead of *S. coelicolor ssgR*. Plasmids GWS7 and pGWS10 were used for expression of *ssgA*. pGWS7 (van Wezel *et al.*, 2000a) contains *ssgA* behind the constitutive *ermE* promoter in the integrative vector pSET152, while pGWS10 is a pHJL401 derivative, harbouring a 1.2 kb insert with *ssgA* preceded by its natural upstream (promoter) region. The insert of pGWS10 was generated by PCR with oligonucleotides Q18 and Q6 (Table 3), and corresponded to the -625/+540 section relative to the start of *ssgA*.

4. Constructs for the expression of His₆-tagged SsgR

The SsgR expression plasmid pGWR11 was constructed by amplifying *ssgR* from the *S. coelicolor* genome with *Pfu* DNA polymerase and oligonucleotides Q2 and Q3 (Table 3), and cloned as an *NdeI*-*HindIII* fragment into pET15b, allowing the production of N-terminally His₆-tagged SsgR; the full sized product was designated SsgR-241. For the expression of a truncated version of SsgR (called SsgR-155), containing the N-terminal 155 amino acids of the protein and lacking the TM signature, we made the pET15b-based expression construct pGWR12. The procedure was the same as for pGWR11, except that oligonucleotides Q2 and R155Bam were used (Table 3), the latter introducing a stop codon

immediately downstream of the CTC codon for Leu155. The PCR product was cloned as an *NdeI-BamHI* fragment into pET15b.

Expression and purification of N-terminally (His)₆-tagged SsgR

Plasmids pGWR11 and pGWR12 were used for the expression of full-sized SsgR (241 aa) and truncated SsgR (155 aa), respectively. For protein expression and purification, BL21 codonplus cells (Stratagene) transformed with either pGWR11 or pGWR12 were grown in LB broth at 30°C to an OD₆₀₀ of about 0.5, SsgR expression was induced by the addition of 0.1 mM IPTG, and the culture was incubated for a further 4-6 h. Cells were collected by centrifugation at 4°C, and pellets resuspended in resuspension buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 300mM NaCl, and 10 mM imidazole). After sonication, the samples were spun down, and the supernatant and pellet analysed on a 10-12% SDS-PAGE gel. Gels were stained with Coomassie Brilliant blue in 7% ethanol/12% acetic acid.

Soluble SsgR-155 was purified using Ni-NTA affinity chromatography according to the Novagen protocol, and eluted with 200 mM imidazole. Samples were dialysed against standard buffer (50 mM Tris pH 7.5, 40 mM NH₄Ac, 10 mM MgCl₂, 1 mM DTT). Inclusion bodies containing SsgR-241 were harvested by centrifugation at 15,000 x g, solubilised and purified using the Pierce 6xHis B-Per kit, according to the manufacturer's instructions. After purification of SsgR-241 under denaturing conditions using Ni-NTA affinity chromatography, the purified protein was dialysed against standard buffer.

Mobility-shift assay

Binding of SsgR to target DNA was studied in the following binding buffer: 20 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM MgCl₂, 1mM EDTA, 1mM DTT, and 5% glycerol. To minimise nonspecific interactions, 50 ng of pBR322 and 100 µg/ml BSA were added. Reaction mixtures (20 µl) contained 3 nM [³²P]-labelled DNA probe and 2 µl of diluted protein fraction (between 1-20 ng). After 10 min incubation at 30°C, 2 µl dye (50% glycerol, 0.25% xylene cyanol, and 0.25% bromophenol blue) was added and samples were analysed on a non-denaturing 6% polyacrylamide gel in 1X TBE buffer. The DNA probe used was produced by PCR using oligonucleotides Q10 and [³²P]-labelled Q11 (Table 3), and corresponded to the -195/+41 region relative to the *ssgA* translational start,

similar to *ssgA*-S1 (Fig. 1A). Electrophoresis was carried out at 4°C (4 h at 15 V/cm). After drying, gels were analysed in a phosphor-imager (BioRad).

PCR conditions

PCRs were performed in a minicycler (MJ Research, Watertown, MA), using *Pfu* polymerase (Stratagene, La Jolla, LA), and the buffer provided by the supplier, in the presence of 5% (v/v) DMSO, with annealing temperature of 58°C. For oligonucleotides see next section and Table 3.

Transcript mapping and probes

Mycelium was grown on MM or SFM agar plates with mannitol (0.5% w/v) as the carbon source, by plating spores onto presterilised cellophane discs. The RNA was purified from the surface-grown mycelium using the Kirby-based protocol (Kieser *et al.*, 2000), except that DNaseI treatment was used in addition to salt precipitation to fully eliminate DNA from the nucleic acid preparations. Phase-contrast light microscopy was used to assess the developmental stage of the

Table 3. Oligonucleotides.

Primer	DNA sequence	5' end	relative to
Q2	5' ctgaattcatatggtgcagcgggcatgcgctgctc	+1	<i>ssgR</i>
Q3	5' ctgaagctttctaactgatggcatcttg	+781	<i>ssgR</i>
Q6	5' ctgaagcttcaccgctgccttgctggccggg	+540	<i>ssgA</i>
Q10	5' ctggaattcagcgggtgcagaacgagg	-195	<i>ssgA</i>
Q11	5' gtacggcatagggatcccggtctcgtag	+82	<i>ssgA</i>
Q16	5' ctggaattcgtcgtcgcgcagcgactggtg	-338	<i>ssgR</i>
Q17	5' ctgggatactactcgtgctcggcgacgcg	+47	<i>ssgR</i>
Q18	5' ctggaattcgcacacatggcgcgctgg	-625	<i>ssgA</i>
R155Bam	5' gctaaggatcctcagaggggctgcaccgggtagcggtc	+465	<i>ssgR</i>
T7-AF	5' <u>tcggctaatacgaactcactatagggatgcggaaagagagctcctc</u>	+41	<i>ssgA</i>
T7-Rrev	5' gcaagttcatgtcgcagctcgt	-358	<i>ssgR</i>
T7-RF	5' <u>tcggctaatacgaactcactatagggatgcggaaagagagctcctc</u>	+1	<i>ssgR</i>
<i>ssgA</i> -RT-for	5' atgagctttctcgtgtccgagg	+1	<i>ssgA</i>
<i>ssgA</i> -RT-rev	5' cagcggcaccagcttgcggtg	+325	<i>ssgA</i>
<i>whiG</i> -RT-for	5' ggaacagctgatcctgcactac	+141	<i>whiG</i>
<i>whiG</i> -RT-rev	5' tcgcgtacgcgcgtccacggtg	+418	<i>whiG</i>
16S-RT-for	5' tcacggagagtttgatcctggctc	+20	<i>rrnA</i>
16S-RT-rev	5' cccgaaggcgtcatcctcacgc	+436	<i>rrnA</i>

Added T7 promoter sequences are underlined, restriction sites used for cloning presented in bold face. Restriction sites: **gaattc**, *EcoRI*; **aagctt**, *HindIII*; **ggatcc**, *BamHI*; **catatg**, *NdeI*. The *BamHI* site in Q11 occurs naturally in *ssgA*, other sites were designed. Location of 5' end of oligonucleotides (T7 promoter sequences not included) is relative to the start of the gene presented in the last column. The database accession number for *whiG* is SC05621, for *rrnA* SC04123.

surface-grown mycelium prior to harvesting and RNA isolation. Appropriate primers were labeled at their 5' ends with (γ - ^{32}P)-ATP by using T4 polynucleotide kinase before DNA probes were produced by PCR, after which high-resolution S1 nuclease mapping was carried out according to previously described protocol (Kieser *et al.*, 2000). Alternatively, (α - ^{32}P)-UTP-radiolabelled RNA probes were produced with T7 RNA polymerase according to the Maxiscript kit (Ambion), and RNA protection assays were carried out using the RPAIII kit (Ambion). For each RNA protection assay, excess of probe was hybridised to 30 μg of RNA. Protected fragments were analysed on denaturing 6% polyacrylamide gels, where desired alongside a DNA sequencing ladder, produced using the T7 sequencing kit (Amersham Pharmacia Biotech), with as sequencing primers the downstream primers used for generating the PCR-based *ssgA* and *ssgR* probes.

Probes used for transcript mapping were produced by PCR, using oligonucleotides described in Table 3; **(1) *ssgR-T7***, a T7 RNA polymerase-generated RNA probe with incorporated (α - ^{32}P)-UTP, produced from a PCR fragment made using oligonucleotides T7-RF and T7-Rrev; the probe was designed against the -358/+1 region (relative to the *ssgR* translational start); **(2) *ssgR-S1***, a DNA probe encompassing the -338/+47 region (relative to the *ssgR* translational start), generated by PCR with oligonucleotides Q16 and ^{32}P -labelled Q17; **(3) *ssgA-T7***, a T7 RNA polymerase-generated RNA probe with incorporated (α - ^{32}P)-UTP, produced from a PCR fragment made using oligonucleotides T7-AF and Q10; the probe was designed against the -195/+41 region relative to the *ssgA* translational start; **(4) *ssgA-S1*** was generated by PCR on plasmid pGWS6, using ^{32}P -labelled Q11 and the 17-mer universal ('forward') pUC primer, and corresponded to the -195/+82 region (relative to the *ssgA* translational start); the probe contains an approximately 50 nt non-homologous 3' extension to discriminate between full-length protection by RNA and experimental artefacts due to probe reannealing.

RT PCR analyses

RT-PCR analyses were carried out using the SuperScript III one-step RT-PCR System (Invitrogen) for the analysis of RNA. RNA was isolated from mycelium grown on SFM agar plates with a cellophane overlay, on which all strains sporulated well (M145, M851) or a bit (*ssgR* mutant). The samples were

prepared after 24 h, 48 h, and 96 h, corresponding to vegetative growth, aerial growth, and sporulation, respectively. For each RT-PCR reaction 1 µg of RNA was used together with 0.5 µM (final concentration) of each primer. The program used was as follows: 30 min cDNA synthesis at 55°C, followed by 40 cycles of: 15" at 94°C (denaturing), 30" at 56°C (annealing) and 60" 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, and stained with ethidium bromide. The following combinations of oligonucleotides were used (Table 3): (a) *ssgA*-RT-for and *ssgA*-RT-rev for *ssgA*; (b) *whiG*-RT-for and *whiG*-RT-rev for *whiG*; (c) 16SRT-for and 16SRT-rev for 16S rRNA (to check the integrity of the RNA preparations). RT-PCR experiments without prior reverse transcription were performed on all RNA samples to assure exclusion of DNA contamination. Data were verified in several independent experiments.

Computer analysis

Frame analysis was performed using the frameplot program (Ishikawa and Hotta, 1999), sequence alignments were performed using Clustal (Higgins *et al.*, 1996), Blast searches were performed at NCBI (<http://www.ncbi.nlm.nih.gov>), and transmembrane helix predictions using the TMPred program (http://www.ch.embnet.org/software/TMPRED_form.html).

RESULTS

ssgR* is important for sporulation of *S. coelicolor

The *ssgR* gene (*S. coelicolor* database reference SCO 3925) encodes a 241 amino acid IclR-type regulatory protein. Fourteen putative *iclR*-like genes could be identified within the *S. coelicolor* genome. An interesting feature of SsgR is its predicted transmembrane (TM) helix, YALGTVCAAIPITVGTTAATM (residues 185-205; high probability, as predicted by the TMPred server). Orientation is most likely N-terminus inside, which is consistent with the presence of a predicted helix-turn-helix (HTH) DNA binding domain in the N-terminal section of the protein (aa 19-40). Highly similar TM domains are also found in the SsgR homologues from *S. avermitilis* (SAV4268) and *S. griseus* (SsfR; AAF61237).

Another IclR-type regulator occurs in *S. coelicolor* with a highly similar putative TM domain (SCO2832, predicted TM sequence YAVGTVCAAVPITAGSAVGCL), which is the closest relative of SsgR in *S. coelicolor* (40% overall amino acid identity). However, its HTH domain is very different from that of SsgR.

To study the possible role of *ssgR* in *S. coelicolor* M145, an in-frame-deletion mutant of *S. coelicolor ssgR* was created, as described in the Materials and Methods section. This removed the approximately 280 bp *NcoI-SphI* fragment of *ssgR*, resulting in an in-frame deletion of the gene corresponding to aa 78-172. This mutant was designated GSR1. As shown in Figure 1, the mutant had a phenotype similar to that of the *ssgA* mutant GSA3; similarly, GSR1 formed aerial hyphae, but failed to produce spores on rich media such as R2YE or MM with glucose as the sole carbon source. Spores were produced on particular media, notably on MM with mannitol or on SFM, although at reduced levels and after prolonged incubation (Figure 1). Such an unusual conditionally white phenotype is also typical of the *ssgA* mutant (van Wezel *et al.*, 2000a).

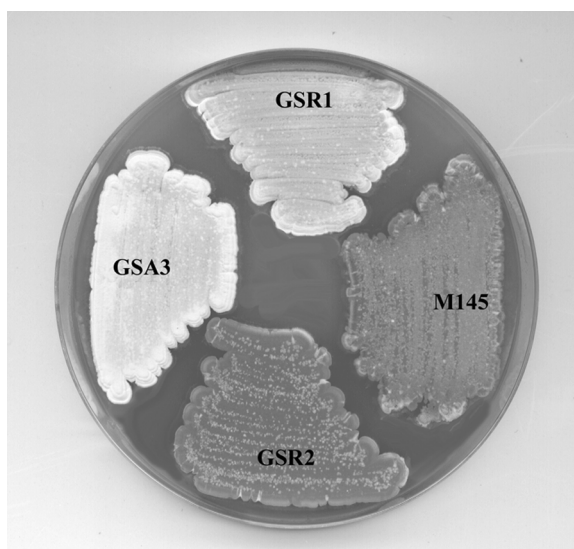


Figure 1. Phenotypes of the *ssgA* and *ssgR* mutants. Sections of the relevant strains were streaked onto SFM plates, and photographed after 7 days. Strains used in this experiment: **M145**, Wild-type *S. coelicolor*; **GSA3**, *ssgA::aadA* disruption mutant; **GSR1**, *ssgR* in-frame deletion mutant; **GSR2**, GSR1 complemented by *ssgR* (pGWR1). Note the slightly grey appearance of the mutants, indicative of a low degree of sporulation on this particular medium.

***ssgR* is transcribed from a single developmentally regulated promoter**

For transcriptional analysis of *ssgR*, and precise localization of the transcriptional start sites, transcript mapping experiments were performed on RNA isolated from *S. coelicolor* M145 grown on MM agar plates with mannitol as the sole carbon source. RNA was isolated at 12-24 hr intervals during 5 days, so as to provide representative samples to analyse transcription. The developmental stage of the samples was monitored using phase contrast microscopy.

Considering the low expression level of *ssgR* (see below) we used an end-to-end ³²P-labelled RNA probe for transcript mapping. RNA protection analysis using probe *ssgR*-T7 (-358/+1, relative to the *ssgR* translational start; Figure 2A) resulted in one major protected band, with a length of approximately 210 nt (Figure 3). Transcription of *ssgR* was upregulated after approximately 64 h, corresponding to the onset of sporulation (few spores were observed by phase contrast microscopy at this point). The experiment was repeated several times, also using RNA derived from cultures grown on SFM instead of MM agar plates. While the mycelium grew significantly faster on SFM than on MM, the developmental dependence of *ssgR* transcription was very similar in all experiments (not illustrated). Promoter probing experiments using the *redD* reporter system (van Wezel *et al.*, 2000c) confirmed the presence of promoter activity immediately upstream of *ssgR* (data not illustrated), ruling out the possibility that the transcripts had arisen from processing of an upstream-located promoter.

The exact transcriptional start point of the *ssgR* transcript was identified by comigration of the protected RNA probe together with a DNA sequencing ladder. The most likely transcriptional start site was identified as one of two A residues around 210 nt upstream of the translational start of *ssgR* (Figure 3B). This transcriptional start site is preceded by the sequence TAGAGT, which fully conforms to the consensus -10 sequence (TAGAPuT) for promoters recognised by the major RNA polymerase σ factor (σ^{hrdB}) of *Streptomyces* (Strohl, 1992). However, we failed to identify a plausible -35 sequence.

Transcriptional analysis of the *S. griseus* *ssgR* orthologue, designated *ssfR*, showed that it is also transcribed from a single promoter, with a promoter sequence that is similar to that of *ssgRp*; the -10 sequences are almost identical (TAGAGT for *S. coelicolor*, TACAGT for *S. griseus*) and of the -50/-8 regions

(relative to the transcriptional start sites), 62% of the nucleotides are identical (S. Horinouchi, pers. comm.). Upstream of the *SsgR* homologue of *S. avermitilis* we identified a sequence that is highly similar to the *ssgR* promoter of

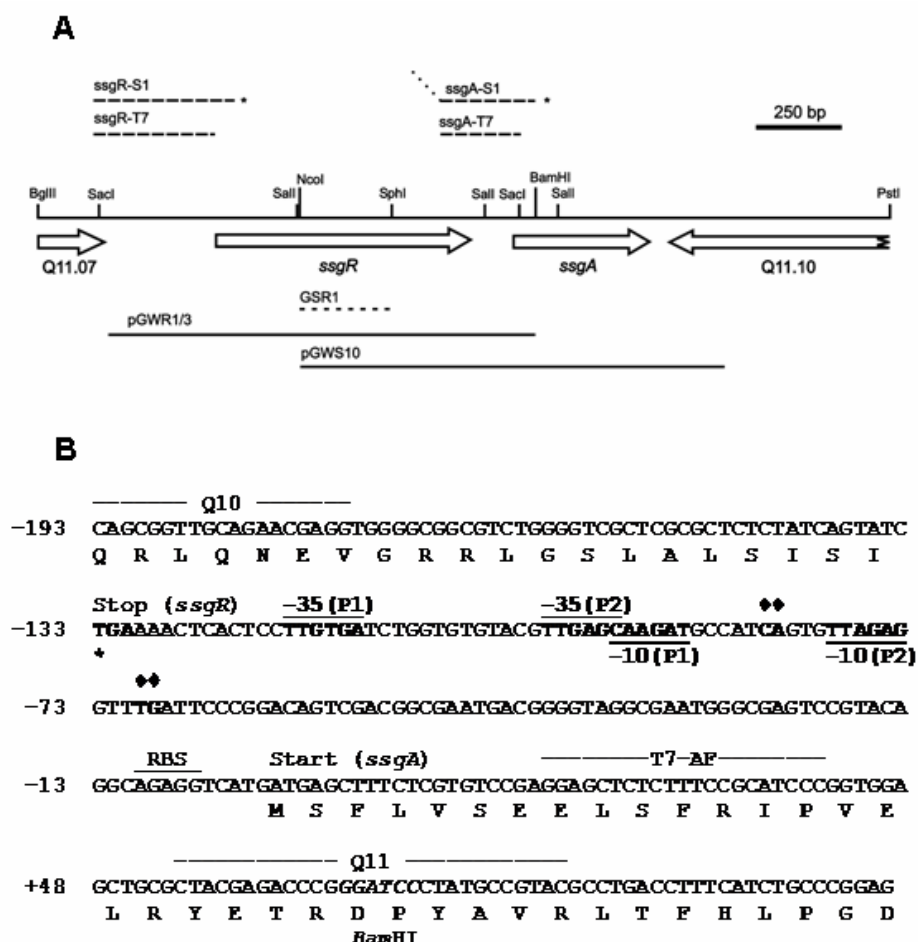


Figure 2. Map of the *ssgRA* gene cluster, and sequence of the *ssgA* promoter region. 2A. Map of *ssgRA*, and location of DNA fragments and probes. The *ssgRA* gene cluster is located on cosmid Q11. The most likely translational start of *ssgR* (ORF Q11.8; accession CAB46964) is a GTG triplet at nt position 4318648 on the genome, that of *ssgA* (Q11.9; accession CAB46963) an ATG triplet at nt position 4319504, resulting in proteins of 241 amino acids (SsgR) and 135 aa (SsgA), respectively. ORFs shown as arrows, indicating the direction of their transcription. Inserts of complementation constructs are shown by solid lines below the map. Dashed lines above the map represent the location of probes used for high resolution transcript mapping (See Materials and Methods, section 'transcript mapping and probes'). Extension (tilted dotted line) indicates 50 nt stretch containing non-homologous vector sequences. The approximately 280 nt deletion in the *ssgR* mutant GSR1 is represented by a dashed line below the sequence. **2B.** DNA sequence of the *ssgRA* intergenic region. Nucleotide numbering refers to the *ssgA* translational start site. ♦, transcriptional start sites (see Figure 4B); putative -35 and -10 sequences are overlined and underlined, respectively. Derived aa sequences corresponding to the end of *ssgR* and the start of *ssgA* are shown below the sequence. **RBS**, putative ribosome binding site. p1 and p2 refer to transcription start sites observed in surface-grown cultures. Important oligonucleotides are indicated (T7-AF and Q11 are complementary to the coding strand).

S. coelicolor, and this sequence therefore constitutes a likely *ssgR* promoter. This putative promoter (presumed -50/-1 region is 80% identical to that of *S. coelicolor ssgRp*) would start around nt position -240 relative to the start of *S. avermitilis ssgR*.

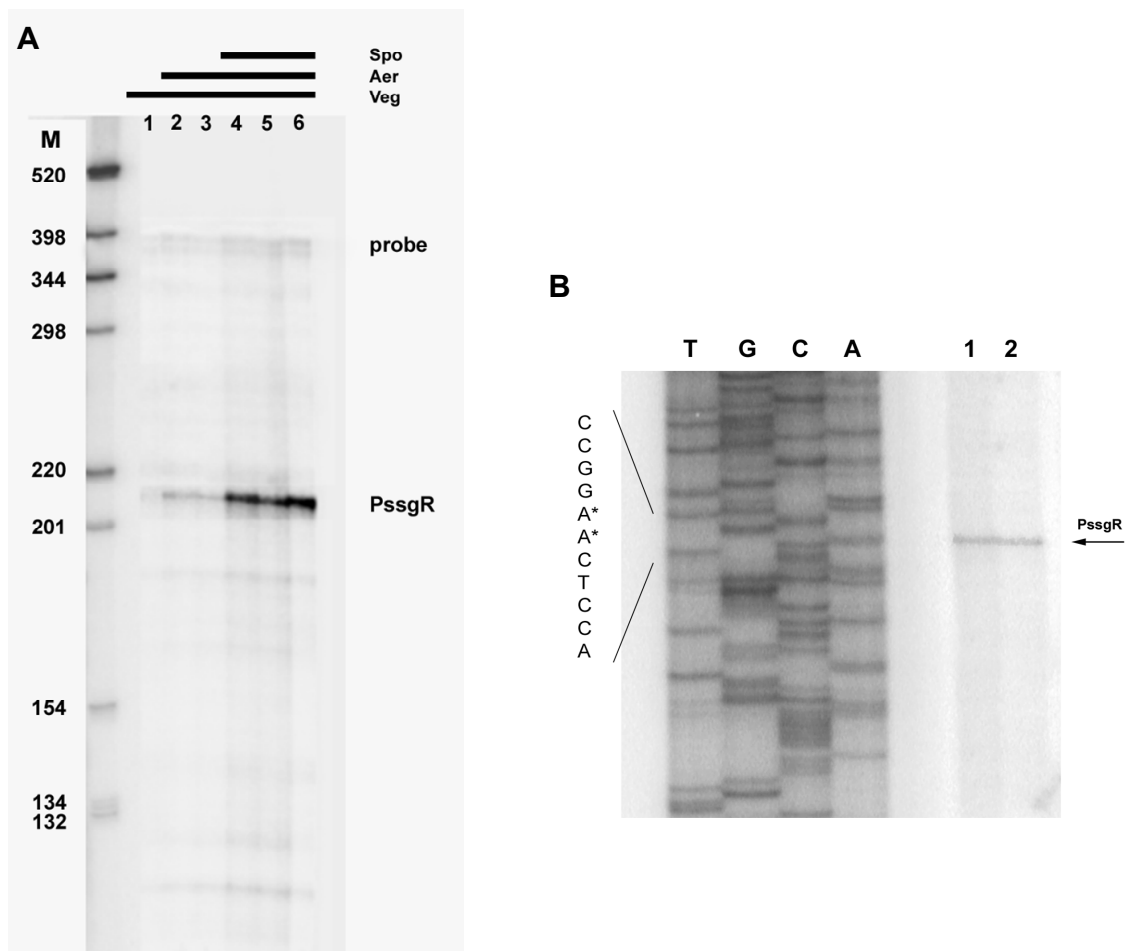


Figure 3. Growth phase-dependent transcription of *S. coelicolor ssgR*. **3A.** Transcript mapping experiment to identify *ssgR* transcripts. RNA was isolated from solid-grown MM + mannitol cultures of *S. coelicolor* M145, and analysed using high resolution transcription analysis. RNA time points: (1) 24 h (2) 36 h (3) 48 h (4) 64 h (5) 80 h (6) 96 h. Sample 2 corresponded to the onset of aerial mycelium formation, and sporulation started after approximately 64 h (sample 4). The RNA probe used for the RNA protection assays was *ssgR*-T7 (Figure 2A). While *ssgR* transcripts were already visible in samples 2 and 3, transcription of *ssgR* was strongly enhanced as soon as sporulation started. The location and developmental regulation of the *ssgR* promoter was verified with different probes and independent experiments (e.g. Figure 7B). **3B.** Determination of the exact transcription start site of *ssgR*. **TGCA**, *ssgR* nucleotide sequence ladder. Lanes 1 & 2 are the same samples as shown in lanes 2 & 3 in Figure 3A. Asterisks indicate the most likely transcriptional start site, coinciding with two A residues at nt positions -210 and -211, relative to the *ssgR* translational start site, respectively.

Transcription of *ssgA* is dependent on *ssgR*

High-resolution mapping of *ssgA* transcripts was performed on the same RNA as was used for the analysis of *ssgR* transcription, with RNA probe *ssgA*-T7 (-195/+41, relative to the *ssgA* translational start; Figure 2AB). Two RNA-protected bands of approximately 125 nt and 110 nt were observed (p1 and p2, respectively; Figure 4A, left panel). The bands appear as double bands, with one nt difference. Such a duplication is often seen, e.g. for the *ftsZ* promoters (Flårdh *et al.*, 2000) and the *sigF* promoter (Kelemen *et al.*, 1996), probably as the result of an experimental artefact. While in all independent experiments it was difficult to detect *ssgR*-derived transcripts, *ssgA* transcripts could be readily detected.

SsgA transcription was induced after approximately 80 h, a time point where sporulation was well underway, while *ssgR* transcription was induced one time point earlier, corresponding to the onset of sporulation. Thus, transcription of *ssgR* is activated at least several hours prior to transcription of *ssgA*. We did not observe full-length protection of the probe in these experiments, which was confirmed by experiments using probe *ssgA*-S1 in Figure 7 (below), which carries a 50 nt non-homologous extension at its 3' end. Comigration of a DNA sequencing ladder showed that the transcriptional start sites corresponded to the approximate nt positions -84 and -69 relative to the translational start of *ssgA*, respectively (Figure 4B). The transcriptional start sites (full sequences in Figure 2B) are preceded by the sequences 5' TTGTGA-18 bp-CAAGAT-3' (for p1) and 5' -TTGAGC-15 bp-TTAGAG-3' (for p2), which show limited similarity to the consensus -35 and -10 sequences (5'-TTGACN-16-18 bp-TAGAPuT-3'; (Strohl, 1992) for promoters recognised by the major RNA polymerase holoenzyme of *Streptomyces*.

Since *ssgR* and *ssgA* mutants are phenotypically highly similar, a possible dependence of *ssgA* transcription on *ssgR* was investigated. For this purpose, transcriptional analysis was performed using RNA isolated from surface-grown MM mannitol cultures of GSR1, the congenic *ssgR* mutant of *S. coelicolor* M145. We repeatedly failed to detect significant levels of *ssgA* transcripts in these samples, suggesting that *ssgA* transcription is directly or indirectly dependent on *SsgR* in *S. coelicolor* (Figure 4A, right panel).

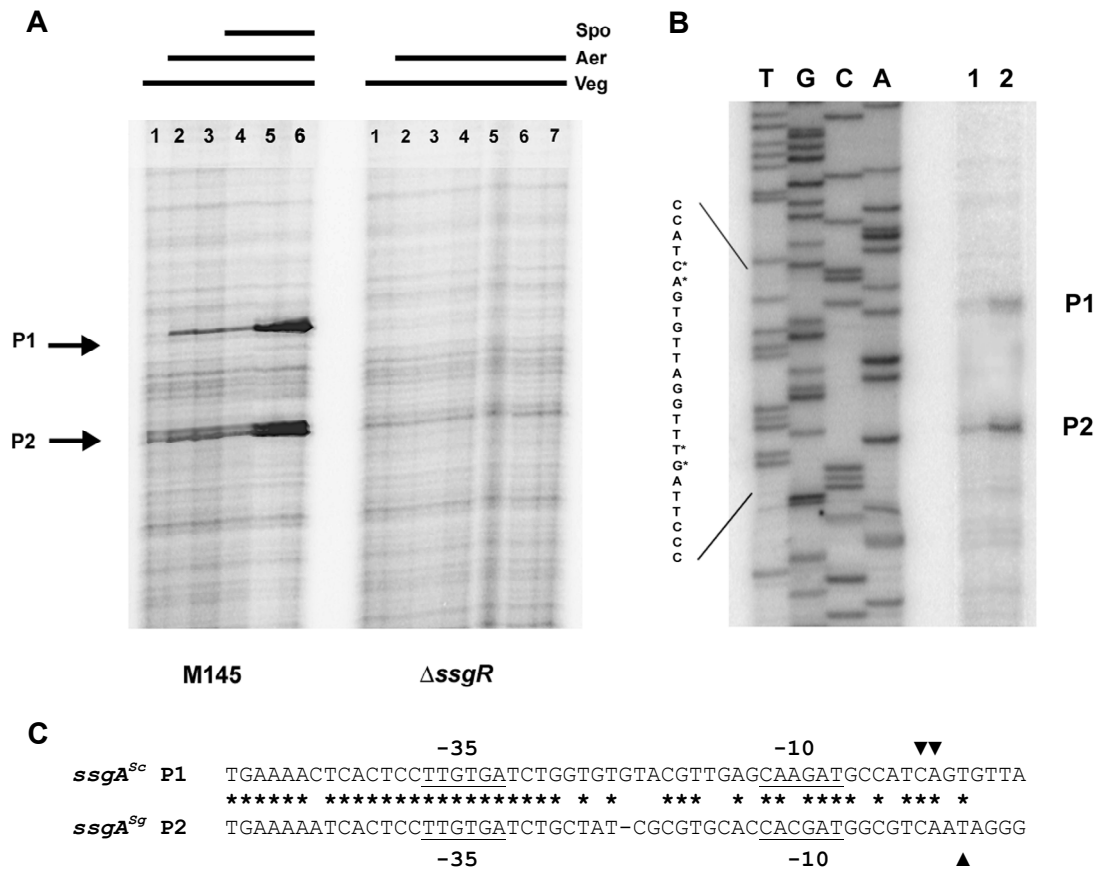


Figure 4. Growth phase-dependent transcription of *S. coelicolor ssgA*, and dependence on *ssgR*. **4A.** Transcript mapping experiment to identify *ssgA* transcripts in *S. coelicolor* M145 and its congenic *ssgR* mutant GSR1. RNA was isolated from solid-grown MM cultures. The RNA probe used for the RNA protection assays was *ssgA*-T7 (Figure 2A). Left panel: transcription in M145. The RNA used was the same as in Figure 3. Bands p1 and P2 correspond to *ssgA* transcripts. The intensity of both transcripts varied in a growth phase-dependent manner, with a maximum in lanes 5 and 6. Right panel: transcription in GSR1. RNA time points: (1) 24 h (2) 36 h (3) 48 h (4) 64 h (5) 80 h (6) 96 h (7) 120 h. Sample 3 corresponded to the onset of aerial mycelium formation. The relatively strong background is due to the use of a T7-generated RNA probe with incorporated nucleotides. The location and developmental regulation of the promoters was verified with several different probes and independent experiments (e.g. S1 mapping, Figure 7). **4B.** Determination of the transcription start sites of *ssgA*. **TGCA**, *ssgA* nucleotide sequence ladder. Lanes 1 & 2 correspond to the same samples as shown in lanes 2 & 3 in Figure 4A. Asterisks indicate the most likely transcriptional start sites for the two transcripts, coinciding with a A residue (for p1) and a G residue (for p2), at nt positions -84 and -69, relative to the *ssgA* translational start site, respectively. **4C.** Alignment of the *S. coelicolor ssgA* p1 and *S. griseus ssgA* p2 promoters. Putative -10 sequences underlined; arrowheads indicate the likely transcriptional start site. Asterisks indicate identical nucleotides between the two promoters. *S. griseus ssgA* p2 start site taken from (Yamazaki *et al.*, 2003).

The integrity of the RNA was confirmed by mapping the transcript of *ssgD* (SCO6722), one of the six *ssgA*-like genes in *S. coelicolor*, using the same RNA as in the experiments for mapping *ssgA* transcripts. The *ssgD* gene is expressed in all growth phases and in an *ssgR*-independent manner, from a single promoter (B. Traag & G.P. van Wezel, unpublished data). We observed no difference between M145 and GSR1 (data not illustrated). The absence of *ssgA*-derived transcripts in the *ssgR* mutant was confirmed by RT PCR experiments (see below).

Expression of SsgA restores sporulation to an *ssgR* mutant

To further assess the dependence of *ssgA* transcription on *ssgR*, we analysed the morphological effect of *ssgR*-independent expression of *ssgA* in the *ssgR* mutant. For this purpose, two constructs were introduced into GSR1, one with *ssgA* preceded by its own (putatively SsgR-dependent) regulatory sequences, and one with *ssgA* positioned behind the SsgR-independent and constitutive *ermE* promoter. In a control experiment, we also introduced *ssgR* expression constructs in the *ssgA* mutant. In the latter case, no effect was expected. As additional controls, *ssgR* and *ssgA* mutants were complemented by wild-type copies of *ssgR* and *ssgA*, respectively. The results are shown in Figure 5A. As was anticipated, the *ssgA* and *ssgR* mutants could be complemented by the introduction of wild-type *ssgA* (on pGWS10, giving transformant GSA4) and *ssgR* (on pGWR1, transformant GSR2), respectively. This underlines that the non-sporulating phenotype of the *ssgR* and *ssgA* mutants is solely due to the respective gene deletions. Interestingly, morphological differentiation of the *ssgR* mutant could be fully restored by the expression of *ssgA* under the control of the SsgR-independent *ermE* promoter (GSR4 in Figure 5A). Therefore, the sporulation deficiency of the *ssgR* mutant is due to the lack of sufficient SsgA. In contrast, introduction of multiple copies of *ssgA* behind its own promoter did not complement the *ssgR* mutant (GSR3 in Figure 5A), providing additional evidence that the natural *ssgA* promoters are both inactive in an *ssgR* mutant background. Finally, as expected, introduction of pGWR1 in the *ssgA* mutant (transformant GSA5) had no apparent effect on development. As a control, all plasmids were transformed to the parental strain M145; these transformants showed normal sporulation, indicating that none of the plasmids had a negative effect on

sporulation. To test if *S. griseus* *ssfR* could also restore sporulation to the *ssgR* mutant, we introduced pGWR5 into GSR1. pGWR5 is essentially the same plasmid as pGWR1, except that it contains *ssfR* from *S. griseus* B2682 instead of *ssgR*. The non-sporulating phenotype of the resulting transformants shows that introduction of *ssfR* fails to complement the *S. coelicolor* *ssgR* mutant (Figure 5B).

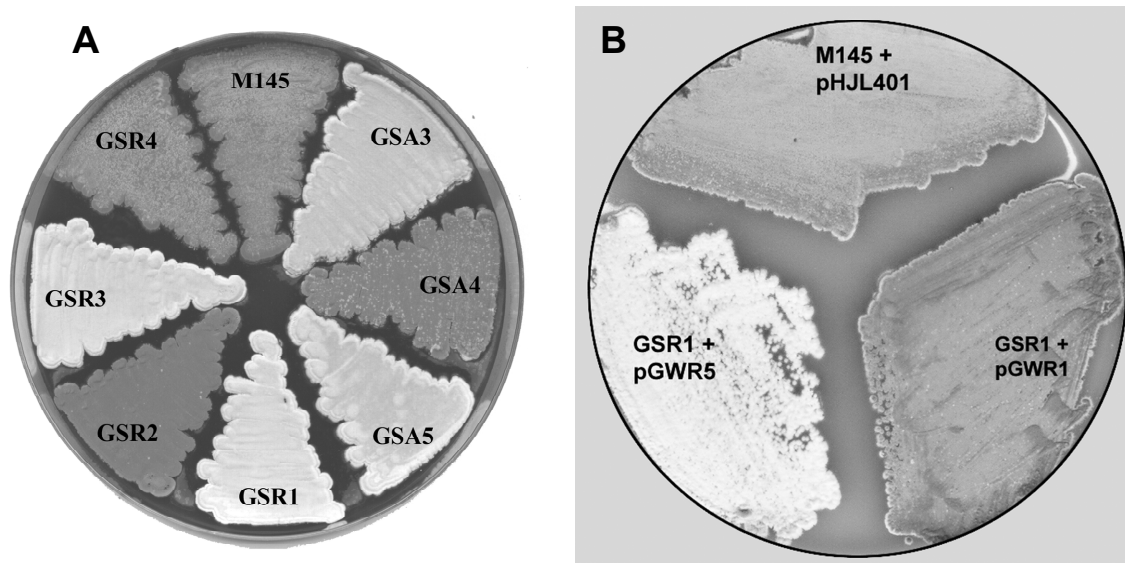


Figure 5. Complementation of the *S. coelicolor* *ssgR* mutant. **A.** Complementation of the sporulation-defective phenotypes of the *S. coelicolor* *ssgA* and *ssgR* mutants. Strains used in this experiment: **M145**, Wild-type *S. coelicolor*; **GSA3**, *ssgA* disruption mutant; **GSA4**, *ssgA* mutant complemented by pGWS7; **GSA5**, *ssgA* mutant harbouring pGWR1; **GSR1**, *ssgR* in-frame deletion mutant; **GSR2**, GSR1 complemented by *ssgR* (pGWR1); **GSR3**, GSR1 harbouring pGWS10 (*ssgA* with its own promoter); **GSR4**, GSR1 complemented by *ssgA* expressed from the SsgR-independent *ermE* promoter (pGWS7). Note that *ssgA* can only restore sporulation to the *ssgR* mutant if it is expressed from a promoter that is independent of intact SsgR. **B.** Complementation experiment using *S. griseus* *ssfR*. Transformants of GSR1 (the *ssgR* mutant of *S. coelicolor* M145) with pGWR5 (containing *S. griseus* *ssfR*) are shown, which fail to sporulate. Controls are full complementation of GSR1 by pGWR1, and wild-type level of sporulation by *S. coelicolor* M145 harbouring control plasmid pHJL401. For both experiments, strains were grown on SFM and incubated at 30°C for 7 days.

SsgR binds directly and specifically to the *ssgA* promoter region

To analyse if SsgR could directly bind to the upstream region of *ssgA*, expression constructs were made to express and purify sufficient quantities of the protein for DNA binding assays (Materials and Methods). Construct pGWR11 was designed to produce full-length SsgR (241 aa, called SsgR-241) in *E. coli*, containing an N-terminal His₆-tag for purification using Ni-NTA chromatography.

However, the protein was fully insoluble, and after purification of the protein from inclusion bodies using denaturing procedures, renaturation resulted in complete precipitation of the protein, with no detectable soluble protein (as judged by SDS-PAGE). The TMPred program identified a likely transmembrane (TM) region in SsgR, encompassing amino acids 185-205 (also present in the homologues of *S. avermitilis* and *S. griseus*). To remove the putative TM domain from the protein, we designed construct pGWR12 to express a shortened SsgR protein (155 aa, called SsgR-155). This resulted in soluble protein, which eluted from a Ni-NTA column in buffer containing 150 mM imidazole. The protein fraction containing pure SsgR-155 was tested for DNA binding activity in a mobility shift assay. Binding was observed to the DNA fragment SsgA-S1, which contains the -195/+45 section relative to the start of *ssgA* (Figure 2AB), producing a single and discrete DNA-protein complex (band C in Figure 6). Complete binding (lane 2) was observed with around 25 ng (approximately 1 pmol) of purified protein; at a 1:12 dilution (lane 4) virtually all DNA was in the unbound state, although some residual binding activity could be observed.

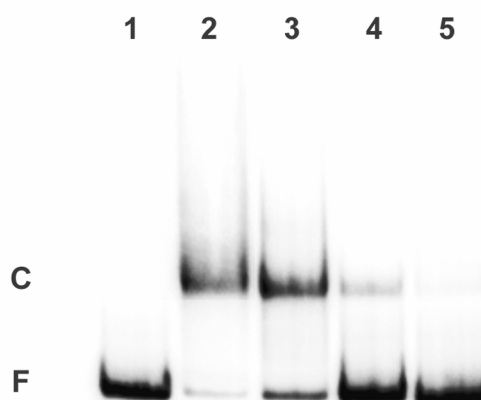


Figure 6. Binding of SsgR to the *ssgA* promoter region

Mobility shift assay showing binding of truncated His₆-tagged SsgR-155 to the *ssgA* upstream region (-195/+41 relative to the start of *ssgA*). The DNA fragment used was ³²P-end-labelled *ssgA*-S1 (Figure 2A). Lanes: (1) control (no protein); (2) 25 ng SsgR-155; (3) 6 ng SsgR-155; (4) 2 ng SsgR-155; (5) same as lane 3, but with 5-fold excess of cold probe. **F**, free (unbound) fragment; **C**, complex of DNA and purified His₆-tagged SsgR-155. Similar results were obtained when a smaller probe was used, which encompassed the -195/-75 region relative to the *ssgA* translational start site.

The mobility shift could be fully reversed by the addition of excess of cold probe (figure 6, lane 5), while the presence of a large excess of pBR322 did not affect binding. A fragment containing a shorter part of the *ssgA* promoter region (up to -75 relative to the start of *ssgA*) bound SsgR with similar affinity, narrowing down the SsgR binding site to the region between -195 and -75 relative to the start of *ssgA*. Specificity of SsgR for the *ssgA* promoter was underlined by its failure to bind to the SsgR promoter or to DNA fragments harbouring the start and middle part of the *ssgR* gene (not shown).

Transcription of *ssgA* and *ssgR* in sporulation (*whi*) mutants of *S. coelicolor*

Since *ssgA* controls the formation of sporulation septa, and expression of both *ssgA* and *ssgR* is induced during sporulation, a possible dependence of their transcription on the *S. coelicolor* sporulation genes *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ* was investigated. RNA from the corresponding *whi* mutants (Table 1) grown on MM agar plates with mannitol as the sole carbon source, was analysed by S1 nuclease mapping. Transcription of *hrdB*, which encodes the principal, essential σ factor of *S. coelicolor*, is expressed at a relatively constant level in *S. coelicolor*, and was monitored as an internal control (data shown in (Kelemen *et al.*, 1998)). S1 nuclease mapping of transcripts with DNA probe *ssgA*-S1 (-195/+82; Figure 2AB) revealed both *ssgA* transcripts in all *whi* mutants, showing that none of these genes is essential for *ssgA* transcription (Figure 7A). Expectedly, transcripts were absent in RNA isolated from vegetative mycelium. Developmental regulation of *ssgA* was also not significantly affected in any of the *whi* mutants, although *ssgA* transcription was slightly but reproducibly upregulated in the *whiH* mutant, which is developmentally stalled in a phase immediately prior to the onset of sporulation-specific cell division (Ryding *et al.*, 1998). Transcriptional analysis of *ssgR* in the *whi* mutants was done with DNA probe *ssgR*-S1, encompassing the -338/+47 region relative to the *ssgR* translational start site (Figure 2A). Similar to *ssgA*, *ssgR* appeared slightly upregulated in the *whiH* mutant, but was otherwise not significantly affected in any of the *whi* mutants (Figure 7B).

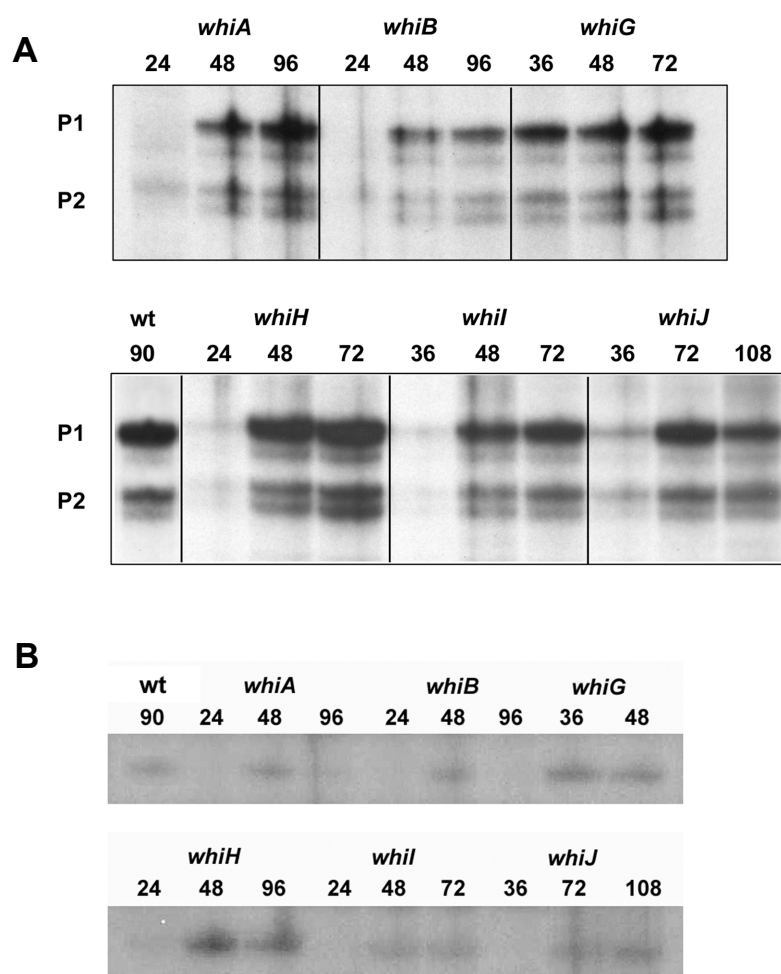


Figure 7. Transcriptional analysis of *ssgA* and *ssgR* in *whi* mutants of *S. coelicolor*. RNA was isolated from surface-grown MM/Mannitol cultures of the *S. coelicolor* sporulation mutants *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ*, and analysed by nuclease S1 mapping. For each mutant, the first time point corresponds to the transition from vegetative to aerial growth, and the second and third time point to aerial growth. 7A. Transcript analysis of *ssgA*; 7B. Transcript analysis of *ssgR*. The probes used were *ssgR*-S1 and *ssgA*-S1, respectively (Figure 2A). As a control we used wild-type *S.coelicolor* A3(2), as this is the congenic parent of the *whi* mutants described in the paper. The results were essentially the same as those shown in Figures 3 and 4 (data not illustrated).

RT-PCR analyses of developmental genes

In an independent set of experiments, we performed transcript analysis on RNA isolated from surface-grown MM Mannitol cultures by RT-PCR (Figure 8). To establish whether *ssgR* is important for earlier stages of aerial development, we analysed the transcription of the crucial regulatory gene *whiG*. The outcome of was very similar for M145 and the *ssgR* mutant, with approximately constant

transcript levels of *whiG* relative to the 16S rRNA in both strains, showing that *whiG* transcription was not significantly affected by the deletion of *ssgR* (Figure 8). Expectedly, there was a strong increase in *ssgA* transcript levels in RNA samples from surface-grown M145 towards the onset of sporulation (48 and 96 h), while we failed to detect *ssgA*-derived transcripts in the *ssgR* mutant. These results correspond well to the transcript mapping experiments presented in Figure 4A. Considering the dependence of *ssgA* on *adpA* (*bldH*) in *S. griseus*, we also analysed *ssgA* transcription in *adpA* mutant M851, which was characterised previously (Takano *et al.*, 2003). Interestingly, SsgA-derived transcripts were readily detected in this mutant; however, presence of *ssgA* transcripts in the 24 h sample (corresponding to vegetative growth) indicates that its regulation may be affected in the *adpA* mutant.

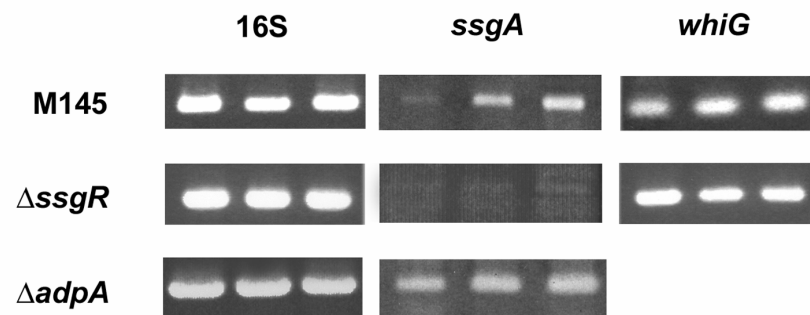


Figure 8. Transcriptional analysis of developmental genes by RT PCR.

Time points were (from left to right) 24h, 48h, and 96h, corresponding to vegetative growth, aerial growth and sporulation, respectively, for all three strains. Strains (vertical axis) were: *S. coelicolor* M145 and its *ssgR* and *adpA* mutant derivatives (GSR1 and M851, respectively). RNA was obtained from mycelium grown at 30°C on SFM plates (using a cellophane overlay). RNA (transcripts) analysed in the mutants are shown on horizontal axis (16S rRNA ("16S"), *ssgA*, and *whiG*). The RT-PCR experiments revealed normal *whiG* transcription in the *ssgR* mutant, and dependence of *ssgA* transcription on *ssgR*, but not on *adpA*. *whiG* transcription was not analysed in the *adpA* mutant. SFM agar plates were used to allow sporulation of the *ssgR* and *adpA* mutants.

DISCUSSION

***ssgR* and *ssgA* are transcribed in a growth-phase dependent manner**

Our results show that *ssgA* and *ssgR* are sporulation genes, transcribed in a growth phase-dependent manner on solid-grown cultures. A constructed *ssgR*

mutant had a phenotype very similar to that of the *ssgA* mutant published previously (van Wezel *et al.*, 2000a), showing a conditional White phenotype (no sporulation except on mannitol-containing media). The *ssgR* gene was transcribed from a single growth-phase-dependent promoter (*ssgRp*) in solid-grown cultures of *S. coelicolor* M145. While steady-state transcript levels of *ssgR* were low under the conditions tested, the activation of *ssgRp* coincided with the onset of aerial mycelium formation, and transcript levels were strongly upregulated at the onset of sporulation. A very similar promoter was responsible for transcription of the *ssgR* orthologue in *S. griseus* (S. Horinouchi, pers. comm.). The sequence TAGAGT separated by 5-6 nt from the transcriptional start site, constitutes a -10 promoter consensus sequence that may be recognised by the principal sigma factor σ^{hrdB} , but a -35 consensus sequence for this probable promoter could not be identified.

ssgA is transcribed from two promoters (*ssgA* p1 and *ssgA* p2), separated by approximately 15 nt, suggesting overlap between them. The absence of read-through indicates that on solid-grown cultures, *ssgA* is transcribed only from these two promoters. Earlier experiments with liquid-grown cultures showed that *ssgA* is expressed at a low level and only after nutritional downshift, and co-regulated with *ssgR* from a full-length transcript (van Wezel *et al.*, 2000a). This experiment was repeated, again showing read-through from the *ssgR* promoter (data not illustrated). This discrepancy remains unexplained, but could reflect interesting differences between liquid- and solid-grown cultures. Both promoters were developmentally controlled, and maximal transcript levels were reached one time point later than those of *ssgR* transcripts, at a time corresponding to sporulation. Such a sporulation-specific expression conforms to a role in the activation of sporulation-specific cell division. Despite the similar developmental regulation and strength of *ssgA* p1 and p2, there is no obvious similarity between the respective promoter sequences.

Comparison to the *S. griseus* *ssgA* promoters revealed almost complete conservation between *ssgA*^{Sc} p1 and *ssgA*^{Sg} p2 (Figure 4C). This sequence is also highly conserved in *S. avermitilis*. However, *ssgA*^{Sc} p2 shows no sequence similarity to *ssgA*^{Sg} p1. Apparently, some elements of *ssgA* regulation are shared between *S. coelicolor* and *S. griseus*, while others are different (see the final paragraph).

Expression of *ssgA* is activated by SsgR

Our failure to detect *ssgA* transcripts in the *ssgR* mutant indicated that the growth-phase-dependent induction of *ssgA* transcription is dependent on SsgR. This is in accordance with the observation that *ssgR* transcription is strongly induced one time point earlier than that of *ssgA*, at a time between aerial hyphae formation and the onset of sporulation; transcripts of *ssgA* itself were induced when sporulation was initiated. Interestingly, *ssgA* transcripts were more abundant than those of *ssgR*, as seen in multiple independent experiments. In further support of activation of *ssgA* transcription by SsgR, introduction of a plasmid expressing *ssgA* from the constitutive and SsgR-independent *ermE* promoter restored a wild-type phenotype to the *ssgR* mutant of *S. coelicolor*, while a similar plasmid harbouring *ssgA* with its natural promoter failed to complement the mutant. This suggests that while it is possible that other genes are regulated by *ssgR*, the sporulation deficiency of the *ssgR* mutant is solely due to lack of SsgA, and that *ssgA* transcription fully depends on SsgR. This is also supported by the observation that fragmentation of *S. coelicolor* in submerged culture, typical of transformants expressing *ssgA*, was induced by the introduction of pGWR3, a multi-copy plasmid harbouring only *ssgR* (data not illustrated). Interestingly, introduction of a plasmid harbouring *S. griseus* *ssfR* did not restore sporulation to the *ssgR* mutant, indicating significant functional differences between the respective gene products. SsgR of *S. coelicolor* belongs to the family of IclR-like transcriptional regulators (Zhang *et al.*, 2002). These proteins are characterised by an N-terminal DNA binding domain, and a C-terminal ligand binding domain, which is also important for oligomerization of the protein. Typically, binding of the substrate induces a conformational change, releasing the protein from its target sequence (Yamamoto and Ishihama, 2003; Zhang *et al.*, 2002). As an exception, the *Streptomyces* SsgR/SsfR proteins have a predicted transmembrane helix. Our experiments suggest that cleavage of this putative TM domain is required for its solubility, as only a truncated version of SsgR (155 amino acids long) was soluble. Whether SsgR also binds a substrate is unclear, but considering that on several occasions it was shown that the C-terminal section of IclR-type proteins is required for interaction between monomers, its removal is expected to affect the mode of binding of SsgR. The truncated SsgR-155 produced a single mobility shift on a DNA fragment

containing the *ssgA* promoter region, indicating that the SsgR dependence of the transcription of *ssgA* is mediated through direct *trans*-activation by SsgR. The binding site was narrowed down to the -195/-75 section relative to the *ssgA* gene. The region around the stop codon of *ssgR* constitutes a possible binding site, as it harbours a for streptomyces unusually A/T-rich sequence (TGAAAACTCACTCC) that shows significant similarity to the consensus sequence TGAAAA(A/T)NNTTTPyPy for IclR-type binding sites (Pan *et al.*, 1996; Zhang *et al.*, 2002). Typically, IclR-type regulators bind to multiple IclR boxes, although we failed to observe additional binding by SsgR. We cannot rule out the possibility that this different behaviour is due to the absence of the C-terminal (interaction) part of the protein, which we had to remove to obtain soluble SsgR protein. We are currently analysing the mode of action and the activation of SsgR in more detail.

Interdependence of *ssgRA* and other developmental genes

In *S. coelicolor* *whiA*, *whiB*, *whiG*, *whiI*, and *whiJ* mutants, the level and timing of *ssgA* and *ssgR* transcription are comparable to those found in the parental wild-type *S. coelicolor* A3(2) as well as those in *S. coelicolor* M145. Considering that *ssgRA* transcripts are found in all "early" *whi* mutants analysed, this strongly suggests that the gene cluster is not controlled by the classical *whi* genes. In a reverse experiment, we also showed that *whiG* is not significantly affected in an *ssgR* mutant, providing further support for the mutual independence of the *ssgRA* cluster on the one hand, and the early *whi* genes on the other. This independence is apparently supported by the observation that the white phenotype of the *ssgA* and *ssgR* mutants is medium-dependent, and that these genes as well as most of the *ssgA*-like genes are under carbon catabolite control, which is not the case for other *whi* genes (Chapter IV of this thesis). Further transcriptional analysis is required to assess whether the observed upregulation of *whiH* in the *ssgA* and *ssgR* mutants is significant. A possible explanation for the necessity of *whi* gene-independent expression of *ssgRA* is that the genes are also involved in the activation of sporulation-specific cell division under conditions where an aerial mycelium is not produced, in particular during submerged sporulation. Indeed, *ssgA* is essential for this process in *S. griseus*

(Kawamoto *et al.*, 1997), and its overexpression results in hyperseptation and a low level of submerged sporulation in *S. coelicolor* (van Wezel *et al.*, 2000a).

Differential regulation of *ssgRA* in *S. coelicolor* and *S. griseus*

How does the situation in *S. coelicolor* compare to that in *S. griseus*? In the latter organism, *ssgA* is dependent on AdpA, an A-factor-dependent transcriptional activator that is essential for development and streptomycin production (Ohnishi *et al.*, 2002). In contrast, *S. coelicolor* *scbA* mutants fail to produce the A-factor-like γ -butyrolactone SCB1, but show normal sporulation (Takano *et al.*, 2001), and *adpA* mutants sporulate normally on mannitol-containing media (Takano *et al.*, 2003). Since such sporulation is not possible in the absence of *ssgA*, its transcription is most probably not dependent on SCB1 or AdpA, which was confirmed by our observation that *ssgA* transcripts could be readily detected in an *S. coelicolor* *adpA* mutant. Rather, in *S. coelicolor* both *ssgA* promoters are directly dependent on activation by SsgR. While in *S. griseus* the activity of *ssgA*^{Sg} p2 (virtually identical to *ssgA*^{Sc} p1, Figure 4C) also depends on SsfR, that of *ssgA*^{Sg} p1 does not, which results in significant expression of *ssgA* in an *ssfR* mutant (Yamazaki *et al.*, 2003). Therefore, regulation is clearly different in *S. griseus*, also illustrated by the significantly higher *ssgA* transcript levels in this organism, which is a prerequisite for submerged sporulation (van Wezel, unpublished data).

In summary, several important differences exist between the regulation of the *ssgA* orthologues in *S. coelicolor* (^{Sc}) and that in *S. griseus* (^{Sg}):

- (1) *ssgA*^{Sc} fully depends on activation by SsgR, while activity of only one of the *ssgA*^{Sg} promoters is reduced in an *ssfR* mutant.
- (2) The SsgR^{Sc} and SsfR^{Sg} proteins may be functionally different, as the gene from *S. griseus* fails to complement the *S. coelicolor* *ssgR* mutant.
- (3) It is unlikely that the A-factor-like molecule SCB1 plays a role in the regulation of *ssgA*^{Sc}, while A-factor is essential for the regulation of *ssgA*^{Sg} (through AdpA). This may at least partially explain the different impact of A-factor on the development of these organisms, because SCB1 (and on mannitol also AdpA) mutants of *S. coelicolor* sporulate normally, while A-factor is essential for sporulation of *S. griseus* (Takano *et al.*, 2001)).

(4) There is no detectable transcription of *ssgA*^{Sc} in submerged culture under normal conditions, while it is strongly expressed in *S. griseus* (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a; van Wezel *et al.*, 2000b).

The expression level of *ssgA* has a major impact on mycelial morphology of both organisms (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a; van Wezel *et al.*, 2000b), and the different expression levels of *ssgA* in these organisms thus provides a possible explanation for their strong morphological differences in submerged cultures, and this may be one of the main reasons why *S. griseus* is able to sporulate in submerged culture, but not *S. coelicolor*. We are currently investigating the exact roles of SsgA and SsgR in the sporulation process, with focus on their cellular localizations, protein structures and molecular modes of action.

Acknowledgements

We are very grateful to S. Horinouchi for sharing unpublished data and for providing valuable comments on the manuscript, to E. Takano for providing *adpA* mutant M851, to R. Amons (LUMC, Leiden) for N-terminal amino acid sequencing of SsgR, and to K. Flärdh, B. Keijser, B. Kraal, E. Takano, and E. Vijgenboom for stimulating discussions. This work was supported by a grant from the Royal Netherlands Academy of Arts and Sciences (KNAW) to GPvW, and from the Dutch applied research council (STW) to BT.

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; Flårdh *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 occurring 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 addressed, 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 Δ glk 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
<i>S. coelicolor</i> M145	Wild type <i>S. coelicolor</i> A3(2)	(Kieser <i>et al.</i> , 2000)
<i>S. coelicolor</i> M512	M145 $\Delta actII$ -ORF4 $\Delta redD$	(Floriano and Bibb, 1996)
<i>S. coelicolor</i> J1915	M145 Δglk	(Kelemen <i>et al.</i> , 1995)
<i>S. coelicolor</i> M512 $\Delta glkA$	M512 and J1915 protoplast fusion	This work
<i>S. coelicolor</i> GSA2	M145 harboring pGWS4SD	(van Wezel <i>et al.</i> , 2000a)
<i>S. coelicolor</i> GSA3	M145 $\Delta ssgA$	(van Wezel <i>et al.</i> , 2000a)
<i>S. coelicolor</i> GSB1	M145 $\Delta ssgB$	(Keijser <i>et al.</i> , 2003)
<i>S. coelicolor</i> J2401	M145 $\Delta whiA$	(Flärdh <i>et al.</i> , 1999)
<i>S. coelicolor</i> J2402	M145 $\Delta whiB$	(Flärdh <i>et al.</i> , 1999)
<i>S. coelicolor</i> J2400	M145 $\Delta whiG$	(Flärdh <i>et al.</i> , 1999)
<i>S. coelicolor</i> J2210	M145 $\Delta whiH$	(Ryding <i>et al.</i> , 1999)
<i>S. coelicolor</i> J2450	M145 $\Delta whiI$	(Ainsa <i>et al.</i> , 1999)
<i>S. coelicolor</i> C77	<i>S. coelicolor</i> A3(2) <i>whiJ</i> locus 77	(Ryding <i>et al.</i> , 1999)
<i>S. griseus</i> B2682	Wild type <i>S. griseus</i>	
<i>S. griseus</i> SY1	<i>S. griseus</i> mutant strain	(Kawamoto and Ensign, 1995a)
<i>S. albus</i> DSM40313	Wild type <i>S. albus</i>	
<i>S. clavuligerus</i> NRRL8165	Wild type <i>S. clavuligerus</i>	
<i>S. collinus</i> DSM40733	Wild type <i>S. collinus</i>	
<i>S. diastatochromogenes</i> Tü1062	Wild type <i>S. diastatochromogenes</i>	
<i>S. filipinensis</i>	Wild type <i>S. filipinensis</i>	
<i>S. fradiae</i> Tü1222	Wild type <i>S. fradiae</i>	
<i>S. granaticolor</i>	Wild type <i>S. granaticolor</i>	
<i>S. lividans</i> 1326	Wild type <i>S. lividans</i>	
<i>S. ramocissimus</i>	Wild type <i>S. ramocissimus</i>	
<i>S. roseosporus</i> ATCC31568	Wild type <i>S. roseosporus</i>	
<i>S. venezuelae</i> ATCC15439	Wild type <i>S. venezuelae</i>	
<i>Streptomyces</i> species Wlb19	Novel soil isolate	This work
<i>Streptomyces</i> species Che26	Novel soil isolate	This work
<i>Streptomyces</i> 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 *EcoRI-BamHI* fragments, with the *BamHI* 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 <i>ssgA</i>	This work
pGWS221	-512/+12 region relative to the start of <i>ssgR</i>	This work
pGWS216	-592/+21 region relative to the start of <i>ssgB</i>	This work
pGWS217	-216/+35 region relative to the start of <i>ssgC</i>	This work
pGWS211	-291/+22 region relative to the start of <i>ssgD</i>	This work
pGWS110	-223/+38 region relative to the start of <i>ssgE</i>	This work
pGWS218	-401/+99 region relative to the start of <i>ssgF</i>	This work
pGWS219	-467/+62 region relative to the start of <i>ssgG</i>	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 OD₆₀₀ 0.3-0.4, from which a 10 ml sample was taken, designated T₀. 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 (T₁) and 60 min (T₂).

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 the 16S 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 4°C 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 30,000x *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* (SsgA₁₄₅) 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	<u>GATGAATTC</u> <i>CAGCATCTGAAA</i> ACTCACTCCTTGTGATC	-140	<i>ssgA</i>
ssgA-seqR	<u>GATCAAGCTT</u> <i>CTGCTGCTGTTCTC</i> (C/G)ATCGC(C/G)CAGA	+709	<i>ssgA</i>
PssgA-for	<u>GTCGAATTC</u> <i>CCACCATGGCGCGCTGGCGCGAC</i>	-619	<i>ssgA</i>
PssgA-rev	<u>CTGGGATCC</u> <i>CCCCGGGTCTCGTAGCGCAGCTC</i>	+70	<i>ssgA</i>
PssgR-for	<u>GTCGAATTC</u> <i>GGACTGCCGTGGTGGGTGAAGTG</i>	-512	<i>ssgR</i>
PssgR-rev	<u>GTCGGATCC</u> <i>CGCCCGCTGCACGGAGCCGATC</i>	+12	<i>ssgR</i>
PssgB-rev	<u>GTCCGAGCTC</u> <i>GCTCTCCCGAGTGATCACTGGTC</i>	-592	<i>ssgB</i>
PssgB-for	<u>GTCGGATCC</u> <i>GCAGCTGACCGTGGTGTGAT</i>	+21	<i>ssgB</i>
PssgC-rev	<u>GTCGGAATTC</u> <i>GTCTGACGCCGGGTTACCCGAGGT</i>	-216	<i>ssgC</i>
PssgC-for	<u>GTCGGATCC</u> <i>TGCACGACCAGGGTCTTGTCAC</i>	+35	<i>ssgC</i>
PssgD-for	<u>GTCGAATTC</u> <i>GTCCCGTGCCTGCGCTGCTTCCC</i>	-291	<i>ssgD</i>
PssgD-rev	<u>GTCGGATCC</u> <i>ACTGCTCGATGACGGTGGAC</i>	+22	<i>ssgD</i>
PssgE-for	<u>GTCGAATTC</u> <i>GAGGTCGGGGCGTTGATGAATC</i>	-223	<i>ssgE</i>
PssgE-rev	<u>GTCGGATCC</u> <i>AGGATGTGGGCTCGTGCCTAC</i>	+38	<i>ssgE</i>
PssgF-for	<u>GTCGAATTC</u> <i>CGCGTGGGCCTGACCGACATGAC</i>	-401	<i>ssgF</i>
PssgF-rev	<u>GTCGGATCC</u> <i>CTCGAGAGCGCCCGTCATCTG</i>	+99	<i>ssgF</i>
PssgG-for	<u>GTCGAATTC</u> <i>CCTCGACCGGGTCTCGTCAAG</i>	-467	<i>ssgG</i>
PssgG-rev	<u>GTCGGATCC</u> <i>AGGACGAGCCTGAGCTCCAG</i>	+62	<i>ssgG</i>

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 *ssgAp*, *ssgEp* or *ssgGp* was low. In contrast, *ssgDp* 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 *ssgDp* 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 *ssgDp* 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*.

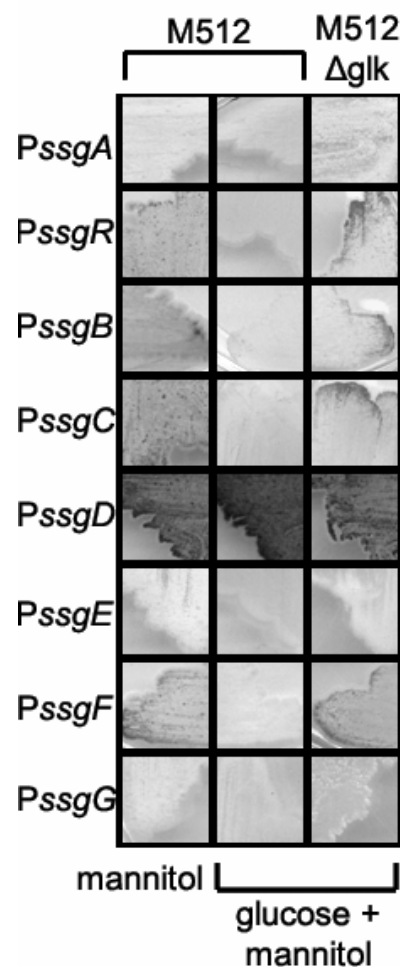


Figure 1. Dependence of the activity of *ssg* gene promoters on different carbon sources (For colour figure see Appendix A). The ability to stimulate Red production by the upstream fragments of *ssgA-G* and *ssgR* (*ssgXp*) was tested on minimal medium containing mannitol (left panel) or mannitol + glucose (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).

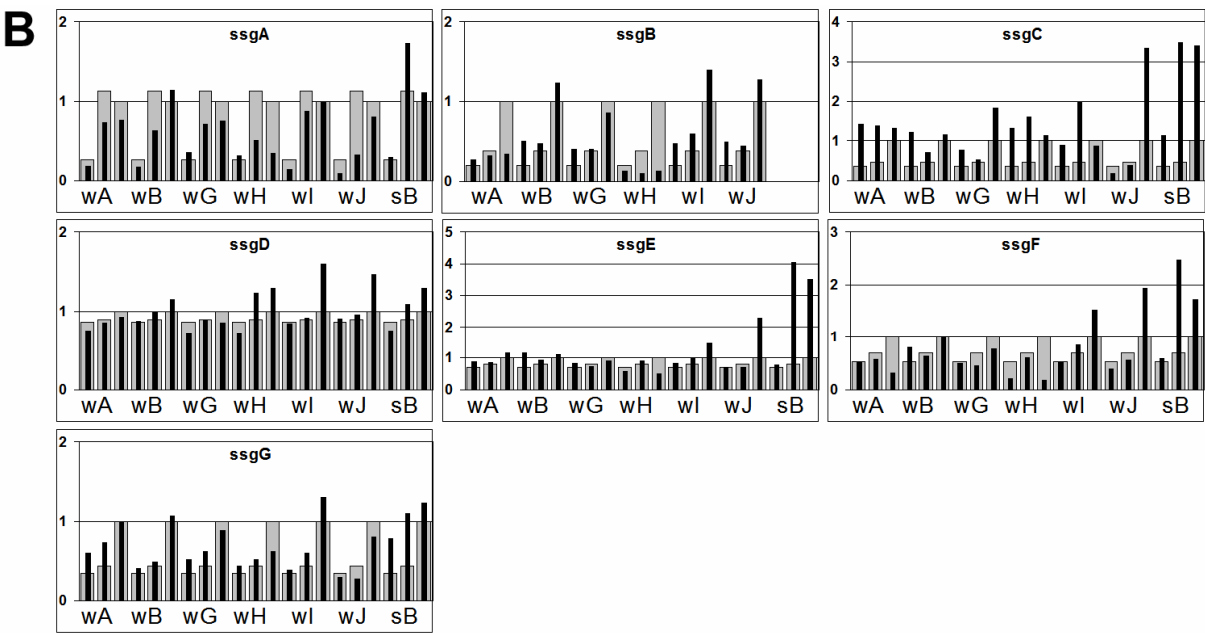
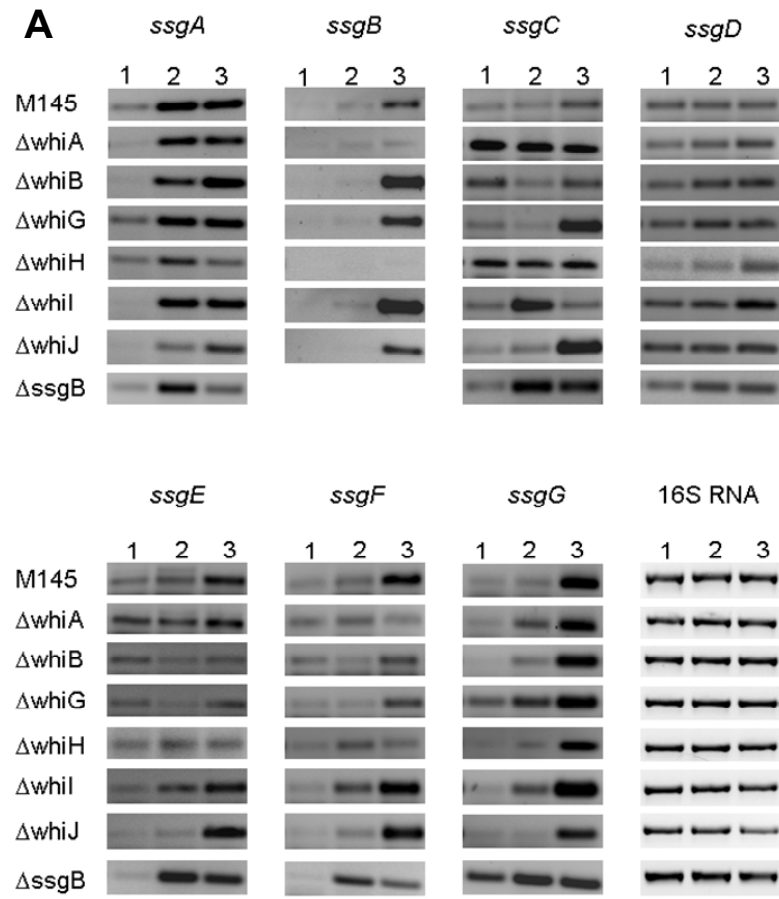


Figure 2. Transcriptional analysis in *S. coelicolor whi* mutants. **A.** Representative picture of semi-quantitative RT-PCR results of *ssgA-G* and 16S rRNA (top) in RNA purified from the parental strain M145 and mutants of *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, *whiJ* and *ssgB* (left). For all strains, 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 subtracted, 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** ($\Delta whiA$), **wB** ($\Delta whiB$), **wG** ($\Delta whiG$), **wH** ($\Delta whiH$), **wI** ($\Delta whiI$), **wJ** ($\Delta whiJ$) and **sB** ($\Delta ssgB$).

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 OD₆₀₀ 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 (T₀), *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 (T₁ and T₂) *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.

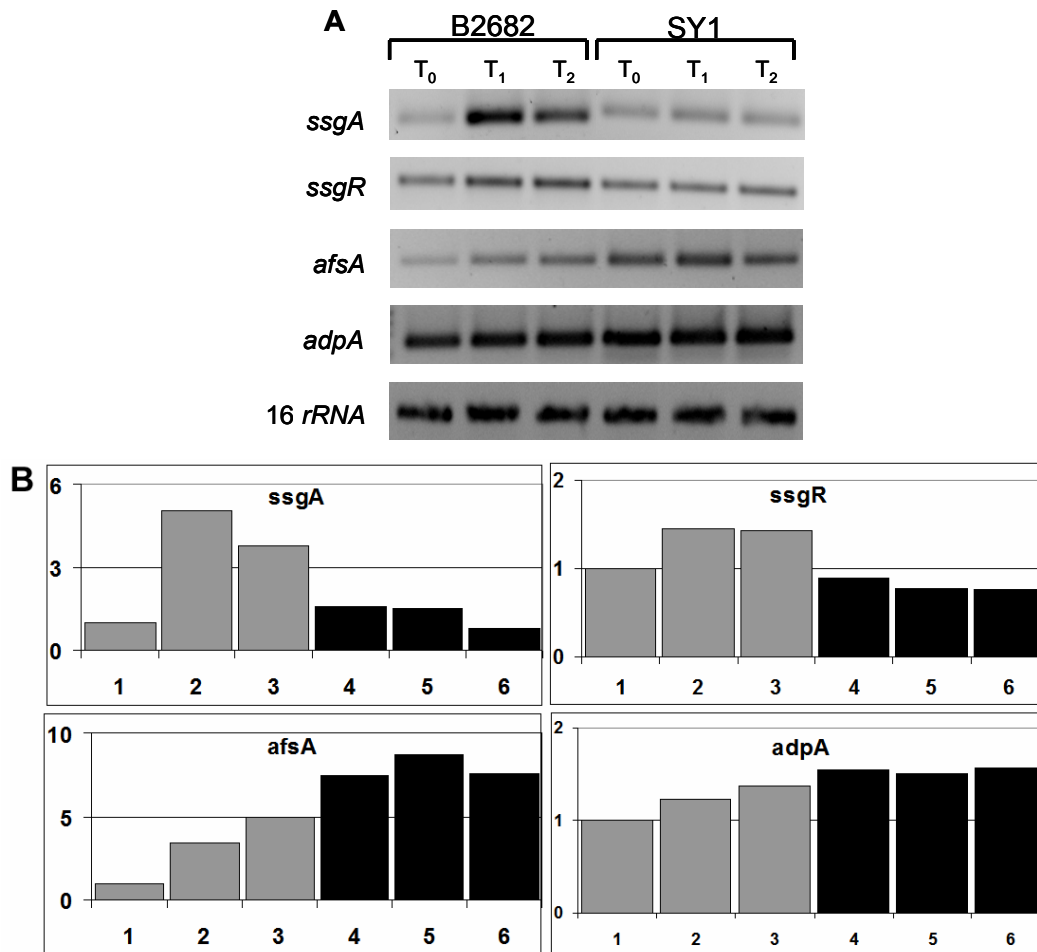


Figure 3. Transcriptional analysis in *S. griseus* B2682 and SY1. A. Semi-quantitative RT-PCR on *ssgA*, *ssgR*, *afsA*, *adpA* and 16S rRNA (control) in RNA purified from liquid cultures of *S. griseus* B2682 and SY1. RNA extracts were purified from rich liquid (pre-)cultures (T₀), and after nutritional shift-down after 30 (T₁) and 60 min (T₂). **B.** Bar graphs showing quantified intensities of the bands, with the background value subtracted. For each gene, bar values are relative to the T₀ value, which was set to one. On the X-axis of each graph, bar numbers 1-3 correspond to T₀, T₁ and T₂ of B2682 (grey bars), and bar numbers 4-6 correspond to T₀, T₁ and T₂ 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).

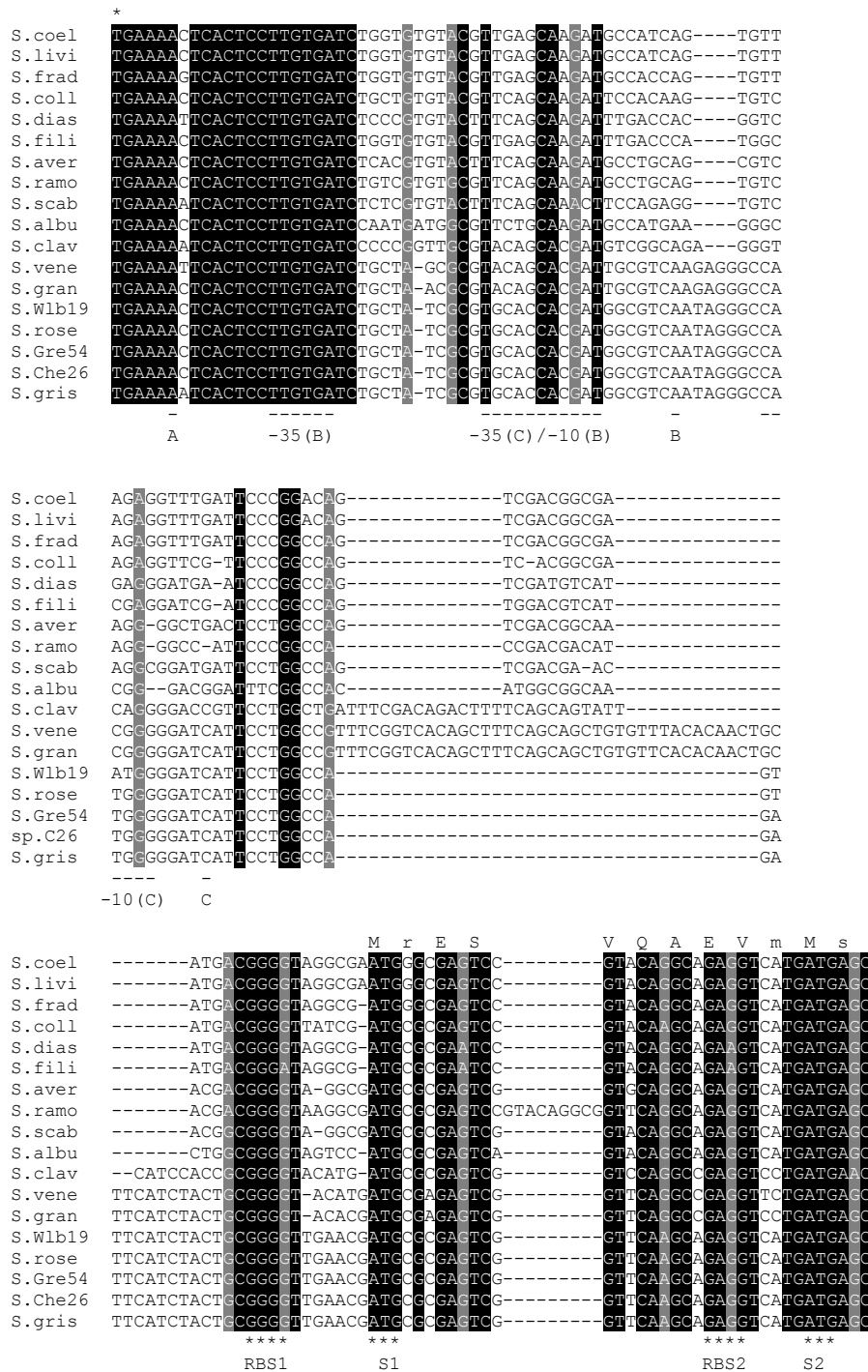


Figure 4. Alignment of *ssgA* promoter regions. Only completely conserved nucleotides are shaded; nucleotides shaded in light grey refer to conserved purines. The two alternative start codons (S1 and S2) for *ssgA* and their respective ribosome binding sites (RBS1 and RBS2) are indicated below the aligned 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 SsgA₁₄₅). Western analysis indeed revealed a protein band of the same length as SsgA₁₄₅ 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 SsgA₁₄₅ 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.

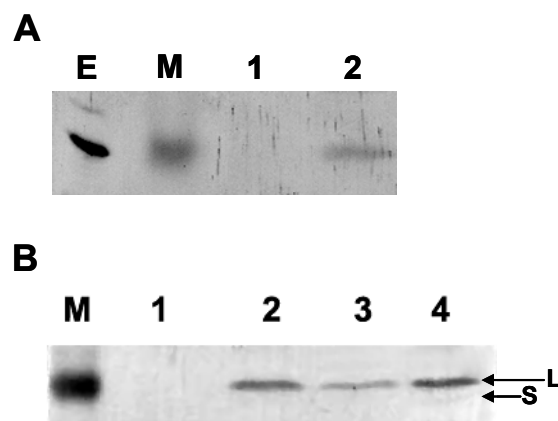


Figure 5. Western blot analysis of *S. griseus* SsgA. Western blots stained with antibodies raised against *S. griseus* SsgA. Lanes (M) pre-stained marker band (approx. 16 kDa; Bio-Rad), (1) GSA3, (2) GSA2, (3) B2682, (4) SY1, (E) *E. coli* purified SsgA₁₄₅. **A.** SsgA detected in GSA2 (lane 2) runs at the same height as the SsgA protein of 145 amino acids (SsgA₁₄₅) 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 SsgA₁₄₅ is produced abundantly. A second faint band is detected in extracts of SY1 (lane 4), which is perhaps SsgA₁₃₅ (arrow with "S").

Interestingly though, an additional fainter band was observed in SY1 below the band corresponding to SsgA₁₄₅. Perhaps this band is the result of modification or proteolysis of SsgA₁₄₅. Another possibility is that this band may reflect the production of SsgA₁₃₅, 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 streptomyces generally share between 80 to 90% end-to-end sequence identity, with usually few differences occurring 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).

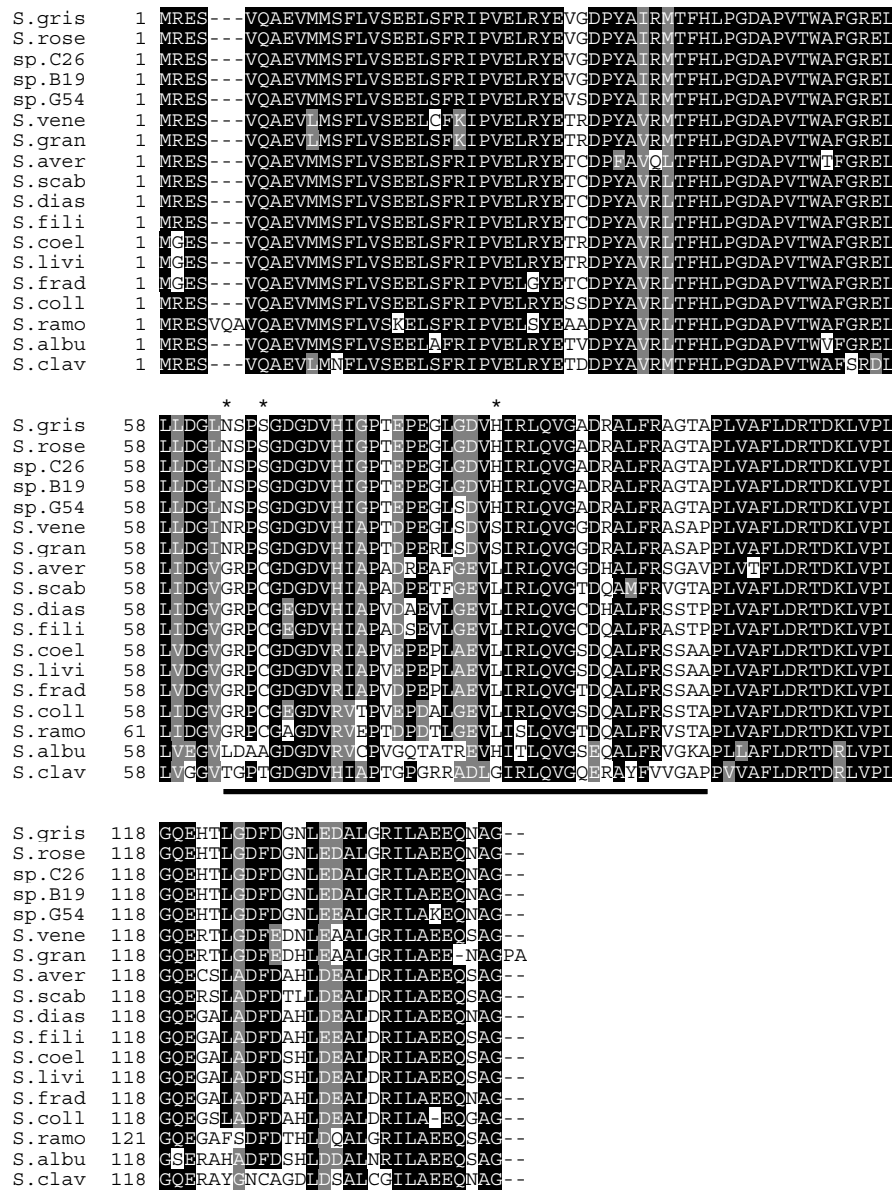


Figure 6. Alignment of SsgA orthologues. Residues conserved in at least 80% of the sequences are shaded; identical residues are shaded in black, residues with similar properties are shaded in light grey. Regions with higher variability (residues 63-102 and residues 121-stop) are underlined. Residues 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.

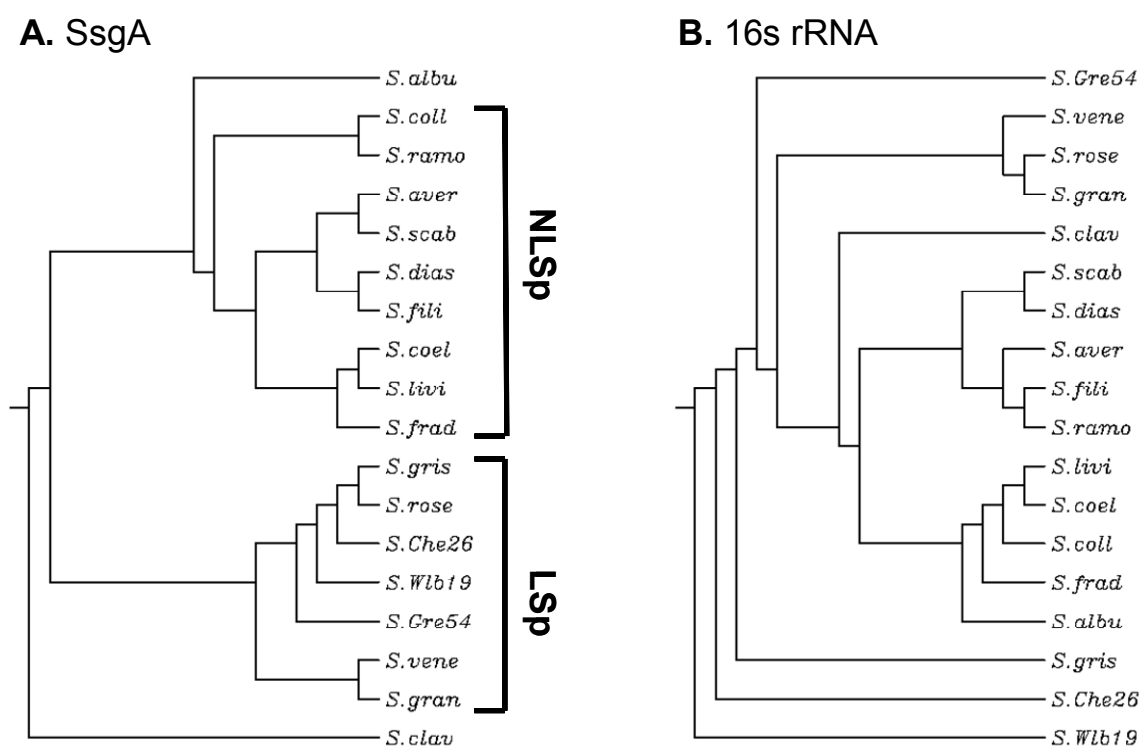


Figure 7. Phylogenetic tree of SsgA proteins and 16S rRNA sequences. Phylogenetic trees are shown for SsgA (A) and 16S rRNA (B) from 18 *Streptomyces* strains (see Results section). Two major branches of SsgA proteins are indicated, namely: SsgA orthologues from strains that produce spores in liquid-culture (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 streptomyces 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 streptomyces) 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, streptomyces, 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.

Chapter V

Characterization of the sporulation control protein SsgA by use of an efficient method to create and screen random mutant libraries in streptomyces

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and Gilles P. van Wezel

Appl Environ Microbiol (2007) 73: 2085-2092

ABSTRACT

Filamentous actinomycetes are commercially widely used as producers of natural products. However, the mycelial life-style of actinomycetes has been a major bottleneck in their commercialization, and screening is difficult due to the poor growth in microtitre plates. We previously demonstrated that the enhanced expression of the cell division activator protein SsgA results in fragmented growth of streptomycetes, with enhanced growth rates and improved product formation. We here describe a novel and efficient method to create, maintain and screen mutant libraries in *Streptomyces*, and its application for the functional analysis of *S. coelicolor ssgA*. The variants were amplified directly from deep-frozen biomass suspensions. Around 800 *ssgA* variants - including single amino acid substitution mutants for over half of all SsgA residues - were analysed for their ability to restore sporulation to an *ssgA* mutant. The essential residues were clustered in three main sections, and hardly any were in the carboxy-terminal third of the protein. The majority of the crucial residues is conserved among all SsgA-like proteins (SALPs). However, the essential residues L29, D58 and S89 were conserved only in SsgA and not in other SALPs of *S. coelicolor*, suggesting an SsgA-specific function.

n.b. At the time of publication, translation of SsgA was thought to start from the third of three in frame ATG start codons (see Chapter IV). The numbering of the amino acid residues in the text and figures of this Chapter differs by 10 residues (e.g. Y25 in this chapter refers to Y35 of SsgA₁₄₅ described in Chapter IV).

INTRODUCTION

Filamentous microorganisms are widely used as industrial producers of products such as antibiotics, anticancer agents, antifungicides and enzymes (Bennett, 1998; Demain, 1991; Hopwood *et al.*, 1995). These organisms include the eukaryotic filamentous fungi (in particular ascomycetes) and the prokaryotic actinomycetes (*e.g.* *Amycolatopsis*, *Nocardia*, *Rhodococcus* and *Streptomyces*). The market capitalization for antibiotics and enzymes totals around 28 and 2 billion dollars per year, respectively. While the industrial potential of actinomycetes is widely recognized, the filamentous life-style makes them less favourable for growth in submerged cultures, producing dense clumps or pellets (Bushell, 1988; Wardell *et al.*, 2002). Other host-specific disadvantages include slow growth rates and high viscosity of the culture broth. The latter necessitates high stirrer speeds, resulting in uncontrolled fragmentation and lysis of the mycelium. Hence, morphological engineering of streptomycetes has become increasingly important for optimization of fermentation processes and improving productivity. For this, understanding the factors that control morphogenesis in submerged cultures is of the utmost importance. On solid-grown cultures, the high G+C Gram-positive streptomycetes have an unusually complex life cycle, with mycelial growth and reproduction through spores (Chater, 1998, 2001; Hopwood, 1999). After spore germination a large vegetative mycelium of branching hyphae is produced. Upon starvation sporogenic aerial hyphae are produced on top of the vegetative mycelial mat, culminating in the simultaneous formation of many sporulation septa and eventually producing chains of mature uninucleoid spores. In submerged cultures most streptomycetes only grow by hyphal extension, although some species produce submerged spores (Kendrick and Ensign, 1983).

The SsgA-like proteins (SALPs) form a family of developmental regulators exclusively occurring in sporulating actinomycetes. The genome of *S. coelicolor* (Bentley *et al.*, 2002) contains seven SsgA-like proteins (SALPs), with specific tasks in the control of the sporulation process, each protein playing distinct and important roles (Keijser *et al.*, 2003; Noens *et al.*, 2005; van Wezel and Vijgenboom, 2004). SsgA plays an important role in the control of morphogenesis in both liquid- and solid-grown cultures and is a known activator

of sporulation-specific cell division (Jiang and Kendrick, 2000b; van Wezel *et al.*, 2000a). Overproduction of SsgA in *S. griseus* and *S. coelicolor* results in mycelial fragmentation (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a). The industrial importance of *ssgA* was recently highlighted for a number of *Streptomyces* species, and increased expression of *ssgA* from *S. griseus* resulted in fragmented growth of *S. coelicolor*, *S. lividans* and *S. roseosporus* and enhanced growth rates in batch fermentations, with strongly improved enzyme production by *S. lividans* (van Wezel *et al.*, 2006). The SALPs do not share significant sequence homology to other proteins and are one of the few protein families that lack any clear known protein motif. At the time of this work no data were available on their three-dimensional structure, however during the writing of this thesis the first crystal structure of a SALP was elucidated (discussed in Chapter II). A mutational analysis of the SsgA protein would provide more insight into the functional importance of individual residues. However, screening a library of perhaps thousands of transformants is feasible only when microtitre plate (MTP)-based screening and colony PCR are an option. While growth of streptomyces in MTPs was reported previously (Minas *et al.*, 2000), PCR-based screening is difficult and often requires mycelium pretreatment (lysis) and DNA isolation steps, and direct screening of transformants was reported only for transformants carrying high copy-number plasmids (Soliveri *et al.*, 1999; Van Dessel *et al.*, 2003).

In this work we describe an efficient method to create, propagate and screen a large strain collection of random *ssgA* mutants, and present a way to amplify and sequence the individual mutant clones directly from deep-frozen samples stored in microtitre plates. For each of the amino acid residues of the SsgA protein the importance for *in vivo* function was analysed.

MATERIALS AND METHODS

Bacterial strains and culturing conditions

The bacterial strains used in this work are listed in Table 1. *Escherichia coli* K-12 strains TG1 (Sambrook *et al.*, 1989) and ET12567 (MacNeil *et al.*, 1992) were used for propagation of plasmids and were grown and transformed using

standard procedures (Sambrook *et al.*, 1989). *Streptomyces coelicolor* M145 was obtained from the John Innes Centre strain collection. GSA3 (van Wezel 2000) is an *ssgA* null mutant of M145, created by the insertion of the spectinomycin and streptomycin resistance cassette *aadA* (Prentki and Krisch, 1984). GSA3 produces aerial hyphae but produces very few spores and only on mannitol-containing media. Introduction of a plasmid carrying *ssgA* and its promoter region fully restored sporulation (Traag *et al.*, 2004).

Table 1. Bacterial strains, plasmids and libraries.

Bacterial strains	Relevant genotype	Reference
<i>E. coli</i> JM109	F' <i>traD36 proA⁺B⁺ lacI^q</i> <i>Δ(lacZ)M15/ Δ(lac-proAB) glnV44</i> <i>e14⁻ gyrA96 recA1 relA1 endA1 thi</i> <i>hsdR17</i>	(Sambrook <i>et al.</i> , 1989)
<i>E. coli</i> ET12567	<i>dam, dcm, hsdM, hsdS, hsdR, cat,</i> <i>tet</i>	(MacNeil <i>et al.</i> , 1992)
<i>S. coelicolor</i> M145	wt (SCP1 ⁻ SCP2 ⁻)	(Kieser <i>et al.</i> , 2000)
GSA3	M145 <i>ΔssgA</i> (::aadA)	(van Wezel <i>et al.</i> , 2000a)
Plasmid/construct	Description	Reference
pIJ2925	Derivative of pUC19 with <i>Bgl</i> II sites flanking the multiple cloning site.	(Janssen and Bibb, 1993)
pHJL401	Shuttle vector with <i>ori</i> for maintenance in <i>E. coli</i> (pUC) and <i>ori</i> for replication in <i>Streptomyces</i> (SCP2*)	(Larson and Hershberger, 1986)
pGWS32	pHJL401 with a 2 kb fragment harbouring the <i>ssgR</i> and <i>ssgA</i> gene cluster (including promoter regions)	This study
pGWS278 (library)	pIJ2925 + random mutagenic PCR product of <i>ssgA</i> (reaction with additional dGTP)	This study
pGWS279 (library)	pIJ2925 + random mutagenic PCR product of <i>ssgA</i> (reaction with additional dTTP)	This study
pGWS280 (library)	pGWS32 replacing the wild-type <i>ssgA</i> sequence from the <i>Bam</i> HI site by inserts from pGWS278 and pGWS279	This study

Preparation of media, protoplast preparation and transformations were done according to (Kieser *et al.*, 2000). SFM (soya flour agar (Kieser *et al.*, 2000)) was used to make spore suspensions and to check for complementation of the GSA3 phenotype by *ssgA* mutant-containing plasmids. R2YE agar plates were used for regenerating protoplasts and selection of transformants.

Creating a library of random mutants

PCRs were performed in a iCycler™ Thermal cycler (Biorad). Oligonucleotides used in this research are listed in Table 2. PCR-mediated random mutagenesis was performed using *Taq* polymerase in the buffer supplied, with 5% dimethyl sulfoxide (DMSO), 200 µM dNTP and 100 pM of each appropriate oligonucleotide, and in the presence of 4.5 mM MgCl₂, 0.1 or 0.5 mM MnCl₂ and an additional 3.2 mM of dATP, dCTP, dGTP or dTTP, to allow occasional errors.

An approximately 540 bp fragment containing the complete *ssgA* sequence of *S. coelicolor* (nt positions +1/+540 relative to the translational start site) was amplified by PCR with primers Q5-Q6 (Table 2) and *Taq* DNA polymerase. A large excess of pGWS32 template (around 50 ng) was used to ensure enough yield within 15 cycles. A control PCR was performed (without additional agents) to make a preliminary judgement on the extent of mutations. Samples were separated on a 1% agarose gel in TAE buffer, correct products were gel-purified and the yield optimized by an additional PCR-amplification using proofreading *Pfu* polymerase (Stratagene, La Jolla, LA) in the buffer supplied.

Table 2.

Primer	DNA sequence (5' to 3')	Location end	5'
Q1	ctg gaattc tagcatcgagggcaggacatcaga	-1450	
Q5	ctg gaattcatatg agctttctcgtgtccgagg	+1	
Q6	ctg aagctt caccgctgccttgctggccgggtc	+540	
<i>ssgA</i> W41A	ccgaaggcc <u>gcg</u> gtcaccggcgcgctc	+105	
<i>ssgA</i> S89A	cgcggcggag <u>gc</u> cacggaacagcg	+254	
<i>ssgA</i> L94A	gaaggccac <u>gcg</u> ggcgcggcg	+270	
<i>ssgA</i> P106A	cctgccccag <u>gcgc</u> caccagcttg	+308	

Oligonucleotides used in this study. Restriction sites used for cloning presented in bold face. Restriction sites: **gaattc**, *EcoRI*; **aagctt**, *HindIII*; **catatg**, *NdeI*. Underlined triplets are present to introduce codons encoding alanine. Location of 5' end of oligonucleotides given is relative to the start of *ssgA*.

Site-directed mutagenesis

PCR-based site-directed mutagenesis was performed using Q5 as forward primer and reverse primers containing specific mutations (Table 2), so as to create fragments of *ssgA* with a specific codon replaced by either GCG or GCC, the most frequently occurring Ala codons in *Streptomyces*. Subsequently, a second PCR was done with the mutated PCR products as forward primers and primer Q6 as reverse primer, resulting in a full length *ssgA* sequence specifying an SsgA mutant with a single amino acid substitution. Using a similar strategy as that described under (1. General cloning vectors), these site-directed *ssgA* mutants were then cloned as *Bam*HI-*Hind*III digested fragments into pGWS32 (Table 1), so as to replace the wild-type copy of *ssgA*. The clones were verified by DNA sequencing.

Plasmids and constructs

1. General cloning vectors

pIJ2925 (Janssen and Bibb, 1993) is a pUC19-derived plasmid used for routine subcloning. For cloning in *Streptomyces* we used shuttle vector pHJL401 (Larson and Hershberger, 1986), containing an *E. coli* pUC19 origin of replication (*ori*) and an *Streptomyces* SCP2* *ori* (Lydiat *et al.*, 1985) (approximately five copies per chromosome). pIJ2925 and pHJL401 contain an ampicillin resistance marker for selection in *E. coli*. For *Streptomyces* transformants thiostrepton was used for selection of pHJL401. Plasmid DNA was isolated from ET12567 (Table 1) prior to transformation to *Streptomyces*.

2. Construction of *ssgA* clone pGWS32

For construction of pGWS32 an approximately 2 kb DNA fragment was PCR-amplified from genomic DNA of *S. coelicolor* M145 using primers Q1-Q6, and was inserted as a *Eco*RI-*Hind*III fragment into pHJL401 digested with the same enzymes. Introduction of this plasmid in the *ssgA* null mutant GSA3 fully restored sporulation (see Results section). pGWS32 was used as a basis for cloning of the plasmid library of *ssgA* variants.

Construction of mutant libraries

Mutations in *ssgA* of *S. coelicolor* were introduced by random mutagenic PCR. PCR-generated mutant libraries were cloned as *EcoRI-HindIII* digested fragments into *EcoRI-HindIII* digested pIJ2925 (Table 1). Subsequently, *E. coli* TG1 cells were transformed by electroporation. Colonies were grown separately in 96-well MTPs in 200 μ L LB medium overnight. All cultures reached similar optical density (fully grown) and were therefore expected to contribute approximately equal amounts of plasmid DNA. After replication of the cultures to fresh MTPs containing 200 μ L LB + 10% glycerol for storage, biomass was pooled and DNA purified in batch. *ssgA* variants were inserted as *BamHI-HindIII* fragments into pGWS32 (Table 1) using the *BamHI* restriction site present approximately 60 bp downstream of the translational start of *ssgA* and the *HindIII* restriction site from the multiple cloning site. After transformation of *E. coli* TG1 cells, colonies were grown separately in 96-well MTPs in 200 μ L LB medium overnight. Cultures were replicated for storage before being pooled and DNA purified in batch. This resulted in plasmid library pGWS280 (Table 1). Prior to transformation of *Streptomyces*, the library DNA was transformed into *E. coli* ET12567 by electroporation and plated on 12 cm square petridishes, giving around 2×10^4 colonies per transformation, after which cells were harvested and DNA was isolated. This DNA was transformed to GSA3 (Table 1) and plated on R2YE agar plates; transformants were selected by screening for thiostrepton resistance.

Recovering ssgA variants and DNA sequencing

To sequence the individual clones of the mutant library, the *ssgA* gene was amplified by PCR in 96-well PCR plates. As template we used 3 μ L of frozen mycelial or spore stock, replicated from deep-frozen 96-deepwell plates using a metal pin replicator (Enzyscreen, Leiden, The Netherlands). Pretreatment of the mycelium and/or spores was not required. All DNA sequencing was performed directly on PCR-amplified DNA fragments by Baseclear BV (Leiden, the Netherlands).

Statistics to determine the cut-off for the analysis of multiple mutants

Of the 790 sequenced clones, 348 *ssgA* mutant clones (approximately 44%) failed to complement the sporulation-deficient phenotype. This figure therefore

illustrates the chance that any clone in the library would be non-complementing. This figure was used to get a statistically relevant indication of the number of independent clones required to judge the importance of a certain residue for protein function. If a particular residue was mutated in n independent clones, the approximate probability of this being coincidental becomes $P=(0.44)^n$. To obtain a reliability score above 0.95, a minimum of four independent clones was required.

Computing

Amino acid sequence alignments were done using Clustal W software (Thompson *et al.*, 1994). Figure 1 was generated using Boxshade software (http://www.ch.embnet.org/software/BOX_form.html). Sequences of SsgA variants were aligned against the wild-type sequence of SsgA by the commercial SeqScape package at BaseClear, allowing rapid identification of amino acid changes. The data were then introduced in Excel (Microsoft).

RESULTS

Alignment of all known SALPs from streptomycetes

As of this moment 24 sequences of SsgA-like proteins (SALPs) from different streptomycetes are available in the databases. These include seven different SALPs in the fully sequenced genomes of *S. coelicolor* (designated SsgA-G), seven in *S. scabies* and six in *S. avermitilis*. Additionally, four orthologues of SsgA have been annotated, namely from *S. albus*, *S. goldenienseis*, *S. griseus* and *S. netropsis*. Hybridization data suggest that orthologues of SsgA, SsgB, SsgD, SsgE and SsgG (*S. coelicolor* nomenclature) occur in most if not all streptomycetes (our unpublished data). Furthermore, several homologues were identified in the genomes of other sporulating actinomycetes, namely *Thermobifida fusca*, *Kineococcus radiotolerans*, *Nocardioideis*, *Acidotherrnus cellulolyticus*, *Salinispora tropica* and several *Frankia* species. Without exception these non-*Streptomyces* orthologues share highest sequence homology with members of the SsgB-SsgG branch of the phylogenetic tree (Noens *et al.*, 2005). Due to the lower sequence homology, we have not considered these orthologues

in this work. An alignment of all *Streptomyces* SALPs (Figure 1) highlighted several conserved areas, with 16 conserved residues in all 24 sequences (Figure 1A).

Creation of a library of ssgA variants

The strict alignment (100% conservation, Figure 1A) highlights four identical residues and 12 residues with similar properties, that are conserved among all SALPs. To further address the question which residues are crucial for SsgA function, we set out to create a mutant library of SsgA. In order to create mutant variants of *S. coelicolor* *ssgA* as basis for a mutant library, we performed mutagenic PCR in four reactions containing an excess of either dATP, dCTP, dGTP or dTTP, and in the presence of 0.1 mM or 0.5 mM MnCl₂ (inducing amplification errors). Reactions containing additional dGTP and dTTP were much more efficient than those with dCTP or dATP (not shown). The PCR products were cloned as *EcoRI/HindIII*-digested fragments into pIJ2925 (Table 1) digested with the same enzymes, and transformed to *E. coli* TG1 by electroporation. As a test, of each transformation five individual clones were sequenced; this revealed the desired 2-7 nucleotide changes in the region of interest when 0.1 mM MnCl₂ was used in the initial PCR reactions, and 15-20 nucleotide changes when 0.5 mM MnCl₂ was used. The products of the latter reaction were discarded. A total of 1056 transformants (528 transformants derived from the excess dGTP PCR and another 528 from the excess dTTP PCR) - representing the maximum number of independent mutants in our library - were grown separately in 96-well MTPs overnight. To ensure a satisfactory recovery of the different mutants, a large excess (at least five-fold) of this number of colonies was grown in the subsequent cloning steps (Figure 2). DNA was purified in batch resulting in plasmid libraries pGWS278 (generated with excess of dGTP) and pGWS279 (generated with excess of dTTP), see Table 1. To transfer the *ssgA* variants from pIJ2925 to a vector that allows expression in *Streptomyces*, pGWS278 and pGWS279 were digested with *Bam*HI-*Hind*III, using the *Bam*HI restriction site naturally occurring in *ssgA* and the *Hind*III restriction site from the multiple cloning site, generating *ssgA* fragments corresponding to all residues from amino acid position 25 onwards (25-135). The fragments were then inserted into pGWS32, a low-copy number *E. coli-Streptomyces* shuttle vector that harbours

the entire *ssgRA* gene cluster and its natural promoters (Table 1). Using the *Bam*HI restriction site for cloning replaced the nucleotides encoding amino acids 25 to 135 by the PCR-generated *ssgA* variants, while the nucleotides encoding the first 24 amino acids remained unchanged (*i.e.*, wild type). After transformation of *E. coli* TG1, colonies were again grown separately in 96-well MTPs and DNA was purified in batch resulting in library pGWS280 (Table 1). A schematic representation of the different cloning steps leading to pGWS280 is given in Figure 2. Protoplasts of the *ssgA* null mutant GSA3 (Table 1) were transformed with pGWS280 and plated on R2YE agar plates. After approximately three days thiostrepton resistant colonies were individually transferred to solid SFM cultures in 24-well plates.

Figure 1. Alignment of SALPs; residues 100% conserved (A) and 50% conserved (B). Identical residues are shaded in black, residues with similar properties are shaded in grey. Nomenclature is based on highest sequence homology to the *S. coelicolor* SALP homologues. Contigs refer to short sequence files extracted from the *S. scabies* database (www.sanger.ac.uk/Projects/S_scabies). The complete genome sequences of *S. avermitilis* (Ikeda *et al.*, 2003) and *S. coelicolor* (Bentley *et al.*, 2002) have been published. Proteins, abbreviations and accession numbers are presented in the Table below. (Figure continued on the following pages).

Alignment name	SALP	Organism of origin	Database or contig number
SsgA_Scoel	SsgA	<i>S. coelicolor</i>	SCO3926
SsgB_Scoel	SsgB	<i>S. coelicolor</i>	SCO1541
SsgC_Scoel	SsgC	<i>S. coelicolor</i>	SCO7289
SsgD_Scoel	SsgD	<i>S. coelicolor</i>	SCO6722
SsgE_Scoel	SsgE	<i>S. coelicolor</i>	SCO3158
SsgF_Scoel	SsgF	<i>S. coelicolor</i>	SCO7175
SsgG_Scoel	SsgG	<i>S. coelicolor</i>	SCO2924
SsgA_Saver	SsgA	<i>S. avermitilis</i>	SAV4267
SsgB_Saver	SsgB	<i>S. avermitilis</i>	SAV6810
SsgD_Saver	SsgD	<i>S. avermitilis</i>	SAV1687
SsgE_Saver	SsgE	<i>S. avermitilis</i>	SAV3605
SsgY_Saver	SsgY	<i>S. avermitilis</i>	SAV570
SsgZ_Saver	SsgZ	<i>S. avermitilis</i>	SAV580
SsgA_Sscab	SsgA	<i>S. scabies</i>	contig: scab0274d04.q1k
SsgB_Sscab	SsgB	<i>S. scabies</i>	contig: scab0975f02.p1k
SsgD_Sscab	SsgD	<i>S. scabies</i>	contig: scab0372d01.q1k
SsgE_Sscab	SsgE	<i>S. scabies</i>	contig: scab0136f09.q1k
SsgG_Sscab	SsgG	<i>S. scabies</i>	contig: sab0313c04.q1k
SsgV_Sscab	SsgV	<i>S. scabies</i>	contig: scab0162c08.q1k
SsgW_Sscab	SsgW	<i>S. scabies</i>	contig: scab0039b09.q1k
SsgA_Salbu	SsgA	<i>S. albus</i>	AF195771
SsgA_Sgold	SsgA	<i>S. goldeniensis</i>	AF195773
SsgA_Sgris	SsgA	<i>S. griseus</i>	BAA21558
SsgA_Snetr	SsgA	<i>S. netropsis</i>	AF195772

A method to create and screen a random mutant library of SsgA in streptomyces

A

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SsgA_Sgoid 1 -----MSFLVS-----EELSFRIPVELRMETC-DPYAVRLTFHLP---GDAPVTV
SsgA_Sscab 1 -----MSFLVS-----EELSFRIPVELRMETC-DPYAVRLTFHLP---GDAPVTV
SsgA_Saver 1 -----MSFLVS-----EELSFRIPVELRMETC-DPFAYQLTFHLP---GDAPVTV
SsgA_Scoel 1 -----MSFLVS-----EELSFRIPVELRMETR-DPYAVRLTFHLP---GDAPVTV
SsgA_Snetr 1 -----MRESVQAEVLSFLVS-----EELSFKIPVELRMETR-DPYAVRMTFHLPL---GDAPVTV
SsgA_Sgris 1 -----MSFLVS-----EELSFRIPVELRMETV-DPYAVRLTFHLP---GDAPVTV
SsgA_Salbu 1 -----MSFLVS-----EELAFRIPVELRMETV-DPYAVRLTFHLP---GDAPVTV
SsgB_Scoel 1 -----MNTTVSCELHLRLVVS-----SESLPVPAGLRMDTA-DPYAVHATFHTG---AEETVEW
SsgB_Sscab 1 -----MNTTVSCELHLRLVVS-----SESLPVPAGLRMDTA-DPYAVHATFHTG---AEETVEW
SsgB_Saver 1 -----MNTTVSCELHLRLVVS-----SESLPVPAGLRMDTA-DPYAVHATFHTG---AEETVEW
SsgG_Scoel 1 ----MHTNPTGPTVVERELELRLVLS-----PESGIPVPARLGMDTD-DPYAVHITFHID---SGHPVHW
SsgG_Sscab 1 ----MRHTVVERELELGLVLS-----PEHTLPVPAKLGRSD-DPYAVHITFHIN---SDRPVHW
SsgY_Saver 1 -----MQLVVP-----HEISAPVSMRLRMEPS-DPYVVRAAFTAVD---SDETVEW
SsgZ_Saver 1 ----MESLKTVMQEVAVQLVVS-----RTYLSLSCMSLRMEPT-DPYVVRATFFTH---TDEPTEW
SsgD_Scoel 1 -----MSTVIEQSVEARLVAA-----APRMPSIPATLHMDRA-DPFAYRMTFFPATLEGVEVOW
SsgD_Saver 1 -----MSTVIEQSVEARLVAA-----APRMPSIPATLHMDRS-DPFAYRMTFFPATLEGVEVOW
SsgD_Sscab 1 -----LSTVIEQSVEARLVAA-----APRMPSIPATLHMDRS-DPFAYRMTFFPATLEGVEVOW
SsgE_Saver 1 ----MSVVEQYARAHVSD-----APEAQDTVPVVLMDPEVDPRSVRIGLPG-----PHEW
SsgE_Sscab 1 ----MSVVEQYARAHVSTD-----TPDDRDTVPVVLMDPDNDASAVRRLPG-----PDEW
SsgE_Scoel 1 ----MSVVEQYARAHLLTDGDLDPQDDGGAIPVVLMDPQLDPSKVCVALPGRGGRAGSGREW
SsgV_Sscab 1 ----MTVTLEQPARALLVTA-----EDREVPVPASLRMRSD-DPLAVHLDFFPADISLNGTMTVW
SsgW_Sscab 1 -MHPAQPTMPTPPALEQARACLITP-----AYEEVPVRTTLRTPD-DPLAVHIDFPAGVSAGVSVTW
SsgF_Scoel 1 MSGDHHGVQAQHSAAQALLPLSLCLSQMTG-ALEWEDVPAEFMDPD-HPLLVITIRFAPEG---APPVW
SsgC_Scoel 1 ----MNTVVHKTIVVQLQAG-----GTADRFPVLAHLMDAA-DPFALTIVVFSHDG---RVLARW
consensus 1
                                     v       y       v       w

SsgA_Sgoid 42 AFGRELLIDGGPRPCGDGVHAPADPETFGEVLRLQV-----GSDQAMFRVGTAPLVAFLDRTDKIV
SsgA_Sscab 42 AFGRELLIDGVGRPCGDGVHAPADPETFGEVLRLQV-----GTDQAMFRVGTAPLVAFLDRTDKIV
SsgA_Saver 42 TFGRELLIDGVGRPCGDGVHAPADREAFGEVLRLQV-----GGDHAFRSGAVPLVTFDRTDKIV
SsgA_Scoel 42 AFGRELLIDGVGRPCGDGVHAPVEPEPLAEVLRLQV-----GSDQALFRSSAAPLVAFLDRTDKIV
SsgA_Snetr 52 AFGRELLDGINRPSGCDGVHAPTDEGLSDVSLRLQV-----GADRALFRAGAPPLVAFLDRTDKSV
SsgA_Sgris 42 AFGRELLDGLNSPSGCDGVHGTPEGLGDVHRLQV-----GADRALFRAGTAPLVAFLDRTDKIV
SsgA_Salbu 42 VFGRELLVEGVLDAAAGCDVRCVPGQTATREVHTLQV-----GSEQALFRVGKAPLLAFLDRTDQGL
SsgB_Scoel 52 VFARDLLAEGLHRPTGTGDVRRWPSRSHGQGVVCLALSS-----PEGEALLEAPARALESLKRTDAAV
SsgB_Sscab 52 VFARDLLAEGLHRPTGTGDVRRWPSRSHGQGVVCLALSS-----PEGEALLEAPARALESLKRTDAAV
SsgB_Saver 52 VFARDLLAEGLHRPTGTGDVRRWPSRSHGQGVVCLALSS-----PEGEALLEAPARALESLKRTDAAV
SsgG_Scoel 58 TFARDLLVEGVFRPSGHDVRRWPSKTEGRSVVCLALSS-----PDGDALLEAPTPQVSAMLERTLRAV
SsgG_Sscab 53 TFARELLVEGVFRPCGCDVRRWPTKVSGRGVVCLALSS-----PDGDALLEAPAAAASAWLERTLRVV
SsgY_Saver 43 IIGRDLLIDGLEGPVGECDISLWPADGPDSDSYLLNP-----PAGTALLKARHEIKTFIQGTEDLV
SsgZ_Saver 54 VLGRDLLADCLRGSAAGCDIRWPAVGRGDKAMYVLGA-----PAGTALLVVPVQDVKTFESAEALV
SsgD_Scoel 55 TFSRELLIACMQEPNGHCDVRRP---YAYDRTVEFHFA-----PEGTAVIHVRSGELRRFLQAAGELV
SsgD_Saver 55 TFARELLASGMEEPVGHCDVRRP---YGYDRTVEFHFA-----PEGTAVVHVRSGELRRFLERTTEL
SsgD_Sscab 55 TFARELLVTGMEESVGHCDVRRP---YGYERLVEFHFA-----PEGTAVVHVHAGEVRRFLEGTIDL
SsgE_Saver 49 AFSRDLLERGLRTPGTGEVRRWP---CGRVQAVVEFHFS-----AQGVAVVEFEAKTLRFELRRTYLAT
SsgE_Sscab 50 TFPRELLERGLRTPPTSGFVSIWP---CGRVQAVVEFHFS-----AQGVAVVQFDTKALIRFLRRTYAT
SsgE_Scoel 60 TFSRELLLEQLRAPAGSGEVRRWP---CGRVQAVVEFHFS-----PQGSVVQFENKALIRFLRRTYAAT
SsgV_Sscab 55 TFSRELLLEKVGAPSGNDVHHWP---CGRLRTVVELHS-----PYGTALLRFEKAALQRFLLRSYGVV
SsgW_Sscab 64 AFARALLAECLTASAGIDVHHWP---CGPAHTVELRS-----PHGMAMIRFDTPTLRFLRRSYAVV
SsgF_Scoel 66 HVGRDLLHEGLRRTSGLDVQVWADTPTDRETAWQVNA-----HGDIAIFSLPVPETEEDWIDRTYLHV
SsgC_Scoel 53 TLDREMVAEGLTRPVGCDVRRRPESRGMWDELRELLGDGRADGERHRAVVFVWAAVAEFLRETHAVV
consensus 71 R 11 G G v v i 1 1 fl

SsgA_Sgoid 106 PLGQERSLADFDALLDEALDRILAEEQNAG
SsgA_Sscab 106 PLGQERSLADFDTLDEALDRILAEEQSAG
SsgA_Saver 106 PLGQESLADFDALHDEALDRILAEEQSAG
SsgA_Scoel 106 PLGQEGALADFDHLDALDRILAEEQSAG
SsgA_Snetr 116 PLGQEQTLDGDFEDSLEAALGKILAEEQNAG
SsgA_Sgris 106 PLGQEHTLGDGFDGNLEDALGRILAEEQNAG
SsgA_Salbu 106 SLGSERAHADFDHLDALNRSIAEEQSAG
SsgB_Scoel 116 PPGTEHRHFDLDQELSHILAES-----
SsgB_Sscab 116 PPGTEHRHFDLDQELSHILAES-----
SsgB_Saver 116 PPGTEHRHFDLDTELSHILAES-----
SsgG_Scoel 122 PPGTEGAQLGIDGGLAELLAR-----
SsgG_Sscab 117 PPGSEFMDLGFDDGLAELLAR-----
SsgY_Saver 107 PRGAEPGHIDLDTSLAHFLAEG-----
SsgZ_Saver 118 PRGTESGHIDWDREVANLFAKG-----
SsgD_Scoel 116 PVGLEHLQLDLHDHDLAELMRGSC-----
SsgD_Saver 116 PVGLEHLQLDLHDHDLAELMRDAC-----
SsgD_Sscab 116 PLGLEHHHVLDLHDHDLAQLMRDAC-----
SsgE_Saver 110 PVPH-----
SsgE_Sscab 111 PVAH-----
SsgE_Scoel 121 AQPVAH-----
SsgV_Sscab 116 PAGREELGPALDRGLTSLRGV-----
SsgW_Sscab 125 PLGGEGLGPAFDDGLASLLDGV-----
SsgF_Scoel 130 PAGTESSRLGTDAFLSKLFDEPEASSR---
SsgC_Scoel 123 RPGREE--VRVDDFLAELTAEG-----
consensus 141

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B

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SsgA_Sgold      1 -----MSFLVS-----EELSFRIPELRYETC-DPYAVRLTFHLP---GDAPVW
SsgA_Sscab      1 -----MSFLVS-----EELSFRIPELRYETC-DPYAVRLTFHLP---GDAPVW
SsgA_Saver      1 -----MSFLVS-----EELSFRIPELRYETC-DPYAVRLTFHLP---GDAPVW
SsgA_Scoel      1 -----MSFLVS-----EELSFRIPELRYETR-DPYAVRLTFHLP---GDAPVW
SsgA_Snetr      1 -----MRESVQAEVMSFLVS-----EELSFRIPELRYETR-DPYAVRLTFHLP---GDAPVW
SsgA_Sgris      1 -----MSFLVS-----EELSFRIPELRYEYVG-DPYAVRLTFHLP---GDAPVW
SsgA_Salbu      1 -----MSFLVS-----EELAFRIPELRYETV-DPYAVRLTFHLP---GDAPVW
SsgB_Scoel      1 -----MNTTVSCEHRLVVS-----SESLPVPAGLRYDTA-DPYAVHATFHTG---AETVW
SsgB_Sscab      1 -----MNTTVSCEHRLVVS-----SESLPVPAGLRYDTA-DPYAVHATFHTG---AETVW
SsgB_Saver      1 -----MNTTVSCEHRLVVS-----SESLPVPAGLRYDTA-DPYAVHATFHTG---AETVW
SsgG_Scoel      1 ----MHTNPTGPTVVEREELRLVLS-----FESGIPVPAELGYHTD-DPYAVHITFHID---SGHPVW
SsgG_Sscab      1 ----MRHTVVEREELGLVLS-----FEHLFPVPAKLGRSD-DPYAVHITFHIN---SRPVPW
SsgY_Saver      1 -----MQLVVP-----HEISAPVSLRYEPS-DPYAVRAAFATVD---DETVEW
SsgZ_Saver      1 -----MESLKTVMQEVAVQLVVS-----RTYLSLMCSLRYEPT-DPYAVRAAFATVD---DETVEW
SsgD_Scoel      1 -----MSTVIEQSVEARLVAA-----APRMPSIPATLHYLRS-DPYAVRMTPAPATLEGVEVCW
SsgD_Saver      1 -----MSTVIEQSVEARLVAA-----APRMPSIPATLHYLRS-DPYAVRMTPAPATLEGVEVCW
SsgD_Sscab      1 -----LSTVIEQSVEARLVAA-----APRMPSIPATLHYLRS-DPYAVRMTPAPATLEGVEVCW
SsgE_Saver      1 -----MSVVEQYARAHVVD-----AEFAQDTPVVLRYDPEVDPSVRIGLPG---PHEW
SsgE_Sscab      1 -----MSVVEQYARAHVVD-----TFDDRDTPVVLRYDPEVDPSVRIGLPG---PHEW
SsgE_Scoel      1 -----MSVVEQYARAHVVD-----TFDDRDTPVVLRYDPEVDPSVRIGLPG---PHEW
SsgV_Sscab      1 -----MTVTLEQPARALVTA-----EDREVPVPSLRYSD-DPLAVHIDFADISLNGMTVW
SsgW_Sscab      1 -MHPAQPTMPTPPALQARACLTTP-----AYEEVVPRTTLRYTPD-DPLAVHIDFADISLNGMTVW
SsgF_Scoel      1 MSGDHHGVQAQHSAAQALPLSLCLSQMTG-ALEWEDVPAEFYTPD-HPLLVITIREAPEG---APFVW
SsgC_Scoel      1 -----MNTVVKHTLVVQLQAG-----GTADRFVLAHLAYLAA-DPFAITVVEVSHDG---RVLAW
consensus      1 t v v m lvvs e s vpv lryet dpyavrltfh gd pv W

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SsgA_Sgold      42 AFGRELLIDGGRPGCGDGVHIAFADPETFGEVLIQLQV-----GSDQAMFVGTAPLVAFDRTDKIV
SsgA_Sscab      42 AFGRELLIDGGRPGCGDGVHIAFADPETFGEVLIQLQV-----GTDQAMFVGTAPLVAFDRTDKIV
SsgA_Saver      42 TFGRELLIDGGRPGCGDGVHIAFADREAFGEVLIQLQV-----GGDHAFVSGAVPLVTFDRTDKIV
SsgA_Scoel      42 AFGRELLVDGGRPGCGDGVRIAPVEPEPLAEVLIQLQV-----GSDQALFSSAAPLVAFDRTDKIV
SsgA_Snetr      52 AFGRELLLDGGRPGCGDGVHIAFADPEGLSDVSIQLQV-----GADRALFAGAPLVAFDRTDKSV
SsgA_Sgris      42 AFGRELLLDGGRPGCGDGVHIAFADPEGLSDVSIQLQV-----GADRALFAGAPLVAFDRTDKIV
SsgA_Salbu      42 VFGRELLVEGVLDAGDGDVVRVCPVGQTATREVTILQV-----GSEQALFVVGKAPLVAFDRTDQGL
SsgB_Scoel      52 VFARDLLAEGHHRPTGTGDVRVWPSRSHGQGVVCTALSS-----PEGEALFAPARALESFILKRTDAV
SsgB_Sscab      52 VFARDLLAEGHHRPTGTGDVRVWPSRSHGQGVVCTALSS-----PEGEALFAPARALESFILKRTDAV
SsgB_Saver      52 VFARDLLAEGHHRPTGTGDVRVWPSRSHGQGVVCTALSS-----PEGEALFAPARALESFILKRTDAV
SsgG_Scoel      58 TFARDLLVEGVFRPGSGHDVVRVWPKTEGRSVVILALSS-----PDGDALFAPAPVSAWLERLTV
SsgG_Sscab      53 TFARELLVEGVFRPGCGDGVVRVWPKTVSGRGVVLALSS-----PDGDALFAPAAVSAWLERLTV
SsgY_Saver      43 ITGRDILLDGLGEGVCEGDTISWADGPFDRSDSYILLNP-----PACTALLMARTHEIKTFLQCTEDLV
SsgZ_Saver      54 VLGDRLLADGLRGSAGCGDIRVWPAVGRGDKAMYIVLGA-----PACTALLFVVPQDVKTFLESAEALV
SsgD_Scoel      55 TFSRELLLAGVQEPNCHGDVVRVP---YAYDRTVLEFHA-----PECTAVIHVRSGLRRLQAGELV
SsgD_Saver      55 TFARELLASGMEEPVCHGDVVRVP---YGYDRTVLEFHA-----PECTAVVHVRSGLRRLRFLRTTELV
SsgD_Sscab      55 TFARELLVTCMEESVCHGDVVRVP---YGYERLVLEFHA-----PECTAVVHVHAGEVRRFLRFLRTELV
SsgE_Saver      49 AFSRDLLEGLRPTGTGTGVVRVWP---CGRVQAVMEFHS-----AOGVAVVEFEAKTLFRFLRRTYLA
SsgE_Sscab      50 TFPRELLERGLRPTTTSCEVSIWV---CGRVQAVMEFHS-----AOGVAVVQFDTKALIRFLRRTYLA
SsgE_Scoel      60 TFSRELLEQLRPAFCSGEVVRVWP---CGRVQAVMEFHS-----FQCCSVVQFENKALIRFLRRTYLA
SsgV_Sscab      55 TFSRELLEKGVGAFSGNGDVHWP---CGRRLTVVLEHS-----PYGTALLRFKAALQRFILRSYGVV
SsgW_Sscab      64 AFARALLAEGITASAGIGDVHLWP---CGPAHTVVELRS-----PHGMAMIRFEDTPTLRRLRIRSYAVV
SsgF_Scoel      66 HVGDRLLHGLRRTSGLGDVQVWADTPTDRETAVLQVNA-----HGDIAIFSLPVPPELEBWIDRTYLV
SsgC_Scoel      53 TLDREVMVAGLTVGVGDVVRVREESRGMWDELRLLELGDGRADGERHRAVVFVWAAAVEAFIRETHAVV
consensus      71 fgRelidG1 p g Gdvrvwp g vli 1 p g allr 1 fldrtd iv

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SsgA_Sgold      106 PLGQERSLADFDALIDEADRLIAEEQNAG
SsgA_Sscab      106 PLGQERSLADFDTLIDEADRLIAEEQSAG
SsgA_Saver      106 PLGQECSLADFDALIDEADRLIAEEQSAG
SsgA_Scoel      106 PLGQEGALADFDHIDEADRLIAEEQSAG
SsgA_Snetr      116 PLGQEQTLGDFEDSLEAALGKILIAEEQNAG
SsgA_Sgris      106 PLGQEHTLGDFDGNLEDAIRILIAEEQNAG
SsgA_Salbu      106 SUGSERAHADFDHIDDAENRSLAEEQSAG
SsgB_Scoel      116 PEGTEHRRHFDLDQELSHILAESE-----
SsgB_Sscab      116 PEGTEHRRHFDLDQELSHILAESE-----
SsgB_Saver      116 PEGTEHRRHFDLDQELSHILAESE-----
SsgG_Scoel      122 PEGTEGAQLGIDDCIAELLAR-----
SsgG_Sscab      117 PEGSEFMDLGFDDCIAELLAR-----
SsgY_Saver      107 PRGAEPGHIDLDTSIAHFIAEG-----
SsgZ_Saver      118 PEGTESGHIDMDREANLFAK-----
SsgD_Scoel      116 PVGLEHLQLDLDHDLAELMRGSC-----
SsgD_Saver      116 PVGLEHLQLDLDHDLAELMRDAC-----
SsgD_Sscab      116 PLGLEHHHVDLDHDLAQLMRDAC-----
SsgE_Saver      110 PVPH-----
SsgE_Sscab      111 PVAH-----
SsgE_Scoel      121 AQPVAH-----
SsgV_Sscab      116 PAGREELGPALDRGITSILRGV-----
SsgW_Sscab      125 PLGGEGLGPAFDCCIASLIDGV-----
SsgF_Scoel      130 PAGTESSRLGTDAISKIFDEPEASSR---
SsgC_Scoel      123 RFGREE--VRVDDFLAEITAEG-----
consensus      141 plg e d d l il

```

After seven days biomass (mycelium and where relevant spores) was individually prepared from each of the GSA3 transformants. Due to the high number of transformants (around 1500) the standard procedures to harvest mycelia and spores was not feasible and a new and much more efficient method was required, which was developed based on the following procedure.

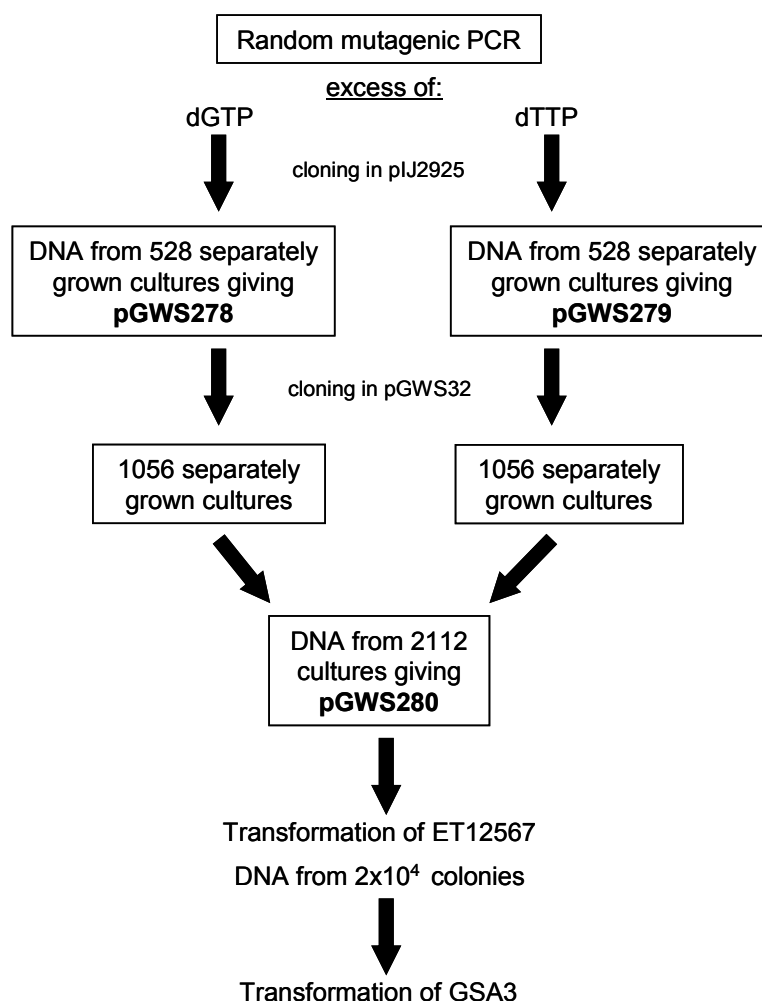


Figure 2. A schematic overview of cloning and propagation in *E. coli* of the plasmid library of *ssgA* variants.

A novel method to prepare and maintain Streptomyces libraries

Spores and/or mycelia were harvested with sterile cotton swabs from the wells of 24-well tissue culture plates in glycerol (20% w/v), and large debris was removed by taking up the suspensions with a syringe through the cotton of the swab. Suspensions were stored separately at -80°C in 96-deep-well MTPs. In this

manner 1440 samples were produced. Using a spring-loaded replicator (Minas *et al.*, 2000) individual transformants from deep-frozen MTPs were replicated onto squared SFM agar plates (12x12 cm) for phenotypic screening. When a functional *ssgA* clone was present in GSA3, the colonies had a typical grey sporulation phenotype, while colonies of GSA3 with an inactive copy of *ssgA* remained white (no grey-pigmented spores); occasionally an intermediate (light grey) phenotype was observed. In this way, the ability of individual clones to restore sporulation to the *ssgA* mutant could be assessed. A typical example is shown in Figure 3a.

The replicator was used to transfer approximately 3 μ L from the deepfrozen suspensions directly to 96-well PCR plates for 'colony PCR' (for details see section "Recovering *ssgA* variants and DNA sequencing" in the "Materials and Methods" section). To optimize the yield all PCRs were amplified once more using proofreading *pfu* DNA polymerase with 2 μ L of the initial reactions as templates. This led to saturated amounts of product for all reactions as confirmed by gel electrophoresis (Figure 3b).

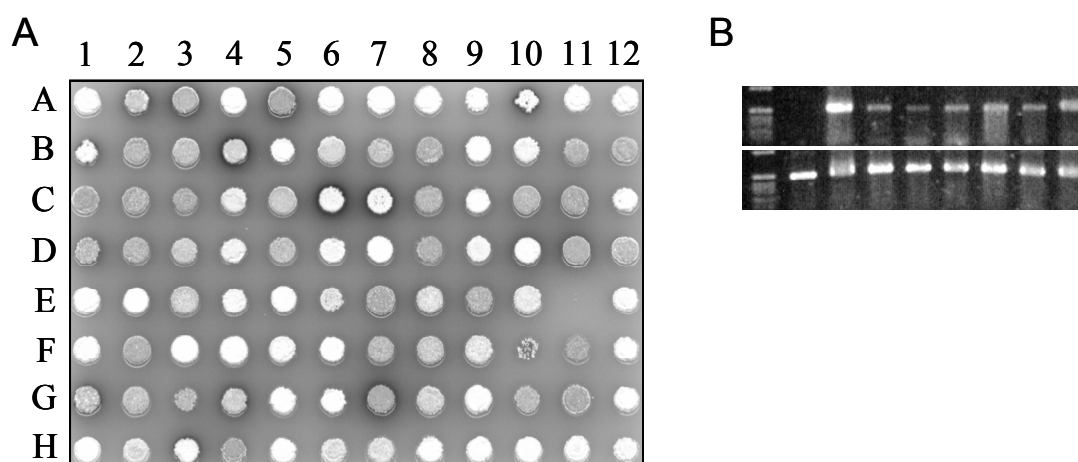


Figure 3A. A typical example of an SFM agar plate containing a replicate of transformants from deepfrozen 96-deep-well MTPs. The colony at position A1 is the control (GSA3 containing pHJL401 without insert), displaying the white, non-sporulating phenotype characteristic of the *ssgA* null mutant. All other colonies represent different GSA3 transformants of the library. Several phenotypes are observed: non-sporulating phenotypes (no complementation) such as in position A4 or E1, sporulating (full complementation) phenotypes such as in position A5 or B2, and intermediate phenotypes (partial complementation) such as in position B6 or H2.

3B. PCR products for DNA sequencing. An initial PCR was done using a few microliters of deepfrozen suspensions of individual mutants as template. The amount of product formed in different reactions was highly variable (upper panel). A second PCR was performed using products of the first PCR as DNA template resulting in saturated amounts for all samples (lower panel).

While successful PCRs on *Streptomyces* biomass were described previously (Van Dessel *et al.*, 2003), these authors used colonies harbouring high-copy-number plasmids (based on pIJ486 (Ward *et al.*, 1986)). Finally, 790 PCR products were sequenced using the same primers as used for amplifications and linked to the respective phenotypes of there transformants.

Functional analysis of the *ssgA* mutants

348 *ssgA* mutant clones (approximately 44%) failed to complement the sporulation-deficient phenotype, while the remaining 442 clones (approximately 56%) restored sporulation either partially or completely. Of these active clones, 145 clones encoded wild-type SsgA, while 297 encoded a functional mutant SsgA. No wild-type sequences were found among the non-complementing clones. This is an important observation, as it indicates that invariably the constructs properly expressed SsgA.

A collection of SsgA mutants with single amino acid substitutions

Out of the 790 analyzed *ssgA* variants 180 specified a single amino acid substitution. For 62 out of the 111 amino acids that were encoded by the part of *ssgA* that was subjected to mutagenesis (residues 25-135) we obtained at least one single amino acid substitution mutant (Table 3). For 13 residues we obtained two different substitutions, and for residue 36 three different ones (D36G, D36N, D36V). Additionally, we substituted residues W41, S89, L94 and P106 for an alanine residue by site-directed mutagenesis (see Materials and Methods section). Of the 66 mutated residues, in total 22 resulted in an inactive SsgA protein. These loss of function mutations (*i.e.* present in non-complementing clones) amongst these single substitution mutants are mostly found in the central part of the protein (covering residues W41 to L98) with small clusters from residues W41 to G51, residues V74 to L78 and residues A85 to F87. Another small group of loss of function mutations is found from residues Y25 to L29. Few loss of function mutations are found in the C-terminal part of the protein, with only two substitutions between residues D99 and G135 resulting in a non-functional SsgA (Table 3; Figure 4).

Analysis of the multiple mutants

The majority of the SsgA variants specified multiple mutations. In this manner we obtained mutants for nearly all residues subjected to mutagenesis, albeit in combination with one or more other mutations. To allow us to also distill information from the multiple mutants on the importance of individual amino acids we used the following approach. The deduced amino acid sequences of all 790 clones were aligned and the total number of times a certain residue was modified, alone or in combination with other mutations, was counted. For each residue the fraction that occurred in clones unable to restore sporulation was determined, resulting in a so-called importance score (Figure 5). As an example,

Table 3. A collection of clones obtained for the library expressing SsgA with single amino acid mutations. Amino acid changes and the ability to restore the sporulation deficient phenotype of the *ssgA* null mutant are indicated. Asterisks indicate point mutants made by site directed mutagenesis.

mutation	sporulation	mutation	sporulation	mutation	sporulation
Y25C	not restored	G57S	not restored	T101A	restored
V27L	not restored	R62G	restored	D102N	restored
R28C	restored	I63T	not restored	K103E	restored
L29P	not restored	P65R	restored	K103M	restored
L29Q	not restored	P70Q	not restored	L104P	not restored
T30A	restored	P70L	restored	L104V	restored
T30S	restored	L71P	restored	V105M	restored
F31L	restored	V74A	not restored	P106A *	restored
F31S	restored	V74E	not restored	L107P	restored
L33P	restored	L75P	not restored	G111D	restored
D36G	restored	I76T	not restored	G111S	restored
D36N	restored	R77G	restored	L113F	restored
D36V	restored	R77Q	restored	L113P	restored
P38L	restored	L78P	not restored	A114T	restored
T40A	restored	Q79R	restored	F116L	restored
W41A *	not restored	S82C	restored	F116S	restored
G44D	restored	A85P	not restored	S118G	restored
R45L	not restored	L86P	not restored	H119P	restored
E46G	restored	F87S	not restored	D121V	restored
L47Q	not restored	S89A *	restored	L124P	not restored
L48P	not restored	S90P	restored	D125V	restored
D50V	restored	A92V	restored	I127T	restored
G51R	not restored	L94A*	not restored	L128P	restored
V52A	restored	V95A	restored	S133C	restored
G53S	restored	L98H	not restored	A134E	restored
R54Q	restored	D99E	restored	G135D	restored
C56R	restored	D99G	restored	G135S	restored

residue D83 was substituted in 12 clones, five of which failed to complement GSA3; hence the *importance score* was 42%. To ensure an accuracy of 95% or more residues that were found mutated in less than four clones were considered below statistical reliability and therefore not included in the analysis (see “Statistics to determine the cutoff for the analysis of multiple mutants” in Materials and Methods). The residues with the highest importance score (80% or higher) are highlighted in Figure 4. While the initial cut-off value was set at four mutations, of all the residues with a high importance score (>80%), none were found in four independent clones; two were found to be mutated in five different clones (P of 0.99) and all others were found to be mutated in six or more independent clones. Many of the important residues are clustered. The two most noticeable clusters are residues 39 to 49 (VTWAFGRELL) and residues 55 to 61 (PCGDGDV). Another relatively clustered group of important residues cover a somewhat larger section, namely residues V74 to L98.



Figure 4. Amino acid sequence of SsgA. Theoretical secondary structure is shown; barrels indicate predicted α -helices, arrows indicate predicted β -sheets. Residues not subjected to mutagenesis in this study (and hence invariably wild-type) are shown in lower case. Residues 100% conserved (identical or similar) between all 24 known *Streptomyces* SALPs (see text for further explanation) are underlined. Identical amino acids are further highlighted in bold face. Bullets above the sequence indicate single amino acid substitutions causing loss of function (Table 3). Asterisks above the sequence indicate amino acids which obtained an importance score of 80% or above from this study (see Results section and Figure 5).

Outside of these three regions, the effect of amino acid substitutions was significantly less obvious (Figure 4). These regions all show high conservation among all SALPs, and therefore most likely relate to a general function, such as target binding. The overall amino acid identity of the SALPs from *S. coelicolor* is between 30 and 50% (Flärdh and van Wezel, 2003).

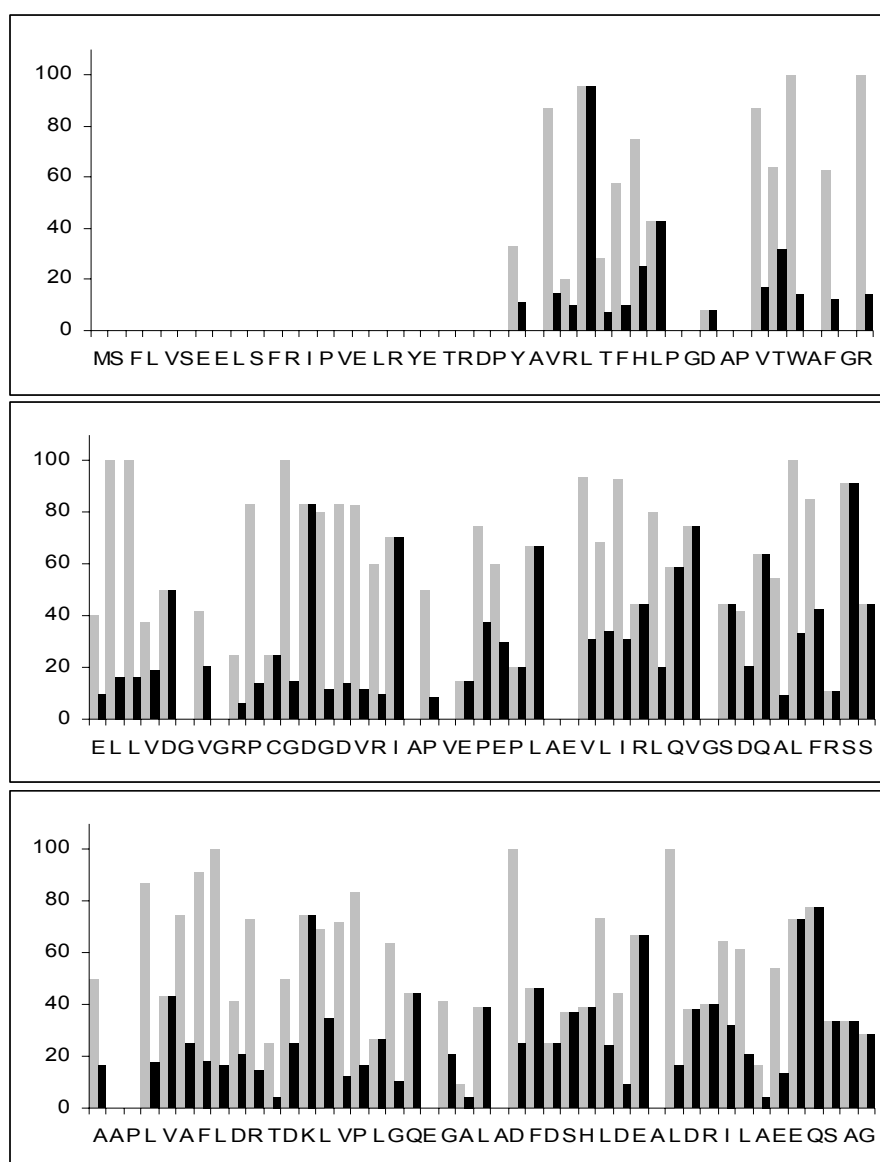


Figure 5. Graph showing the importance score (grey bars) and specificity score (black bars) in percentages on the y-axis. The amino acid sequence of SsgA is shown on the x-axis. SsgA-importance score represents the frequency that a certain mutation occurs in a non-functional *ssgA* clone, were 100% would indicate that an amino acid is essential for SsgA function. By dividing the importance score by the conservation value amino acids are highlighted primarily important for the function of SsgA and less important for the other SALPs. 100% would indicate that an amino acid is essential for SsgA function and unique for the SsgA protein. Residues for which not enough data was obtained (substituted in less than four clones; see Results section) no bars are presented.

An important question is, *can we identify residues that are important for SsgA but not conserved among the other SALPs?* Such residues may be involved in a unique aspect of SsgA function. For this purpose we calculated a specificity score (Figure 5). We divided the importance score obtained from our library by the degree of conservation (Figure 5), expressed as the occurrence of a particular residue in SsgA in all other SALPs of *S. coelicolor*, with a conservation value of 1 if a residue is unique for SsgA, and a value of 7 if a residue occurs in all seven *S. coelicolor* SALPs. In the example described above, we calculated an importance score of 42% for residue D83. This residue occurred in two SALPs, namely SsgA and SsgF, and hence the *specificity score* is 21%. Among the 111 possible candidates, residues L29, D58 and S89 obtained a high specificity score, *i.e.* they were found to be important for SsgA function, but were not significantly conserved among the other SALPs. Expectedly, L29 and D58 are well conserved among all SsgA orthologues (L29 is substituted by a methionine in *S. griseus* and *S. netropsis*). However, S89 is less well conserved (replaced with valine in *S. albus*, *S. goldeniensis* and *S. scabies*, and alanine in *S. griseus* and *S. netropsis*).

DISCUSSION

At the time this work started little structural information was available on the SsgA protein and its relatives (SsgA-like proteins or SALPs). The SALPs do not share significant sequence homology to any known protein family. However, the proteins play a major role in the control of development (Noens *et al.*, 2005), and have been used to efficiently improve growth and product formation (van Wezel *et al.*, 2006). Learning more about the intrinsic properties of the protein is the next logical step in understanding the way the protein(s) function(s). For this purpose, we designed a new mutational analysis strategy to identify the residues that are most important for the function of SsgA. One of the main bottle-necks of working with streptomycetes is the lack of a good system for batch handling of many individual *Streptomyces* colonies, mainly due to the fact that cultivation in microtitre plates and cloning of DNA directly from colonies is very difficult when working with filamentous obligate anaerobe bacteria.

Producing a mutant library in streptomycetes with high efficiency

In this work we detail the creation of a plasmid library of random *S. coelicolor* *ssgA* mutants, and discuss a new method to efficiently maintain the colonies and screen the library. The PCR-based random mutant library resulted in a maximum of over 1000 different *E. coli* clones, a figure that represents the maximum number of different mutant clones. Since the standard procedure of harvesting mycelium and spores is time consuming (and thus costly), the daunting task of producing a library of thousands of transformants required a novel approach. We were able to produce 1440 individual transformants, stored as spore and/or mycelium suspensions in 96-deep-well MTPs at -80 °C, in a short time. Using a spring-loaded replicator (Minas *et al.*, 2000) these suspensions were efficiently replicated to SFM agar plates to analyze the phenotype (sporulating or non-sporulating) of transformants of the *ssgA* null mutant GSA3 harbouring *ssgA* mutant clones (Table 1). The same replicator was used to transfer a few microliters of deep-frozen samples into PCR mixtures. In this way, a total of 790 *ssgA* variants were amplified and sequenced while steps involving DNA purification or pre-treatment of the mycelium were eliminated.

Amino acid clusters important for SsgA function

A comparison of the data obtained from the single-substitution mutants and the data from the complete library similar results were obtained. However, while mutants with the single-amino-acid mutations Y25C, G51R, I63T, P70Q, L75P, A85P and L104P were all inactive, the respective residues were not highlighted by the analysis of multiple mutants. This apparent discrepancy is at least in part explained by the lack of sufficient data for some residues. For instance, the single mutation G51R failed to complement the *ssgA* null mutant, but since G51 was mutated in fewer than four clones it was excluded from the analysis. Alternatively, the differences can be explained by the nature of particular amino acids changes; SsgA with P70Q was inactive, while SsgA with P70V was active and the corresponding clone restored sporulation to an *ssgA* mutant.

We identified several residues that are required for proper SsgA function, or at least for its ability to activate sporulation-specific cell division. A combination of all data highlights three particularly interesting regions, namely clusters of residues V39 to G51 and residues P55 to I63, and a larger region

covering residues V74 to L98. These regions are of great interest as they possibly represent one or more motif(s) essential for the function of SsgA. The penalty for mutations in the C-terminal third of the protein is much lower, highlighted by the fact that only two of the single mutants in this region inactivate SsgA, both of which have a leucine residue changed into a much more bulky proline residue (L104P and L124P).

Most of the important residues considered show high conservation among all SALPs. Therefore, we compared SsgA to the other six SALPs of *S. coelicolor*, to try and identify residues that are important for the function of SsgA, but not conserved among the other SALPs. Thorough analysis revealed that particularly residues L29, D58 and S89 stood out as candidates. Of these, L29 and D58 were particularly highly conserved among the SsgA orthologues of different organisms, while S89 was less well conserved. For residue L29 both the L29P and L29Q single mutations failed to restore sporulation to GSA3. For residues D58 and S89 no single mutants were obtained, but a S89A substitution introduced by site-directed mutagenesis did result in a functional protein, restoring sporulation to GSA3. Hence, out of all the residues identified as crucial for SsgA function in sporulation, only two residues are of particular importance for the function of only SsgA itself.

As discussed in Chapter II, the crystal structure of SsgB from *Thermobifida fusca* was recently resolved, which revealed structural similarity to a class of ssDNA/RNA-binding proteins. *S. coelicolor* SsgA was modelled to the available crystal structure (Ashley Deacon and Qingping Xu, pers. comm.), and the important aa residues highlighted by the study described in this Chapter were mapped to SsgA (Appendix B). Most were found to be located in the buried hydrophobic core of the structure. This is at least supportive of a highly conserved structure among SALPs. Further studies are required to learn more about the structure-function relationship.

In conclusion, we designed a new method to efficiently create, maintain and screen mutant libraries for the industrially important streptomycetes. The technology was successfully applied to create a diverse mutant library of SsgA, with half of all SsgA residues mutated in single mutants, and a large collection of multiple mutants. With the help of this library we can better our understanding of the role of SsgA in the control of growth and development of streptomycetes and

will provide new means to exploit SsgA in strain development, to further improve streptomycetes as industrial production hosts.

Acknowledgements

We are grateful to Wouter Duetz and to Bas Reichert for discussions. This work was supported by a grant from the Royal Netherlands Academy of Arts and Sciences (KNAW) to GPvW.

Chapter VI

Functional and phylogenetic studies of the developmental cell division control protein SsgB in actinomycetes

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A. Mieke Mommaas, and Gilles P. van Wezel

ABSTRACT

The SsgA-like protein (SALP) SsgB is essential for early stages of morphogenesis of streptomycetes. Here we show that SsgB plays a crucial role in developmental cell division, and is possibly involved in septum-site localization. Gene synteny and functional overlap strongly suggest that SsgB is the archetype of the SALP family and probably occurs in all morphologically complex actinomycetes. Complementation studies showed that *ssgB* from two spore-forming (*i.e.* *Salinispora tropica* and *Saccharopolyspora erythraea*) and two non-spore-forming actinomycetes (*i.e.* *Acidothrmus cellulolyticus* and *Kineococcus radiotolerans*) restored sporulation-specific cell division to *S. coelicolor ssgB* mutants, indicating that SsgB orthologues have a universally conserved function in actinomycete morphogenesis. Several abnormalities were observed in such heterologously complemented mutants, most notably the variable spore sizes and shapes. While nucleoid distribution appeared normal, even in chains comprised of very small and large spores, several lysed spores were observed, suggesting weakening of the cell wall. Analysis of a number of developmentally expressed penicillin-binding proteins (PBP) and autolytic enzymes revealed a temporal correlation in the gene expression profiles of *ssgB* and *ftsI*-like *pbp* genes.

INTRODUCTION

In bacteria, the cell division ring typically consists of polymeric rings of the tubulin homologue FtsZ (the Z-ring), which acts as a scaffold and recruits other cell division proteins. In *E. coli* and in *Bacillus*, formation of the Z-ring is driven towards mid-cell and away from segregated chromosomes (Errington *et al.*, 2003; Goehring and Beckwith, 2005; Rothfield *et al.*, 2005). During sporulation-specific cell division in *Streptomyces*, up to a hundred Z-rings are simultaneously laid down in an aerial hypha, at which time mid-cell simply does not exist. In addition, Z-rings are formed over non-segregated chromosomes, and separation of nucleoids is not observed until septal constriction has started (Flårdh, 2003; Schwedock *et al.*, 1997). It is unclear, how correct septum-site localization is achieved in aerial hyphae of *Streptomyces*. For example, actinomycetes lack clear homologues of many important cell division factors involved in Z-ring stability (e.g. FtsA and ZipA), nucleoid occlusion (SlmA and Noc), or septum localization (e.g. MinC and MinE) (Letek *et al.*, 2008). Several actinomycete-unique protein families have been identified that play a role in the control of cell division in streptomycetes, such as for example CrgA-like proteins and SsgA-like proteins (Chater and Chandra, 2006). CrgA-like proteins comprise a family of small integral membrane proteins widespread throughout the order of *Actinomycetales*, thought to play a role in the inhibition of Z-ring formation during *Streptomyces* development (Del Sol *et al.*, 2006). SsgA-like proteins or SALPs have thus far been identified in a limited number of actinomycetes, most of which have a complex morphogenesis. The genomes of *Thermobifida*, *Kineococcus*, *Nocardioides*, *Acidothermus* and *Salinispora* all contain a single SALP-encoding (*ssg*) gene, while multiple homologues occur in *Saccharopolyspora* (two), *Frankia* (three to five) and *Streptomyces* (six to eight). Interestingly, there is an apparent correlation between the complexity of the morphology and the number of SALPs found in actinomycetes, with one SALP protein in actinomycetes that produce single or no spores, and multiple SALPs in actinomycetes that produce an aerial mycelium and spore chains or sporangia (Traag and van Wezel, 2008). SALPs control specific steps of sporulation-specific cell division in streptomycetes, affecting cell wall-related events such as germination, septum localization and synthesis, thickening of the spore wall and

autolytic spore separation (Noens *et al.*, 2005). One plausible role for SALPs is controlling the activity or recruitment of cell-wall modifying enzymes, such as penicillin-binding proteins (PBPs) or autolytic enzymes (Noens *et al.*, 2005; Traag and van Wezel, 2008). PBPs are key enzymes for the synthesis of the bacterial peptidoglycan, both during growth and during cell division (Errington *et al.*, 2003; Holtje, 1998; Stewart, 2005). The best-studied PBPs are PBP2, which is required specifically for lateral cell-wall synthesis in *E. coli* (Den Blaauwen *et al.*, 2003), and FtsI (PBP3), which is part of the divisome and is essential for synthesis of the septal peptidoglycan (Botta and Park, 1981). The separation of mature spores is done by autolytic enzymes or autolysins, such as amidases, lytic transglycosylases and endopeptidases, which facilitate the breaking down of the peptidoglycan matrix (Heidrich *et al.*, 2002). SsgA, SsgB and SsgG are involved in the early stages of sporulation septation (Noens *et al.*, 2005). Mutants of *ssgA* and *ssgB* have fully non-sporulating (*whi*) phenotypes, although *ssgA* mutants produce spores on mannitol-containing media (Jiang and Kendrick, 2000b; van Wezel *et al.*, 2000a). Fluorescence microscopic studies using SsgA-GFP demonstrated that SsgA localizes at sites where the cell wall is restructured, such as at germination sites, growing tips and division sites (Noens *et al.*, 2007). *ssgB* mutants have a non-sporulating (*whi*) phenotype on all media, and produce smooth aseptate aerial hyphae and large white colonies (Keijser *et al.*, 2003). Phylogenetically the closest SALP-relative to SsgB is SsgG. *ssgG* mutants still produced many spores, however a significant number of sporulation septa were missing at irregular intervals, suggesting its involvement in septum-site localization (Noens *et al.*, 2005).

In this study we analyse the gene synteny around the *ssg* genes from different actinomycetes. We show that *ssgB* is most likely the SALP archetype and that there is significant functional overlap between its orthologues from different actinomycetes. A correlation between *ssgB* and developmentally transcribed *pbp* and autolysin genes was investigated. Results presented here shed new light on the role of this highly conserved SALP in septation.

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.

Table 1. *Streptomyces* strains and plasmids

Strain or plasmid	Description	Reference
Strains		
M145	Wild type <i>S. coelicolor</i> A3(2)	(Kieser <i>et al.</i> , 2000)
GSA3	M145 Δ <i>ssgA</i>	(van Wezel <i>et al.</i> , 2000a)
GSB1	M145 Δ <i>ssgB</i>	(Keijser <i>et al.</i> , 2003)
Δ SCO3156	M145 carrying Tn5062 insertion in SCO3156	This study
Δ SCO3771	M145 carrying Tn5062 insertion in SCO3771	This study
Δ SCO3772	M145 carrying Tn5062 insertion in SCO3772	This study
Δ SCO4132	M145 carrying Tn5062 insertion in SCO4132	This study
Δ SCO5466	M145 carrying Tn5062 insertion in SCO5466	This study
Plasmids		
pHJL401	<i>Streptomyces/E. coli</i> shuttle vector (5-10 and around 100 copies per genome, respectively)	(Larson and Hershberger, 1986)
pGWS271	pHJL401 harboring <i>ssgB</i> ^{SalTr} and its upstream region	This study
pGWS294	pHJL401 harboring <i>ssgB</i> ^{Sery} and its upstream region	This study
pGWS295	pHJL401 harboring <i>sacS</i> ^{Sery} and its upstream region	This study
pGWS298	pHJL401 harboring <i>ssgB</i> ^{Accl} fused to the <i>S. coelicolor ssgB</i> promoter	This study
pGWS299	pHJL401 harboring <i>ssgB</i> ^{Krad} fused to the <i>S. coelicolor ssgB</i> promoter	This study

M145 was used for transformation and propagation of *Streptomyces* plasmids. Preparation of media for streptomycete growth, protoplast preparation and transformation were done according to standard procedures (Kieser *et al.*, 2000).

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.

Plasmids and constructs

The plasmids described in this work are summarised in Table 1.

1. General cloning vectors

pIJ2925 (Janssen and Bibb, 1993) is a pUC19-derived plasmid used for routine subcloning. For cloning in *Streptomyces* we used the shuttle vectors pHJL401 (Larson and Hershberger, 1986), for which maintenance in streptomycetes occurs via the SCP2* *ori* (Lydiate *et al.*, 1985)(5 copies per chromosome). Plasmid DNA was isolated from ET12567 prior to transformation to *Streptomyces*. For selection of plasmids in *E. coli* ampicillin was used, and chloramphenicol was added for ET12567 transformants. For selection in *S. coelicolor* we used thiostrepton for pHJL401.

2. Construction of pGWS271, pGWS294 and pGWS295

Approximately 900 bp fragments harboring the *ssgB* orthologue and flanking regions from *Salinispora tropica* (designated *ssgB*^{SalTr}) or from *Saccharopolyspora erythraea* (*ssgB*^{Sery}) (Table 2) were amplified by PCR using oligonucleotides SalTr_BF/SalTr_BR or Sery_BF/Sery_BR (Table 3), respectively. PCR products were cloned as *EcoRI/BamHI*-digested fragments into pHJL401 digested with the same enzymes, resulting in pGWS271 (containing *ssgB*^{SalTr}) and pGWS294 (containing *ssgB*^{Sery}). The second SALP-encoding gene from *Saccharopolyspora erythraea* (designated *sacS*^{Sery}; Table 2) was amplified by PCR using oligonucleotides Sery_2F/Sery_2R, and cloned into pHJL401 as before, resulting in pGWS295.

3. Construction of pGWS298 and pGWS299

Fragments harboring the *ssgB* gene orthologues from *Acidothermus cellulolyticus* (*ssgB*^{Acell}) and *Kineococcus radiotolerans* (*ssgB*^{Krad}) (Table 2) fused to the *S. coelicolor ssgB* promoter region (-171/-1 region relative to the first nucleotide of *ssgB* (Kormanec and Sevcikova, 2002b) were made by gene synthesis (Genscript Corporation, USA). Sequences were designed as such that the promoter sequence was fused to the most likely start codon (nucleotide position 1524044

for *ssgB*^{Acell} or 3147086 for *ssgB*^{Krad} on their respective genomes), based on an alignment with *S. coelicolor* SsgB. *EcoRI* and *HindIII* digested inserts were cloned into pHJL401 digested with the same enzymes, resulting in pGWS298 (containing *ssgB*^{Acell}) and pGWS299 (containing *ssgB*^{Krad}).

Table 2. *ssgB* genes from actinomycetes

Genome tag or contig number	Organism	GeneID	Name in this work
SCO1541	<i>Streptomyces coelicolor</i>	1096967	
SAV6810	<i>Streptomyces avermitilis</i>	1217088	
scab0975f02.p1k	<i>Streptomyces scabies</i>	-	
SGR5997	<i>Streptomyces griseus</i>	-	
FRAAL2127	<i>Frankia alni</i> ACN14a	4235729	
Francci3_1359	<i>Frankia</i> sp. CcI3	3904606	
Franean1_5158	<i>Frankia</i> sp. EAN1pec	5675752	
SACE_1961	<i>Saccharopolyspora erythraea</i>	4941816	<i>ssgB</i> ^{Sery}
SACE_5535*	<i>Saccharopolyspora erythraea</i>	4945278	<i>sacS</i> ^{Sery}
Acel_1369	<i>Acidothermus cellulolyticus</i>	4486262	<i>ssgB</i> ^{Acell}
Krad_3069	<i>Kineococcus radiotolerans</i>	5336467	<i>ssgB</i> ^{Krad}
Noca_2368	<i>Nocardioideis</i>	4595986	
Tfu_2111	<i>Thermobifido fusca</i>	3581046	
Strop_1600	<i>Salinispora tropica</i>	5058058	<i>ssgB</i> ^{SalTr}
Sare_1560	<i>Salinispora arenicola</i>	5704920	

* this is the second *ssg* gene from *Saccharopolyspora erythraea*, not *ssgB*-like

PCR conditions

PCRs were performed in a minicycler (MJ Research, Watertown, MA), using *Pfu* polymerase (Stratagene, La Jolla, LA), and the buffer provided by the supplier, in the presence of 5% (v/v) DMSO. The standard program used was as follows: 2 min at 94°C, followed by 30 cycles of: 60 sec at 94°C (denaturing), 60 sec at 58°C (annealing) and 90 sec at 72°C (elongation). The reaction was completed by 5 min incubation at 72°C. Oligonucleotides used in this work are listed in Table 3.

Microscopy

1. Phase-contrast light microscopy

Strains were grown against cover slips inserted into solid SFM agar plates as previously described (Noens *et al.*, 2007). After seven days spore formation was

assessed by phase-contrast light microscopy. For visualization a Zeiss standard 25 phase-contrast light microscope was used.

2. Fluorescence microscopy

DNA in spore chains was stained with a mixture of two fluorescent dyes: the membrane-impermeant Propidium Iodine (1 µg/ml) (Sigma) and the membrane-permeant Syto-82 Orange (8 µM) (Invitrogen, Molecular Probes). Samples were analysed with an Olympus BH2 fluorescence microscope using a 495 nm band-pass filter and a 515 nm long-pass filter. In this way, a single image can be made where DNA-bound PI emits a red-colored and DNA-bound Syto-82 emits a green-colored fluorescent signal.

3. Electron microscopy

Morphological studies on surface-grown aerial hyphae and/or spores by cryo-scanning electron microscopy (cryo-SEM) were performed using a JEOL JSM6700F scanning electron microscope, as described previously (Keijser *et al.*, 2003).

Table 3. Oligonucleotides

Primer name	DNA sequence (5' to 3')*	Location 5' end [†]
Sery_BF	<u>CTGGAATT</u> CGCGAGCGGGAATTCGATCT	-399
Sery_BR	<u>CTGAAGCTT</u> CGGCTGGGCTCTTGATCTC	+563
Sery_2F	<u>CTGGAATT</u> CGCAACCTTCTGAACGCCGTG	-408
Sery_2R	<u>CTGAAGCTT</u> CGATTCCAGTGCCCGGGAAG	+496
SalTr_BF	<u>CTGGAATT</u> CTCCACTGAGGATCGCTCCGTCAC	-296
SalTr_BR	<u>CTGAAGCTT</u> CATCACCCACCGGCTGAGC	+569

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

[†] Relative to the first nucleotide of of the respective gene

RNA purification and semi-quantitative RT-PCR analysis

For transcriptional analysis of the *pbp* and autolysin genes (see Results section), total RNA was extracted from mycelium harvested from solid MM with mannitol (0.5% w/v) at three time points corresponding to vegetative growth, aerial growth, and late aerial growth (spore formation). RNA extractions were made of the wild type *S. coelicolor* M145, of its *ssgB* mutant GSB1 and from the early sporulation (*whi*) mutants *whiA*, *whiG* and *whiH*. For experimental details on RNA extraction and semi-quantitative reverse-transcriptase PCR (RT-PCR) analysis see the "Materials and Methods" section of Chapter IV of this thesis.

Construction of PBP and autolysin gene mutants

Derivatives of the cosmids carrying a Tn5062 insertion in the genes SCO3156, SCO3157, SCO3771, SCO3772, SCO4132 and SCO5466 (see "Results" section), generated by *in vitro* transposition (Bishop *et al.*, 2004), were a kind gift from Dr. Paul Dyson (Swansea, UK). These recombinant cosmids were transferred to *E. coli* ET12567 containing the conjugative plasmid pUZ8002, allowing direct conjugational transfer of the mutant cosmids to *S. coelicolor* M145. Mutants were obtained by simultaneous screening for loss of the cosmid sequences (kanamycin sensitive) and the presence of the apramycin resistance cassette *aacC4* inserted in the gene of interest.

Computer analysis

Sequence alignments were performed using ClustalW (Higgins *et al.*, 1996). Comparison of the genetic loci of *ssgB* genes (Table 2) was done using Blast, performed at <http://www.ncbi.nlm.nih.gov/>. For genome accession numbers of SALP-carrying actinomycetes see Table 1 of Chapter II of this thesis.

RESULTS

Genetic evidence for *ssgB* orthologues in all SALP-containing actinomycetes

The seven SALP paralogues of *S. coelicolor* (SsgA-G) have an end-to-end sequence identity ranging from approximately 30 to 60%. Considering the relatively small size of these proteins (120-140 aa), they likely share a comparable overall protein-fold with slight structural differences. However, these differences give rise to a distinct role for all seven in the control of sporulation (Noens *et al.*, 2005). With this in mind, it was important to see whether SALPs from distantly-related actinomycete genera over time have obtained a specialized function tailor-made to their host, or if orthologues with a conserved function could be identified. Analysis of the genetic loci of the single *ssg* genes of *Thermobifida*, *Kineococcus*, *Nocardioides*, *Acidothrmus* and *Salinispora* revealed that all loci resembled the gene organization around *ssgB* in *Streptomyces*. In fact, gene synteny suggests that all SALP-containing actinomycetes may have an

ssgB orthologue (Figure 1). This gene synteny includes a gene for tRNA^{val} upstream and at least three tRNA genes (usually tRNA^{gly}, tRNA^{cys} and tRNA^{val}) downstream in all but *Salinispora*. In pathogenic bacteria, pathogenicity islands acquired by horizontal transfer are often situated at tRNA loci, which appear to be common sites for the integration of foreign DNA sequences (Ochman *et al.*, 2000). Other genes typically found in the direct vicinity of *ssgB* are SCO1540, encoding a putative membrane protein, which precedes *ssgB* in *Streptomyces*, *Saccharopolyspora*, *Kineococcus*, *Salinispora* and *Thermobifida*, and SCO1531 for a threonine-tRNA synthetase (SCO1531), which is located further upstream in *Streptomyces*, *Acidothermus*, *Kineococcus*, *Nocardoides* and *Thermobifida* (Figure 1). These observed similarities are unique to the *ssgB* locus, as comparable parallels were not observed for other *ssg* gene homologues.

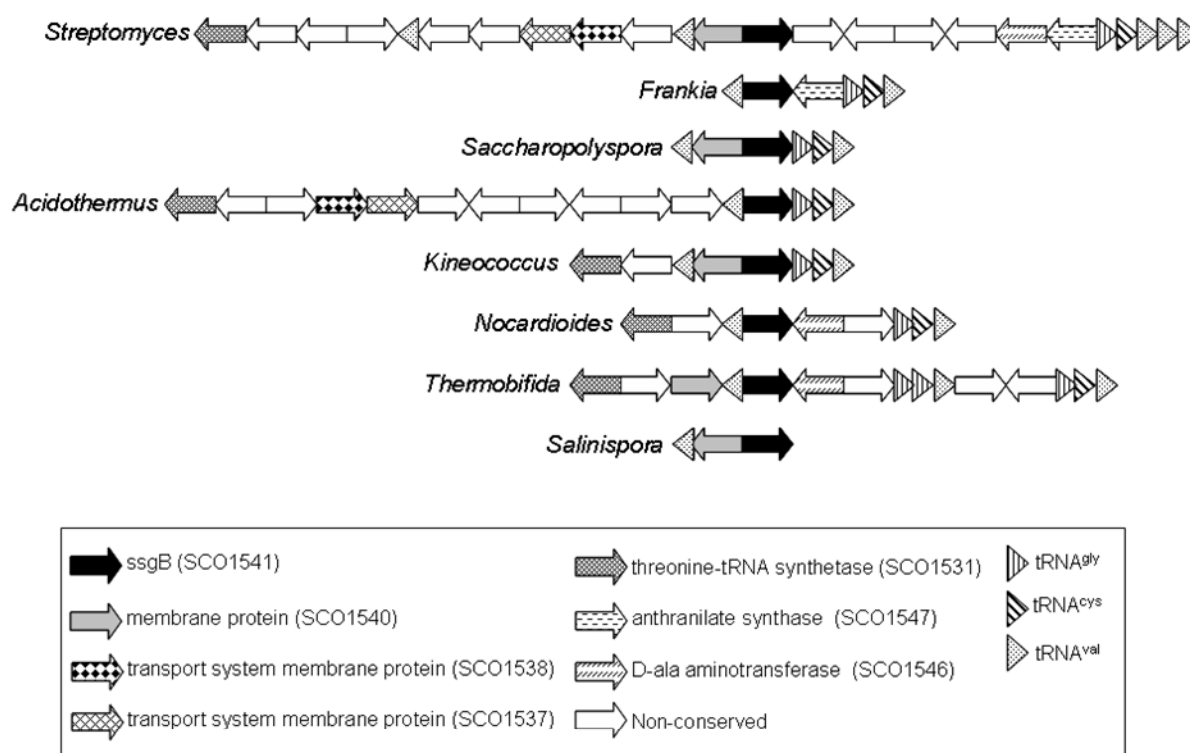


Figure 1. Genetic locus of *ssgB* orthologues from actinomycetes. Genes flanking *Streptomyces ssgB* which are conserved in the flanking regions of *ssgB* orthologues from other SALP-containing actinomycete genera are highlighted. Gene products for conserved genes are given in the box at the bottom.

SsgB orthologues are by far the most conserved SALPs in streptomycetes. They are identical except for amino acid (aa) position 150 (Gln in *S. coelicolor* and *S. scabies* or Thr in *S. avermitilis* and *S. griseus*), although many nucleotide changes occur that do not lead to changes in their aa sequences. Similar high conservation is observed for the putative SsgB orthologues from the plant symbiote *Frankia* and from the salt-water actinomycete *Salinispora*. Those from the two *Salinispora* species differ only at aa position 137 (Asn or Ser), and in the *Frankia* species two orthologues are identical while the third has three aa changes (two conserved Ile/Val changes, and more importantly Ser or Ala at aa position 105).

SsgB orthologues from morphologically distinct actinomycetes restore sporulation to a *S. coelicolor* *ssgB* mutant

What is the extent of the functional overlap between the *ssgB* genes from different actinomycetes? A number of actinomycetes carrying *ssgB* genes are, similar to *Streptomyces*, filamentous bacteria capable of undergoing cell differentiation resulting in the production of spores. Plasmids pGWS271 and pGWS294 (Table 1) contain the putative *ssgB* genes from the single-spore forming *Salinispora tropica* (*ssgB*^{SalTr}) and the oligo-spore-forming *Saccharopolyspora erythraea* (*ssgB*^{Sery}) and their upstream flanking regions, respectively. pGWS271 or pGWS294 were introduced into the *S. coelicolor* *ssgB* mutant GSB1 (Keijser *et al.*, 2003) by transformation. After seven days of growth on SFM agar plates both transformants produced a small amount of the grey spore pigment, which increased over time, in contrast to transformants carrying the empty vector pHJL401 which remained white. Seven days old colonies were examined for spore formation by phase-contrast light microscopy. Colonies expressing *ssgB*^{SalTr} or *ssgB*^{Sery} both produced a significant amount of spores. In the presence of *ssgB*^{SalTr} many single spores and some spore chains were observed (Figure 2A.2), while in the presence of *ssgB*^{Sery} many spore chains were seen (Figure 2A.3). In these transformants, most spore chains had spores of various sizes, and some spores appeared round and swollen compared to wild-type spores (Figure 2A.1). These results indicate that *ssgB* orthologues from sporulating actinomycetes are at least capable of initiating sporulation-specific cell division in *Streptomyces*. To analyse the function of the *ssgB* orthologues

from the morphologically much more distinct *Acidotherrnus cellulolyticus* (unicellular) and *Kineococcus radiotolerans* (unicellular or symmetrical multi-cell clusters), the *ssgB* mutant GSB1 was transformed with plasmids pGWS298 (expressing *ssgB*^{Acell}) or pGWS299 (expressing *ssgB*^{Krad}) (Table 1). Considering the morphological differences between these actinomycetes, the *ssgB* genes were fused by gene synthesis to the promoter sequence of *S. coelicolor ssgB*, in order to ensure proper transcriptional control of these foreign orthologues during development.

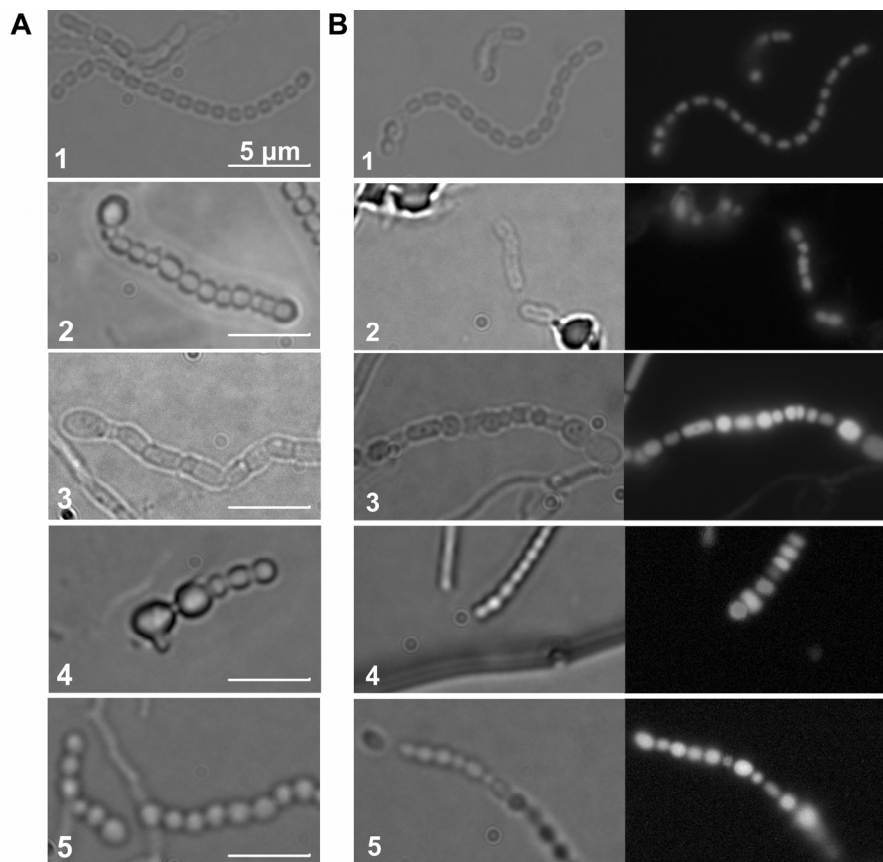


Figure 2. Analysis by phase-contrast and fluorescence microscopy (For colour figure see Appendix A). Samples were prepared from seven days old surface-grown cultures of the parental strain M145 harboring the empty vector (**1**), the *ssgB* mutant harboring pGWS271 (*ssgB*^{SalTr}) (**2**), pGWS294 (*ssgB*^{Sery}) (**3**), pGWS298 (*ssgB*^{Acell}) (**4**) or pGWS299 (*ssgB*^{Krad}) (**5**). Note that all pictures have the same magnification. **A.** Phase-contrast light microscope pictures of spore chains. Notice the regular size of wild type spores (1) and the highly variable size of spores of the transformants (2-5). **B.** DNA was visualized by simultaneous staining with the membrane-impermeant dye Propidium Iodide (red) and the membrane-permeant dye Syto-82 (green). Light microscope images on the left correspond to fluorescent images on the right. Wild type spore chains appear in green, indicating an intact cell wall. Spore chains of the transformants contain several spores with permeated cell walls, and therefore appear in red. **Bar is 5 µm.**

Perhaps surprisingly, colonies of transformants containing *ssgB*^{Krad} had a light grey appearance, suggesting that even this distantly related orthologue could restore sporulation-specific cell division to the *ssgB* mutant. Transformants containing *ssgB*^{Acell} produced significantly less pigment, barely distinguishable from the parental strain. As before, seven days old colonies were examined by phase-contrast light microscopy. Colonies containing *ssgB*^{Acell} produced only occasional single spores and short spore chains (Figure 2A.4). Colonies expressing *ssgB*^{Krad} produced a significant amount of spores, and many long spores chains (ten or more spores) were observed (Figure 2A.5). As before, spores produced by these two transformants varied greatly in size. These results clearly show that the common function of SsgB orthologues is not limited to filamentous and/or spore-forming bacteria, but conserved in morphologically distinct actinomycetes.

The genome of *Saccharopolyspora* contains a second SALP-encoding gene, here designated *sacS*^{Sery} (Table 2), the genetic locus of which does not resemble that of any other *ssg* gene. Introduction of pGWS295, which contains *sacS*^{Sery} (Table 1), into the *ssgB* mutant did not restore spore formation, even after prolonged incubation, as judged by phase-contrast light microscopy (results not shown). Finally, all five above described plasmids were introduced into the *ssgA* mutant GSA3 (van Wezel *et al.*, 2000a), and found to be unable to complement its conditional white phenotype (results not shown). These results support the observation that the complementation by introduction of the foreign *ssgB* genes (presented above) is in fact specific for SsgB.

Scanning electron microscopy

Surface-grown colonies of *S. coelicolor* M145, its *ssgB* mutant derivative GSB1 containing control plasmid pHJL401, and *ssgB* mutant transformants harboring either pGWS271 (*ssgB*^{SalTr}), pGWS294 (*ssgB*^{Sery}), pGWS295 (*sacS*^{Sery}), pGWS298 (*ssgB*^{Acell}) or pGWS299 (*ssgB*^{Krad}) were analysed by cryo-scanning electron microscopy (cryo-SEM). *S. coelicolor* M145 produced abundant and regular spore chains (Figure 3A), while pHJL401 control transformants of the *ssgB* mutant produced typical long non-coiling aerial hyphae without spores (Figure 3B). In agreement with phase-contrast light microscopy, aerial hyphae of *ssgB* mutants containing *ssgB*^{SalTr}, *ssgB*^{Sery} or *ssgB*^{Krad} produced a significant amount of spores

(Figure 3C,D,H), while we failed to detect spores in transformants containing *ssgB*^{Acell}, although some indentations that suggest septum formation were observed (Figure 3G).

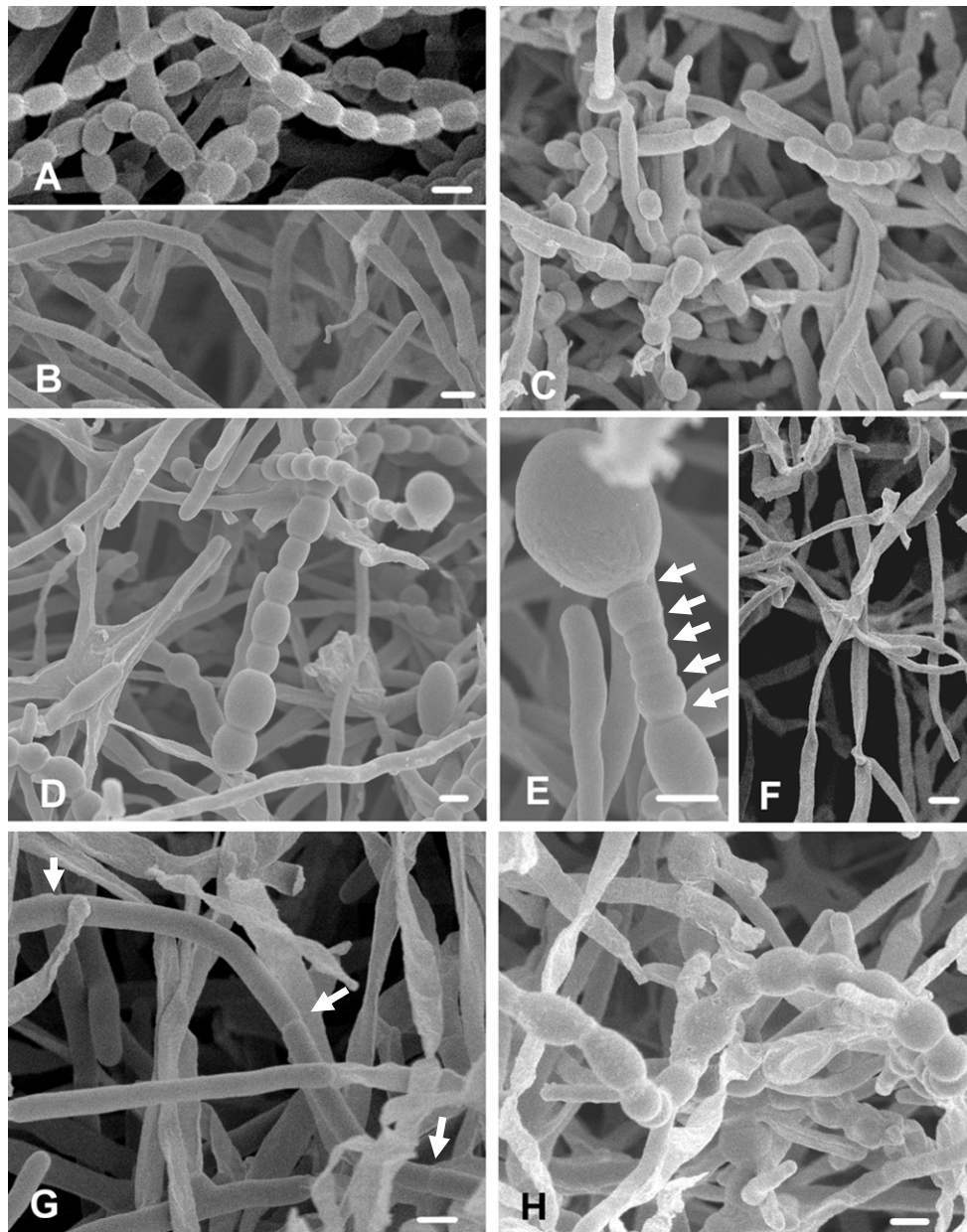


Figure 3. Analysis by cryo-scanning electron microscopy. Seven days old surface-grown colonies of the wild type M145 (A), the *ssgB* mutant (B), and *ssgB* mutant transformants containing pGWS271 (*ssgB*^{SalTr}) (C), pGWS294 (*ssgB*^{Sery}) (DE), pGWS295 (*sacS*^{Sery}) (F), pGWS298 (*ssgB*^{Acell}) (G) or pGWS299 (*ssgB*^{Krad}) (H) were analysed. **Bar is 1 µm** (at the bottom right of each picture). Arrows indicate septum-like indentations in the aerial hyphae.

Expression of *ssgB*^{SalTr} resulted in the production of many single spores and some short spore chains. Some variation in size was observed. However, most spores had the characteristic oval-shape (Figure 3C). Expression of *ssgB*^{Sery} or *ssgB*^{Krad} resulted in the formation of predominantly long spore chains. Spores produced by these transformants were highly variable in size, and often appeared round and significantly swollen (Figure 3D,H), with some extreme examples observed (Figure 3E). *ssgB* mutant transformants containing *sacS*^{Sery} produced only aseptate aerial hyphae (Figure 3F), once again supporting the specific function of the *ssgB* orthologues.

DNA segregation during sporulation

Considering the observed aberrant spore shapes and sizes, we analysed nucleoid distribution in the spore chains of the same transformants. DNA was visualized by simultaneous staining with propidium iodide (PI) and Syto-82 Orange (Syto). PI is a fluorescent dye that cannot penetrate the membrane of living cells and is therefore used to stain DNA in dead or “leaky” cells, while the membrane-permeant Syto is used to stain DNA in living cells. Using a 515 nm long-pass filter, DNA-bound PI emits a red-colored and DNA-bound Syto emits a green-colored fluorescent signal. In mature spore chains of the parental strain M145 well-segregated chromosomes were observed (Figure 2B.1). As expected, the DNA in these wild-type spores was stained by Syto and appeared in green, confirming that the cell wall of these spores was intact. In *ssgB* mutant transformants containing *ssgB*^{SalTr}, *ssgB*^{Sery}, *ssgB*^{Acell} or *ssgB*^{Krad} DNA was distributed over all spores (Figure 2B.2-5), suggesting that the DNA segregation machinery is functional. Even in spore chains consisting of both tiny and very large (swollen) spores, mostly observed in the presence of *ssgB*^{Sery} and *ssgB*^{Krad}, all compartments were fluorescently stained. However, nearly all spore chains consisted of both green and a few red-colored spores, indicating that a number of spores in these transformants had a permeated cell wall (Figure 2B.2-B5).

Analysis of potential functional partners of SsgB

The *S. coelicolor* genome encodes several PBP and autolysin enzymes and a number of these are developmentally controlled, suggesting a role specifically during sporulation (Hao and Kendrick, 1998; Noens *et al.*, 2005). The global

expression profiles for the *S. coelicolor* genome obtained previously by microarray analysis (Noens *et al.*, 2005) allowed us to select genes for cell wall-related enzymes with an expression profile similar to that of *ssgB*. Selected candidates are listed in Table 4.

Table 4. Potential functional partners of SsgB

Genome tag	Gene product description
SCO2090	FtsI, transpeptidase involved in septal cell wall synthesis
SCO2608	PBP2, transpeptidase involved in lateral cell wall synthesis
SCO3156	FtsI-like transpeptidase
SCO3157	FtsI-like transpeptidase
SCO3771	FtsI-like transpeptidase
SCO3772	secreted hydrolase/esterase
SCO4132	SLT lytic transglycosylase (Noens <i>et al.</i> , 2005)
SCO5466	cell-wall hydrolase, implicated in the control of spore-wall thickness (Piette <i>et al.</i> , 2005)

The *ftsI* mutant has a non-sporulating phenotype (J.R. McCormick, pers. comm.), while *pbp2* mutants sporulate abundantly on R2YE and SFM agar plates (Noens, 2007). Using knock-out cosmids carrying a transposon inserted into the genes of interest (see Materials and Methods section), mutants were created for the other selected candidate genes and, in addition, SCO3772. SCO3772 is separated by 18 nucleotides from SCO3771, strongly suggesting that these genes are co-transcribed. Despite different attempts we failed to make mutants for SCO3157. For all other genes several independent knock-out mutants were obtained. The mutants were plated on R2YE and SFM agar plates together with the parental strain M145, and incubated at 30 °C (Figure 4). On both media, vegetative growth and aerial mycelium formation was seemingly unaffected in the mutant strains, although on R2YE agar the SCO3771 mutant appeared to produce more aerial mycelium, as judged visually by its white appearance. After five days, all strains produced an abundance of grey pigment on SFM agar, although the mutant strains were less grey than the parental M145. Impression preparations were made as previously described (Noens *et al.*, 2005) and examined by phase-contrast light microscopy, which revealed the formation of long chains of uniform spores in the wild type and the mutant strains.

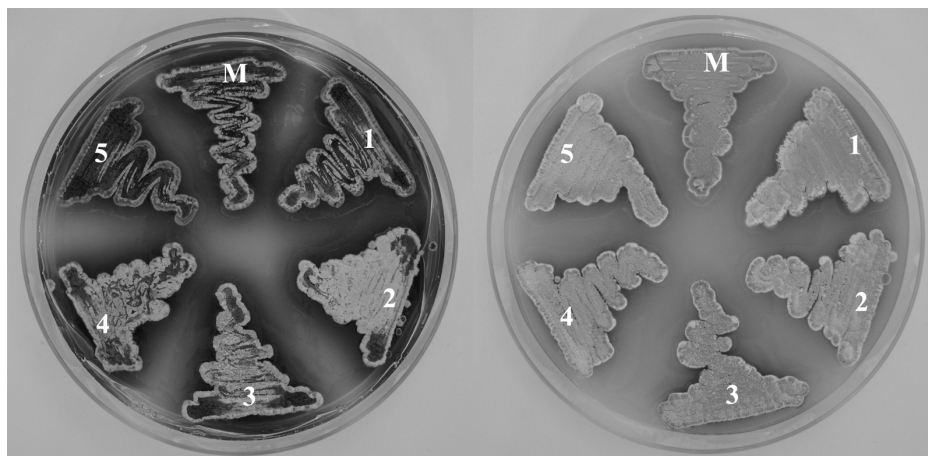


Figure 4. Mutational analysis of developmentally controlled PBP- and autolysin-encoding genes. The parental strain *S. coelicolor* M145 (M), and its mutant derivatives Δ SCO3156 (1), Δ SCO3771 (2), Δ SCO3772 (3), Δ SCO4132 (4) and Δ SCO5466 (5) (Table 1), grown for five days on R2YE (left) and SFM (right) agar plates.

Transcription of the selected *pbp* and autolysin genes was analysed by semi-quantitative RT-PCR, on total RNA purified from surface-grown cultures at different time points from the parental strain M145 and the *ssgB* mutant GSB1 (Figure 5). This was compared to transcription in the early sporulation mutants *whiA*, *whiG* and *whiH* to distinguish between possible defects due to deletion of *ssgB* or to a general failure to sporulate. Transcript levels of SCO3156, SCO3157, SCO4132 and SCO5466 were not significantly affected in any of the mutants (Figure 5a) and quantification of the bands revealed that all differences were less than two-fold as compared to wild type levels (Figure 5b). Transcription of *ftsI* and *pbp2* were down-regulated in several *whi* mutants, and most severely in the *ssgB* mutant, namely three-fold and five-fold lower than in M145, respectively. SCO3771 was detected at comparable levels in the wild type and the *whiG* and *whiH* mutants, and about two-fold up-regulated in the *whiA* mutant during aerial growth (Figure 5b). Interestingly, transcription of SCO3771 was around seven-fold up-regulated in the *ssgB* mutant during aerial growth (Figure 5b).

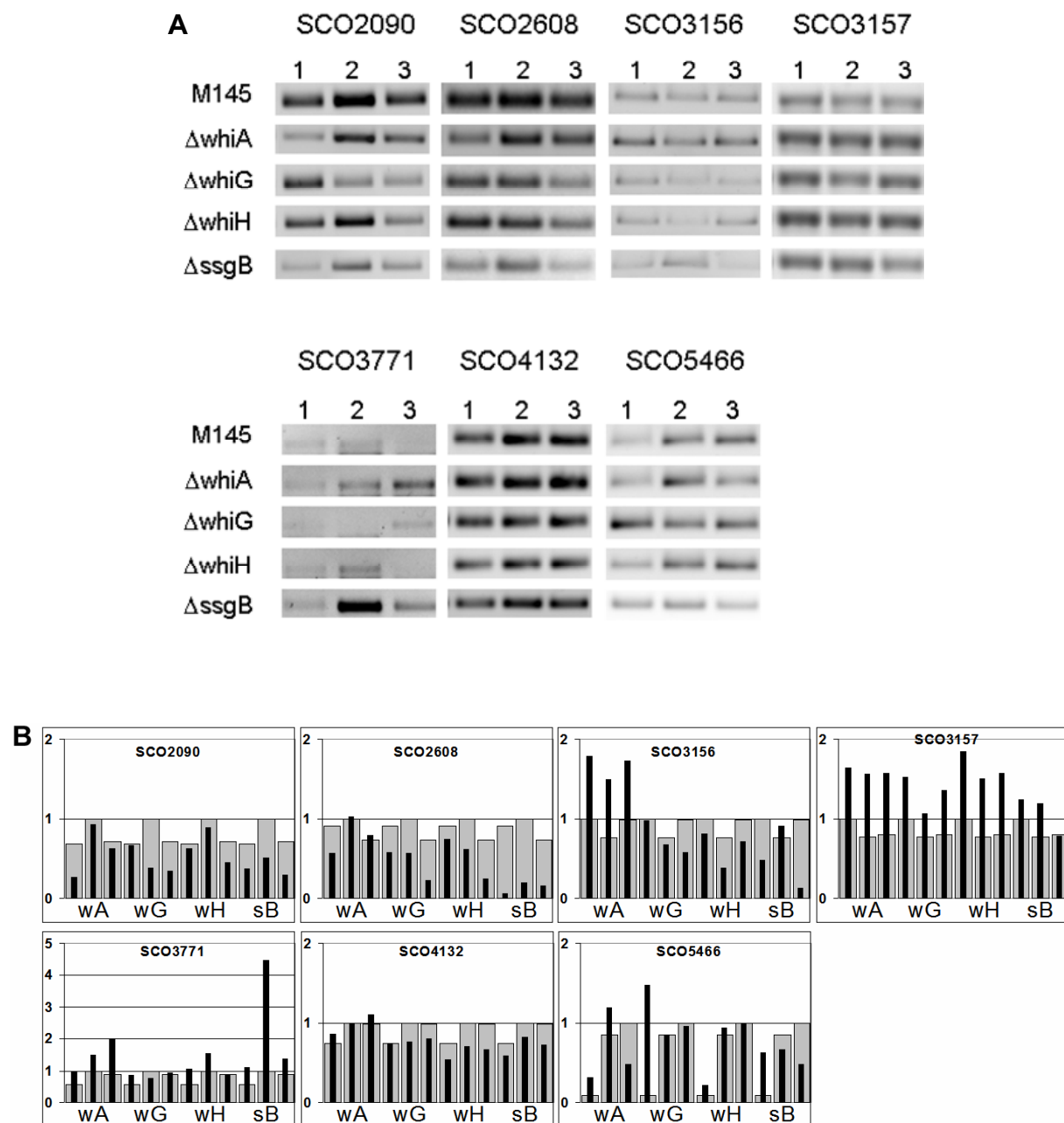


Figure 5. Transcriptional analysis of *pbp* and autolysin genes. **A.** Representative picture of semi-quantitative RT-PCR results of *pbp* and autolysin genes (top) in RNA purified from the parental strain *S. coelicolor* M145 and its sporulation-mutant derivatives $\Delta whiA$, $\Delta whiG$, $\Delta whiH$ and $\Delta ssgB$ (left). Samples **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 5B, corrected for background noise (for details see Chapter IV of this thesis). For each gene, the bar values are relative to the highest value in the wild-type strain (M145; by default set to one). 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** ($\Delta whiA$), **wG** ($\Delta whiG$), **wH** ($\Delta whiH$), and **sB** ($\Delta ssgB$).

DISCUSSION

Functional SsgB orthologues in distinct actinomycetes

All SALP-containing actinomycetes have one *ssg* gene with a locus comparable to that of *Streptomyces ssgB*, and gene synteny strongly suggests that *ssgB* is the archetype of the SALP family and that other *ssg* genes have been derived from spread and/or duplication of *ssgB*. The presence of several tRNA genes in the vicinity of these *ssgB* gene orthologues suggests an original acquisition of the *ssgB* ancestor through horizontal gene transfer. A universally conserved function for SsgB was anticipated, and indeed the orthologues from the filamentous and spore-forming actinomycetes *Salinispora tropica* (*ssgB*^{SalTr}) and *Saccharopolyspora erythraea* (*ssgB*^{Sery}) restored the ability to commence sporulation-specific cell division in the *S. coelicolor ssgB* mutant. Furthermore, the orthologues from the non-spore-forming *Acidothermus cellulolyticus* (*ssgB*^{Acell}) and *Kineococcus radiotolerans* (*ssgB*^{Krad}) also restored sporulation-specific cell division to some extent, indicating that functional SsgB orthologues occur in morphologically distinct actinomycetes. While the intrinsic property to direct sporulation-specific cell division was restored, especially *ssgB*^{Acell} transformants produced precious few spores. Suggestively, all SsgB orthologues cluster together in the so-called SsgBG branch of the SALP phylogenetic tree (Traag and van Wezel, 2008), with the exception of the orthologues from *Acidothermus* and *Nocardioides*, indicating that these two orthologues have diverged significantly over time. As supportive evidence for the specificity of the observed complementation, the non-SsgB SALP of *Saccharopolyspora erythraea* (*sacS*^{Sery}), did not restore sporulation. As a second control, none of the four *ssgB* orthologues could restore sporulation-specific cell division to *ssgA* mutants.

SsgB plays a crucial role in septation

SALPs have been suggested to recruit cell wall modifying enzymes (e.g. PBPs or autolysins) (Noens *et al.*, 2005; Traag and van Wezel, 2008). Of the seven developmentally expressed cell-wall-related enzymes (five PBPs and two autolysins), only *ftsI* mutants have a strictly non-sporulating phenotype (J.R. McCormick, pers. comm.). FtsI is recruited to the divisome and is a transpeptidase essential in septal peptidoglycan synthesis (Botta and Park,

1981). Transcriptional analysis of the same *pbp* and autolysin genes revealed that transcription of another *ftsI*-like gene, SCO3771, was strongly up-regulated in the *ssgB* mutant, while similar up-regulation was not observed in three other early *whi* mutants (*i.e.* *whiA*, *whiG* and *whiH*). These results indicate a temporal correlation in the gene expression profiles of *ssgB* and *ftsI*-like genes and highlights the role of SsgB in septation.

Even though sporulation-specific cell division was restored to *ssgB* mutants by the expression of the foreign SsgB orthologues, a number of abnormalities were observed. Several single spores and short spore chains were observed, and spores had variable sizes. Separation of nucleoids was observed in the transformants, suggesting that the DNA segregation machinery was functional. However, spores appeared to contain an amount of DNA proportional to their size, which implies that large compartments contained more than a single chromosome. A number of spores were fluorescently stained by the dye propidium iodine that cannot penetrate live cells, indicating that several of the spores had lysed. The variable spore sizes perhaps resulted from an aberrant septum-site localization and the irregular distances at which constriction occurred in spore chains observed by scanning electron microscopy suggests problems of this nature. However, cross sections of the large spore compartments should be examined by transmission electron microscopy in order to properly analyse septum formation, including unfinished septa that may have remained undetected. In bacteria, the position of the septum is determined by the localization of the cytokinetic Z-rings (Errington *et al.*, 2003). Interestingly, *ssgB* and the developmental promoter of *ftsZ* are both dependent on the sporulation sigma factor gene *whiH* (Chapter IV of this thesis; (Flärdh *et al.*, 2000)), resulting in simultaneous up-regulation of both genes in sporogenic aerial hyphae. Furthermore, co-expression studies of fluorescent protein fusions of SsgB and FtsZ indicate that initially SsgB localizes prior to Z-ring formation in aerial hyphae (Joost Willemsse and Gilles van Wezel, unpublished data). The functional relationship between SsgB and Z-ring formation/localization is currently under investigation.

In conclusion, here we shed new light on the relatively little studied cell division process in streptomycetes and show that SsgB plays a crucial role in sporulation-specific cell division. SsgB may perhaps function as an alternative to

one of the many important cell division proteins absent from actinomycete genomes (*e.g.* MinC, SlmA or NocA) (Letek *et al.*, 2008). The almost complete conservation of SsgB orthologues within all SALP-containing actinomycete genera from which multiple genomes have been sequenced (*Streptomyces*, *Frankia* and *Salinispora*) supports the importance of SsgB. The ability of several foreign SsgB orthologues to initiate sporulation-specific cell division in *Streptomyces* suggests that perhaps they fulfil a similar role in other actinomycetes, including some non-spore forming ones (*i.e.* *Acidothrmus*, *Kineococcus* and *Nocardioides*).

Acknowledgments

We are grateful to Paul R. Jensen for the kind gift of genomic DNA from *Salinispora tropica*. We thank Jos Onderwater and Evelien Rozema for help with scanning electron microscopy.

Chapter VII

Summary and discussion

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Nederlandse samenvatting

Streptomycetes are filamentous bacteria belonging to the order *Actinomycetales*, members of which most likely share their last common ancestor approximately one billion years ago (Embley and Stackebrandt, 1994). Actinomycetes come in a wide variety of morphologies, such as coccoid (e.g. *Micrococcus*), rod-shaped (e.g. *Mycobacterium*) or mycelium-forming (e.g. *Streptomyces*). SsgA-like proteins (SALPs) each play a distinctive and important role in the control of morphogenesis in streptomycetes (Noens *et al.*, 2005). Unique to actinomycetes, SALPs have thus far been identified in a limited number of actinomycete-genera from various taxonomic suborders. Interestingly, a clear correlation exists between the complexity of the morphology and the number of SALPs found in actinomycetes, with one SALP protein occurring in actinomycetes that produce single or no spores and multiple SALPs in actinomycetes that produce an aerial mycelium and spore chains or sporangia (Chapter II). In this thesis, the regulation of SALPs and their function in morphological differentiation of actinomycetes, in particular of *Streptomyces*, are addressed.

SsgA and the control of *Streptomyces* morphogenesis

The transcriptional regulation of *ssgA* in the model organism *Streptomyces coelicolor* grown on solid-surface was extensively studied (Chapter III), and revealed major differences compared to the regulation of its orthologue in *Streptomyces griseus* (Yamazaki *et al.*, 2003). Life cycle-dependent transcription of *ssgA* in *S. coelicolor* and *S. griseus* originates from two transcriptional start sites. While one of these promoters is essentially the same in both species for both sequence and location, the second one is markedly different (Chapter III). Sequence homology strongly suggests that this conserved promoter is common to all *Streptomyces* species (Chapter IV). In *S. coelicolor*, both *ssgA* transcripts are *trans*-activated by and dependent on SsgR. SsgR binds to a DNA fragment upstream of *ssgA in vitro*, and most likely to a 33 nucleotides A/T-rich sequence surrounding the stop codon of *ssgR* (Chapter III). This sequence is highly conserved in 18 different streptomycetes (Chapter IV). In contrast, *ssgA* transcription in *S. griseus* is dependent on the A-factor-pathway-controlled AdpA (Horinouchi and Beppu, 1994; Ohnishi *et al.*, 2005), while overall transcription is less strongly affected by the cognate SsgR (named SsfR in this species). Interestingly, mutation of the *ssgR* orthologue in this species strongly reduced

transcription originating from the conserved transcriptional start site of *ssgA* (Yamazaki *et al.*, 2003). Considering the sequence conservation of the common promoter and the putative SsgR binding site, and the SsgR-dependence of the common promoter in *S. coelicolor* and *S. griseus*, we anticipate that this promoter may be controlled by SsgR in perhaps all *Streptomyces* species. SsgA is an important determinant for sporulation of *S. griseus* in submerged culture (Kawamoto and Ensign, 1995a; Kawamoto *et al.*, 1997). The additional transcriptional activation by AdpA in *S. griseus* is at least one explanation why this species is able to sporulate in submerged culture, while *S. coelicolor* is not. In line with this, 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). In liquid-grown cultures of wild-type *S. griseus*, *ssgA* transcription is induced upon nutritional depletion, a condition known to induce submerged sporulation. However, transcript levels of *adpA* and *ssfR* are not enhanced in this manner (Chapter IV). This suggests that *ssgA* transcription is repressed in complex liquid media, and induced upon nutritional shift-down. The hyper-sporulating *S. griseus* strain SY1 produces SsgA at high levels in complex liquid media (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a). *ssgA* transcript levels were no longer upregulated by nutritional shift-down in this strain. The mutation causing enhanced SsgA protein levels in complex medium is still unknown, and elucidating this should provide important information as to how *ssgA* responds to the nutritional state of the environment.

Excitingly, phylogenetic analysis of the *ssgA* gene products from 18 streptomycete species revealed a clear correlation between the ability of particular streptomycetes to produce submerged spores and their cognate SsgA proteins (Chapter IV). This strongly suggests that, in addition to the role of transcriptional control, particular amino acid (aa) residues play a role in the ability of SsgA to induce spore formation in liquid-grown cultures. SsgA has been suggested to stimulate septation by modifying the cell wall at specific locations (Noens *et al.*, 2007). Overexpression of *S. griseus* SsgA strongly increases the degree of fragmentation in liquid-grown mycelium (van Wezel *et al.*, 2000a), which is directly proportional to the frequency of septation (van Wezel *et al.*, 2006). As discussed in Chapter II, this mycelium is also much more sensitive to

heat, osmotic stress (e.g. high sucrose concentrations) and SDS-treatment than the parental strain, suggesting that SsgA overexpression results in a weakened cell wall. Interestingly, similar overexpression of *S. coelicolor* SsgA is far less effective (Gilles van Wezel, unpublished data). This highlights a major discrepancy between the functions of *S. coelicolor* SsgA and *S. griseus* SsgA, relating to their ability to induce septation in liquid-grown mycelium. This prompted the development of a method to efficiently create, maintain and screen a library of random *S. coelicolor* *ssgA* variants, in order to study the role of specific aa residues in the function of SsgA (Chapter V). The residues essential for solid-surface sporulation were found clustered in three main sections of SsgA. The majority of these residues is conserved among all SALPs. However, three essential residues (i.e. L29, D58, and S89) were conserved only in SsgA, suggesting these are involved in an SsgA-specific function. The implications of these results and the use of this library with regards to the ability to induce submerged sporulation are currently under investigation. Most of the important aa residues highlighted by the mutational analysis are located in the buried hydrophobic core of the structure of *S. coelicolor* SsgA (Appendix B), which was modelled to the available crystal structure of SsgB from *Thermobifida fusca*. As discussed in Chapter II, the recently elucidated structure of SsgB from *Thermobifida fusca* revealed structural similarity to a class of ssDNA/RNA-binding proteins. If indeed SALPs interact with RNA (or ssDNA), one possibility is that their activity is modulated by interaction with such molecules. Bacterial genomes generally encode several non-coding RNA's (ncRNA) which control a variety of cellular processes (Vogel and Sharma, 2005), including modulating protein activity (Wassarman and Storz, 2000). A recent study highlighted several previously unpredicted ncRNA's in *Streptomyces* (Panek *et al.*, 2008). This possibility requires investigation.

Expression of SALPs during early and late developmental checkpoints

The decision to enter the developmental phase of the life cycle is irreversible, and the formation of aerial hyphae and spores is an energy-consuming process. The onset of development is therefore tightly controlled in a nutrient-dependent manner (Chater and Losick, 1997). This is for example illustrated by the negative effect glucose and other type I carbon sources have on sporulation (Kwakman

and Postma, 1994). The developmental SALP-encoding (*ssg*) genes (*i.e.* *ssgABCEFG*) are all subjected to carbon catabolite repression (Chapter IV), providing novel insight into how nutrient availability controls the later stages of development (*i.e.* sporulation). In contrast, *ssgD* is expressed at a high level already during the earliest stages of growth, and its expression is not affected by glucose. This strongly suggests a role for *ssgD* during vegetative growth. However, mutation of *ssgD* only noticeably affected the integrity of the cell wall in aerial hyphae and spores, and under the conditions tested no clear defects were observed in vegetative hyphae (Noens *et al.*, 2005).

On solid-surface, colony differentiation is controlled by several important sporulation genes, which include six genes essential for the development of aerial hyphae up to the point of septum formation, namely: *whiA*, *whiB*, *whiG*, *whiH*, *whiI* and *whiJ*, all encoding transcription factors (Chater, 1972; Chater and Chandra, 2006). *ssgRA* are transcribed independently of these six early *whi* genes, while *ssgB* transcript levels were strongly down-regulated in *whiA* mutants, and abolished in *whiH* mutants (Chapter III and IV). Of all *S. coelicolor* SALP null mutants, *ssgA* and *ssgB* mutants have a non-sporulating (*whi*) phenotype. *ssgB* mutants have a strict *whi* phenotype on all media, and produce large white colonies (Keijser *et al.*, 2003; Sevcikova and Kormanec, 2003). The absence of *ssgB* transcription may therefore provide a structural explanation for the sporulation-deficient phenotype of *whiH* mutants. This possibility is currently under investigation. On the other hand, *ssgA* mutants have a conditional *whi* phenotype, capable of producing spores on mannitol-containing media, but not in the presence of glucose (Jiang and Kendrick, 2000b; van Wezel *et al.*, 2000a). Such a conditional phenotype is rare among *whi* mutants. This, and the observation that its expression is *whi*-independent, strongly suggest that SsgA provides an alternative to solid-surface sporulation, underlining its important role in submerged sporulation (see above).

SsgB plays a crucial role in septation

Clear similarities were observed in the genetic locus of *ssg* genes in all SALP-containing actinomycetes to that of *ssgB* in *Streptomyces*. This led to the proposition that *ssgB* is the archetype of the SALP family, and that other *ssg* genes have been derived from spread and/or duplication of *ssgB* (Chapter VI).

The presence of several tRNA genes in the vicinity of *ssgB* in all actinomycetes suggests that *ssgB* was originally acquired through horizontal gene transfer, as tRNA loci are implicated as common sites for the integration of foreign sequences (Ochman *et al.*, 2000). *Streptomyces ssgB* mutants produce smooth aseptate aerial hyphae and are deficient in sporulation (Keijser *et al.*, 2003; Sevcikova and Kormanec, 2003). Plasmid-borne *ssgB* orthologues from *Acidothermus cellulolyticus*, *Kineococcus radiotolerans*, *Saccharopolyspora erythraea* and *Salinispora tropica* to some degree all restored sporulation-specific cell division in aerial hyphae of the *ssgB* mutant, indicating that SsgB has a universally conserved function in actinomycete morphogenesis. However, several defects were observed in sporulation in the complemented strains, including abnormal septal spacing and highly variable spore sizes. In all bacteria the division ring consists of polymeric rings of FtsZ molecules (the Z-ring). Interestingly, *ssgB* and the developmental promoter of *ftsZ* are both dependent on the sporulation sigma factor gene *whiH* (Chapter IV; (Flårdh *et al.*, 2000)), resulting in simultaneous up-regulation of both genes in sporogenic aerial hyphae. Moreover, co-expression studies of fluorescent protein fusions indicate that SsgB is recruited prior to Z-ring formation in aerial hyphae (Joost Willemse and Gilles van Wezel, unpublished data). The possibility that SsgB affects septum-site localization through direct interaction with FtsZ is currently under investigation (see “Future research” section). If no direct interaction with FtsZ is found, considering the universally conserved function of SsgB, any putative interacting partner is also likely to be conserved in the genomes of all SALP-containing actinomycetes. The genome sequences of *Acidothermus cellulolyticus* and *Thermobifida fusca* can prove very useful on the search for or elimination of such candidate partners, especially because of their considerably smaller sizes (around 2.4 and 3.6 Mbp, respectively), which allows searching for the proverbial needle in a considerably smaller haystack.

SALPs and the evolution of actinomycete morphogenesis

Functional SsgB orthologues occur in morphologically very distinct actinomycetes (Chapter VI). Multiple SALPs occur in actinomycetes that produce aerial hyphae and complex multispore structures, namely in the streptomycetes (spore chains), in *Frankia* species (sporangia) and in *Saccharopolyspora erythraea*

(short spore chains) (Chapter II). Functional analysis in streptomycetes and phylogenetic evidence strongly suggests that SALPs are crucial for (spore) septum formation in all SALP-containing actinomycetes, that at least two SALPs are required to produce more than one spore septum simultaneously, and that multiple (three or more) SALPs are required to coordinate the production of long spore chains or sporangia.

Where do SALPs fit in actinomycete evolution? SsgB occurs in all sequenced genomes belonging to different taxonomic families within the suborder *Frankineae* (i.e. *Acidothermus*, *Frankia* and *Kineococcus*), suggesting that *ssgB* arose early on in the evolution of this taxon. Genetic evidence further suggests that original acquisition occurred through horizontal gene transfer, providing a plausible explanation for the complete absence of SALP homologues from a considerable number of other fully sequenced actinomycete genomes (e.g. *Corynebacterium*, *Mycobacterium*). Multiple SALPs occur exclusively in aerial mycelium-forming actinomycetes, but it is unclear whether this morphological trait has been derived from a common origin. Whole genome comparisons of the *Frankia* and *Streptomyces* revealed a great deal of similarity, as if to suggest their mycelial morphology had a common origin, even though a phylogenetic tree of 16S rRNA implied an ancient common ancestry (Ventura *et al.*, 2007). Conversely, whole genome comparison revealed that *Saccharopolyspora erythraea*, although formerly identified as *Streptomyces erythreus*, is only distantly related to *S. coelicolor* (Oliynyk *et al.*, 2007). Nevertheless, multiple (two or more) SALPs occur exclusively in actinomycetes which produce multispore structures, suggesting that the acquisition of additional SALPs, through spread and/or gene duplication, occurred relatively recently in actinomycete evolution.

Future research

As a logical extension to the work presented in this thesis, a number of research lines can be considered. First, the role of SsgA during submerged sporulation should be investigated further. A great deal is now known about the transcriptional regulation of *ssgA*, but it is unclear how *ssgA* transcription is induced in liquid-grown cultures of *S. griseus* by nutritional shift-down. Uncovering the mutation resulting in enhanced expression of SsgA in the hyper-

sporulating strain SY1 will undoubtedly provide valuable insight into this question. In light of recent observations, the relationship between particular aa residues of SsgA and its ability to induce submerged sporulation should be investigated. This will improve our understanding as to how SsgA stimulates (submerged) sporulation. The random mutant library described in Chapter V, can prove valuable for this purpose.

Second, multiple SALPs occur exclusively in actinomycetes that produce spore chains or sporangia, and as suggested above, these are perhaps involved in coordinating the formation of multiple septa and/or spores. Single mutants of *ssgC-G* still produce spore chains, although several defects were observed in septum-site localization, DNA segregation/condensation, spore-wall synthesis and autolytic spore separation (Noens *et al.*, 2005). Combinations of SALP mutants in for example *S. coelicolor* may result in streptomycetes producing single spores or short spore chains. Conversely, it would be very interesting to see if multiple SALPs can trigger the production of multispore structures in actinomycetes that normally produce few or no spores. For such experiments *Saccharopolyspora* is a good candidate as the basic machinery to produce short spore chains is present. The single-spore forming *Micromonospora* could be used in a similar fashion, to test if the expression of SALPs from *S. coelicolor* could lead to spore-chain formation.

Finally, a number of important questions regarding the mode-of-action of SALPs need to be addressed in the future in order to enhance our understanding of this still rather mysterious protein family. The overall sequence similarity between SALP homologues is highly suggestive of an essential common aspect in their specialized functions. Live-cell imaging (*e.g.* FRET-FLIM) and biochemical methods (two-hybrid screening) should elucidate the SALP interaction partners. In view of evidence presented in Chapter VI, a possible direct interaction of SsgB with FtsZ is currently under investigation. Another question is, how are SALPs themselves recruited? SsgA and SsgB are most likely recruited prior to Z-ring formation (Joost Willemse and Gilles van Wezel, unpublished data), and hence their localization is by definition not dependent on the divisome. The dynamic and organized localization of SsgA in aerial hyphae (Noens *et al.*, 2007) is suggestive of a relationship with the symmetrical structure of the cell (*i.e.* the *Streptomyces* cytoskeleton).

Nederlandse samenvatting

Streptomyces is een Grampositieve groundbacterie behorend tot de orde van de actinomyceten, waarvan de laatste gemeenschappelijke voorouder waarschijnlijk één miljard jaar geleden leefde (Embley and Stackebrandt, 1994). In de eerste fase van de levenscyclus, produceert *Streptomyces* een netwerk van vertakkende hyfen, het zogenaamde vegetatieve mycelium. Wanneer de omstandigheden ongunstig worden, *e.g.* bij een te kort aan voedingsstoffen, ondergaan kolonies een morfologische ontwikkeling, waarbij luchthyfen worden gevormd op het vegetatieve myceliumnetwerk. Deze luchthyfen worden door tientallen septa verdeeld in ketens van mononucleoïde sporen, welke uiteindelijk fysiek worden gescheiden. SsgA-achtige eiwitten (SALPs) zijn eiwitten die uniek voorkomen in actinomyceten en elk een belangrijke rol spelen bij de controle van de sporulatie in *Streptomyces* (Noens *et al.*, 2005). Het onderzoek zoals dat in dit proefschrift beschreven staat, was gericht op de rol van de (gen)expressie en de functie van deze relatief onbekende familie van eiwitten bij de morfologische ontwikkeling.

SsgA en de controle van *Streptomyces* morfogenese

SsgA stimuleert septumvorming in *Streptomyces* hyfen en bepaalt daarmee wanneer sporulatie op vaste voedingsbodems en in vloeibare culturen plaatsvindt (Jiang and Kendrick, 2000b; Kawamoto and Ensign, 1995a; Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a). Transcriptie-analyse van *ssgA* tijdens de groei van de modelstreptomyceet *S. coelicolor* op vaste voedingsbodems toonde aan dat de groeifase-afhankelijke transcriptie van *ssgA* door twee promotoren wordt verzorgd en dat de transcriptie geheel afhankelijk is van de DNA-bindende regulator SsgR (hoofdstuk III). De sequenties van de meest waarschijnlijke bindingsplek van SsgR en één van de twee promotoren (p2 in *S. coelicolor*) vertonen sterke overeenkomsten in alle onderzochte streptomyceten (hoofdstuk IV). Het is daarom aannemelijk dat SsgR de transcriptie van *ssgA* tot op zekere hoogte activeert of stimuleert in alle streptomyceten. In *Streptomyces griseus* daarentegen, wordt transcriptie van *ssgA* geactiveerd door de A-factor-afhankelijke regulator AdpA (Horinouchi and Beppu, 1994; Ohnishi *et al.*, 2005) en speelt de ortholoog van SsgR in deze soort een minder belangrijke rol van betekenis (Yamazaki *et al.*, 2003). In vloeibare culturen van *S. coelicolor* is er

geen waarneembare transcriptie van *ssgA*, terwijl deze sterk tot expressie komt in *S. griseus* (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a). De verschillende regulatie van *ssgA* geeft daarom tenminste één verklaring waarom *S. griseus* in staat is tot sporulatie in vloeibare culturen, terwijl *S. coelicolor* dit niet is. In vloeibare culturen van *S. griseus* wordt transcriptie *ssgA* sterk gestimuleerd bij de overgang van complex naar minimaal medium (*nutritional shift-down*), een conditie waarbij ook sporulatie geïnduceerd wordt. De transcriptieniveaus van *adpA* en *ssgR* blijven daarentegen hierbij onveranderd (hoofdstuk IV). De hypersporulerende *S. griseus* mutant SY1 produceert aanzienlijk meer SsgA eiwit in complexe media (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a). *S. griseus* SY1 bevat geen mutaties in de genen voor *ssgA*, *ssgR* en die voor de A-factor afhankelijke cascade *afsA*, *arpA* en *adpA*. Dit alles wijst op een nog onbekend controle mechanisme van *ssgA* expressie in complex vloeibare media. Het identificeren van de mutatie in SY1 zal belangrijke informatie opleveren over deze regulatie.

Overexpressie van *S. griseus* SsgA induceert fragmentatie van mycelium in vloeibare culturen (van Wezel *et al.*, 2000a). Verder is dit mycelium gevoeliger voor hitte, osmotische stress en behandeling met natriumdodecylsulfaat (SDS) dan de oorspronkelijke stam, hetgeen suggereert dat overexpressie van SsgA resulteert in een verzwakte celwand (besproken in hoofdstuk II). Vergelijkbare overexpressie van *S. coelicolor* SsgA daarentegen is aanzienlijk minder effectief (Gilles van Wezel, niet-gepubliceerde data). Dit duidt op een groot verschil in functie van SsgA in deze twee soorten in vloeibare culturen. Fylogenetische analyse van de *ssgA* genproducten van 18 streptomyceten onthulde een duidelijke correlatie tussen de mogelijkheid van bepaalde stammen tot sporenvorming in vloeibare media en de aminozuursequentie van SsgA (hoofdstuk IV). Om meer inzicht te krijgen in de rol van specifieke aminozuurresiduen in de functie van SsgA is een methode ontwikkeld waarmee een verzameling van 1500 gemuteerde SsgA-varianten is gemaakt. Hierbij zijn specifieke mutaties opgehangen aan de activiteit van het eiwit (hoofdstuk V). De aminozuurresiduen die van belang zijn voor sporulatie op vaste voedingsbodems zijn geclusterd in drie gedeeltes van het eiwit. Het overgrote deel van deze residuen is geconserveerd binnen alle SALP eiwitten, terwijl drie residuen (*i.e.* L29, D58, and S89) alleen in SsgA geconserveerd zijn. Deze laatste zijn

waarschijnlijk betrokken bij een SsgA-specifieke functie. De verdere implicaties hiervan met betrekking tot de rol van SsgA bij de sporulatie in vloeibare media worden op dit moment onderzocht. De kristalstructuur van de SsgB ortholoog van *Thermobifida fusca* (op basis van de resultaten in hoofdstuk V), werd tijdens het schrijven van dit proefschrift opgelost en openbaar gemaakt door het *Joint Center for Structural Genomics* (besproken in hoofdstukken II en VII). Dit onthulde verrassende structurele overeenkomsten met een klasse van ssDNA/RNA-bindende eiwitten. Het combineren van deze nieuwe driedimensionale gegevens met de mutatie-analyse van SsgA zal ongetwijfeld veel belangrijke informatie opleveren over de relatie tussen structuur en functie.

SALP-expressie in *S. coelicolor* tijdens de levenscyclus

Morfologische ontwikkeling is een energieconsumerend proces en de beslissing om hiertoe over te gaan is voor *Streptomyces* onomkeerbaar. De overgang naar de ontwikkelingsfase wordt daarom streng gecontroleerd (Chater and Losick, 1997). Dit wordt geïllustreerd door het remmende effect van glucose en andere type I koolstofbronnen op de sporulatie (Kwakman and Postma, 1994). Expressie van de groeifase-afhankelijke *ssg* genen (*i.e.* *ssgABCEFG*) wordt onderdrukt door glucose. Daarentegen komt *ssgD* hoog tot expressie voor het begin van de ontwikkelingsfase en wordt zijn expressie niet onderdrukt door glucose (hoofdstuk IV). Dit suggereert dat SsgD ook een rol speelt tijdens de vegetatieve groei. Mutatie van *ssgD* tastte echter ogenschijnlijk alleen de celwand van luchthyfen en sporen aan (Noens *et al.*, 2005).

Tenminste zes sporulatie- of *whi* ("white") genen zijn essentieel voor de ontwikkeling van luchthyfen op vaste voedingsbodems tot aan de vorming van septa, namelijk *whiA*, *whiB*, *whiG*, *whiH*, *whiI* and *whiJ*, allen coderend voor transcriptiefactoren (Chater, 1972; Chater and Chandra, 2006). Transcriptie-analyse van de *ssg* genen in deze zes essentiële *whi*-genen, toonde aan dat *ssgRA* onafhankelijk worden getranscribeerd van deze genen (hoofdstuk III). Transcriptie van *ssgB* was sterk gereduceerd in een *whiA* mutant en vond niet plaats in een *whiH* mutant (hoofdstuk IV). *ssgB* mutanten hebben een klassiek niet-sporulerend fenotype en produceren geen septa in luchthyfen (Keijser *et al.*, 2003; Sevcikova and Kormanec, 2003). De afwezigheid van *ssgB* transcripten is een mogelijke verklaringen voor het gebrek aan sporulatie in *whiH* mutanten.

Aan de andere kant vertonen *ssgA* mutanten een voorwaardelijk “white” fenotype en zijn ze in staat sporen te vormen op mannitol-bevattend medium, maar niet in de aanwezigheid van glucose (Jiang and Kendrick, 2000b; van Wezel *et al.*, 2000a). Tezamen met het feit dat *ssgA* transcriptie “*whi*-onafhankelijk” is, suggereert dit dat SsgA een alternatieve route naar sporulatie mogelijk maakt, hetgeen zijn essentiële rol in sporulatie in vloeibare media onderstreept.

De geconserveerde functie van SsgB in actinomyceten

ssgB is de meest waarschijnlijke archetypen van de SALP familie en orthologen komen voor in alle SALP-bevattende actinomyceten (hoofdstuk VI). De aanwezigheid van een aantal tRNA genen in de loci van alle *ssgB* orthologen, suggereert dat *ssgB* origineel verkregen is door horizontale genoverdracht, daar tRNA loci preferentieel worden gebruikt voor de integratie van vreemd DNA (Ochman *et al.*, 2000). Andere *ssg* genen zijn waarschijnlijk ontstaan door verspreiding en/of duplicatie van *ssgB*. De *ssgB* genen van vier ver verwante actinomyceten (*i.e.* *Acidothermus cellulolyticus*, *Kineococcus radiotolerans*, *Saccharopolyspora erythraea* en *Salinispora tropica*) waren in staat de sporulatie-specifieke celdeling in luchthyfen van de *ssgB* mutant van *S. coelicolor* te herstellen (hoofdstuk VI). Dit geeft aan dat SsgB een universeel geconserveerde functie heeft bij de morfogenese van actinomyceten. Een aantal imperfecties werden waargenomen in het sporulatieproces in aanwezigheid van deze vreemde SsgB orthologen, namelijk: een verzwakte sporenwand, een afwijkende septumafstand en een variabele sporengrootte. De afwijkende afstand tussen de septa impliceert dat SsgB tijdens sporulatie betrokken is bij de localisatie van celdelingssepten. In alle bacteriën is de eerste stap in septatie de localisatie van polymeerringen van FtsZ (Z-ringen), de bacteriële homoloog van tubuline. Interessant genoeg zijn transcriptie van *ssgB* en de sporulatiepromoter van *ftsZ* beide afhankelijk van het sporulatiegen *whiH* (hoofdstuk IV; (Flärdh *et al.*, 2000)). Verder wordt SsgB vóór de formatie van Z-ringen gerecruteerd (Joost Willemse en Gilles van Wezel, niet-gepubliceerde data). De relatie tussen SsgB en FtsZ wordt op dit moment in meer detail onderzocht.

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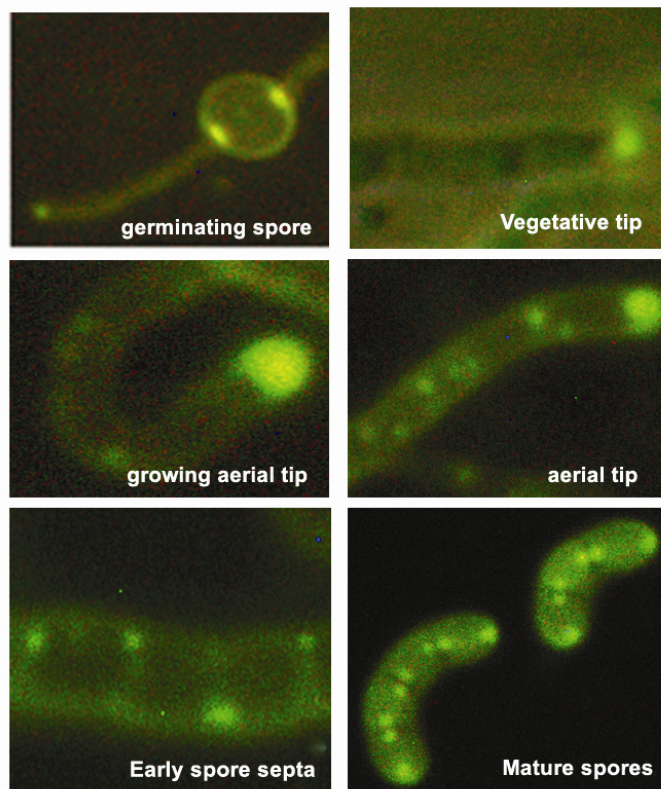
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LIST OF ABBREVIATIONS

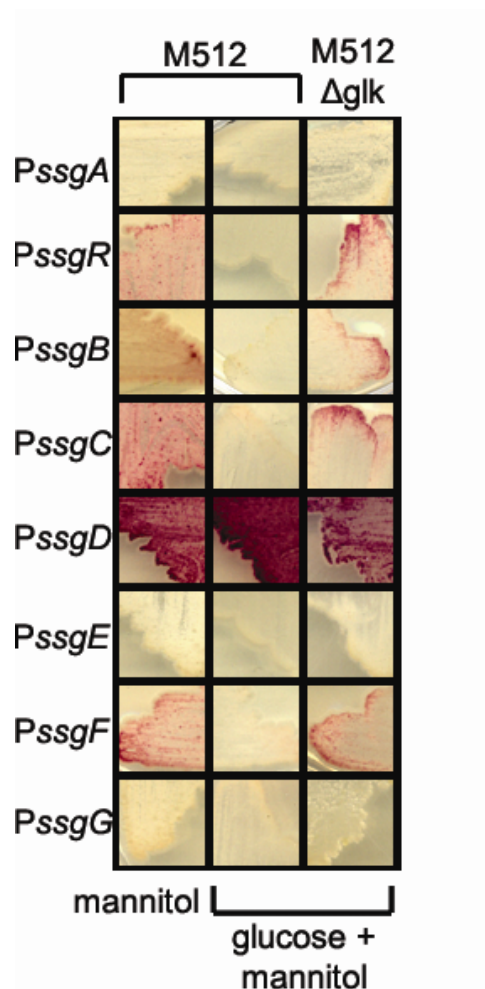
aa	amino acid
AdpA	A-factor dependent protein
AfsA	A-factor synthesis
ArpA	A-factor receptor protein
<i>bld</i>	<i>bald</i> (colony phenotype)
CCR	Carbon catabolite repression
CrgA	Coordination reproductive growth (inhibitor of Z-ring formation)
DMSO	dimethyl sulfoxide
FRET-FLIM	Förster resonance energy transfer -Fluorescence lifetime imaging
FtsZ	Filamentation temperature sensitive (cell division regulator)
GFP	Green fluorescent protein
GlkA	Glucose kinase
HTH	helix-turn-helix
IclR	Isocitrate lyase regulator
Min	“mini-cell” (phenotype; septum site determining)
MM	minimal medium
MTP	microtitre plate
Ni-NTA	nickel nitrilotriacetic acid
Noc	nucleoid occlusion (septum site determining)
nt	nucleotide
ORF	open reading frame
PBP	Penicillin-binding protein (cell wall synthesis)
RBS	ribosome binding site
RT-PCR	Reverse transcriptase -polymerise chain reaction
SALP	SsgA-like protein
<i>Sc</i>	<i>Streptomyces coelicolor</i>
SCB	<i>Streptomyces coelicolor</i> butyrolactones
SDS-PAGE	sodium dodecyl sulphate -polyacrylamide gel electrophoresis
SFM	soy flour mannitol (sporulation medium)
<i>Sg</i>	<i>Streptomyces griseus</i>
SLT	Small lytic transglycosylase
SsgA	Sporulation of <i>Streptomyces griseus</i> (sporulation regulator)
TM	trans membrane
<i>whi</i>	<i>white</i> (colony phenotype)

Appendices

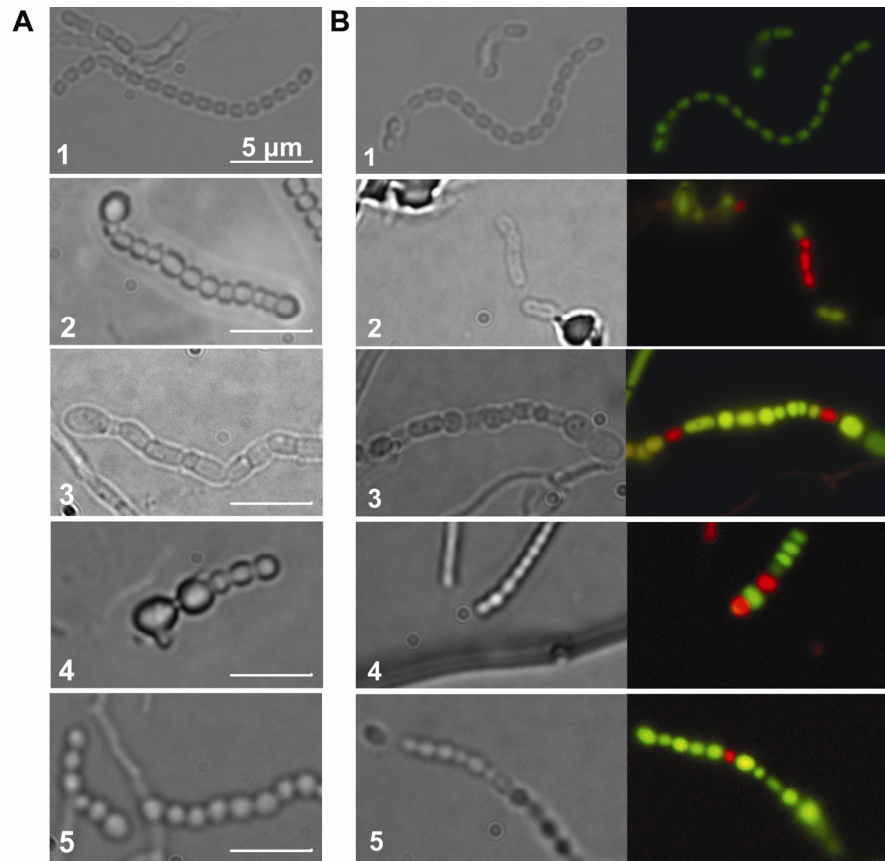
APPENDIX A. Colour figures



Chapter II. Figure 4. Dynamic localization of SsgA-GFP. Fluorescence micrographs of SsgA-GFP localization during the several stages of development of *S. coelicolor*. The width of the vegetative hyphae is around 400 nm (A, B), that of aerial hyphae and spores is around 800 nm (C-F). For further details see Noens *et al* (2007).



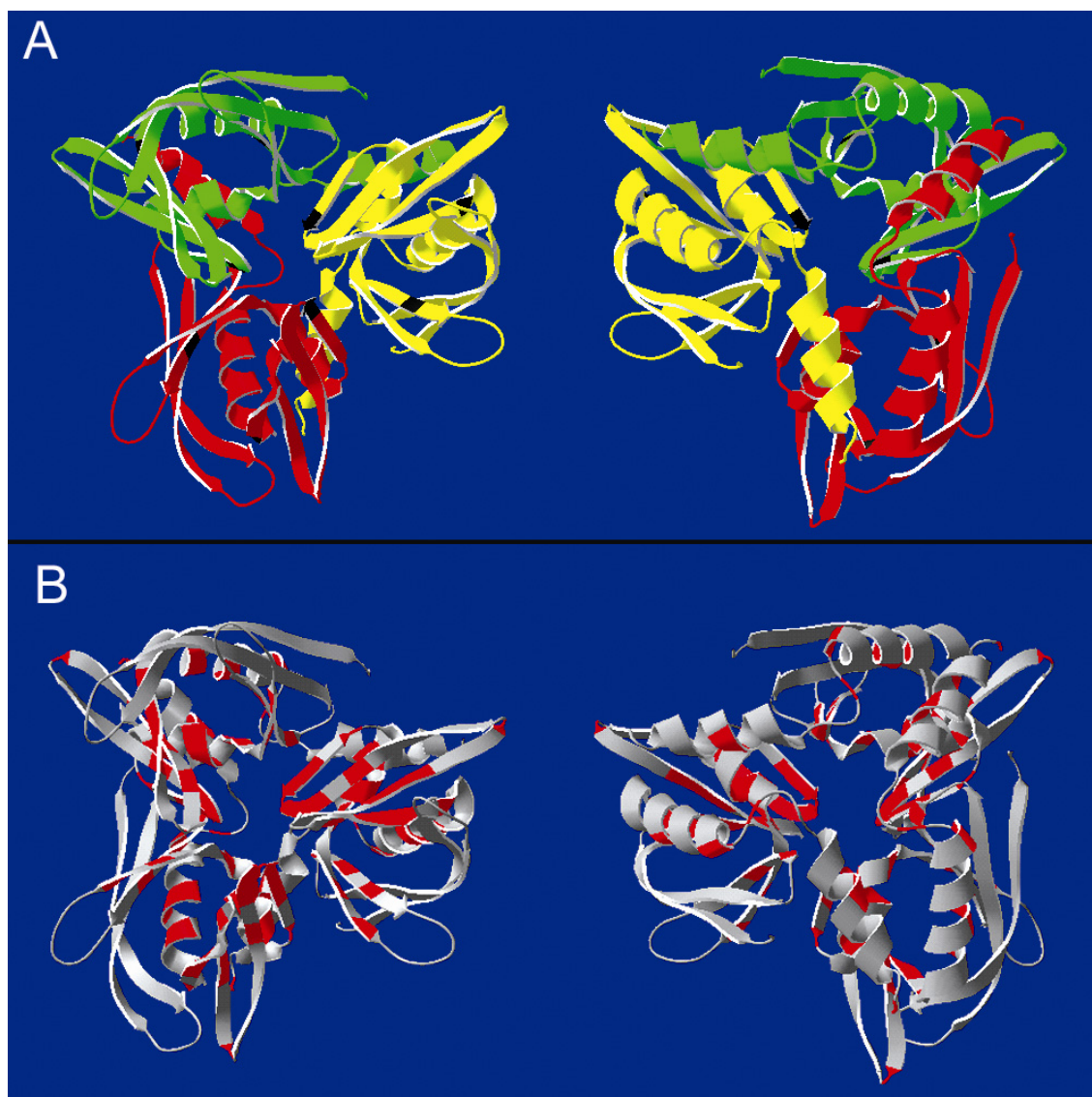
Chapter IV. Figure 1. Dependence of the activity of *ssg* gene promoters on different carbon sources. The ability to stimulate Red production by the upstream fragments of *ssgA-G* and *ssgR* (*ssgXp*) was tested on minimal medium containing mannitol (left panel) or mannitol + glucose (middle and right 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).



Chapter VI. Figure 2. Analysis by phase-contrast and fluorescence microscopy.

Samples were prepared from seven days old surface-grown cultures of the parental strain M145 harboring the empty vector (**1**), the *ssgB* mutant harboring pGWS271 (*ssgB*^{SalTr}) (**2**), pGWS294 (*ssgB*^{Sery}) (**3**), pGWS298 (*ssgB*^{Acell}) (**4**) or pGWS299 (*ssgB*^{Krad}) (**5**). Note that all pictures have the same magnification. **A.** Phase-contrast light microscope pictures of spore chains. Notice the regular size of wild type spores (1) and the highly variable size of spores of the transformants (2-5). **B.** DNA was visualized by simultaneous staining with the membrane-impermeant dye Propidium Iodide (red) and the membrane-permeant dye Syto-82 (green). Light microscope images on the left correspond to fluorescent images on the right. Wild type spore chains appear in green, indicating an intact cell wall. Spore chains of the transformants contain several spores with permeated cell walls, and therefore appear in red. **Bar is 5 µm.**

APPENDIX B. The predicted structure of *S. coelicolor* SsgA



Ribbon presentation of the three-dimensional structure of *S. coelicolor* SsgA modelled to the available crystal structure of SsgB from *Thermobifida fusca* at 2.60 Å resolution (PDB 3CM1 and Ashley Deacon and Qingping Xu, pers. comm.).

A. Front (left) and back (right) view of the predicted SsgA trimer. The three different chains are indicated in green, yellow and red. The amino acid residues L29, D58 and S89 (aa numbering according to Chapter V) that are essential for SsgA function but not conserved among other SALPs, are highlighted in black. **B.** Amino acid residues with a high *importance* score and single aa substitutions causing loss of function are highlighted in red. For details see Chapter V.

Acknowledgements

If I would have to sum up the time I spent during my Ph.D. in Gilles' group in one word, it would be.. impossible! One word just won't cut it. I've learned so much and at the same time had so much fun. I'm proud of what I have accomplished so far and of where I'm about to go, but none of it would have been possible without the help and companionship of the people I met, first in Genexpress and later in the MicDev group. Therefore, I would like to mention the following people, in chronological order: Gilles, Sharief, Sjoerd, Hugo, Elke, Bart, Nicolas (I have about 1500 reasons to mention you), Maryse, Sebastien, Rinus, Emmanuela (Manu), Sylwia, Ellen, Erik, Luisa, Axelle, Anmar, Joost, Riley (I hope you know how important you were to me during my last months in the lab) and Katie (she used to be a Katherine). I'm sure I forgot some names, for which I'm truly sorry, but know that you were appreciated.

There are some people in particular I would like to acknowledge. Elke, the past few years you have been my buddy in and out of the lab. I honestly couldn't think of anyone I would rather share an office and lab with. Your knowledge of the lyrics to some of our favourite songs is unmatched.. and in no way limited to the actual words of the songs. I was proud to be one of your paranimfs, and I'm happy that you've accepted to be my paranimf. Sharief, what would I have done without our analyses of Ajax matches and F1 races. I learned a lot from working with you in the lab, but you were also a great friend outside of the lab. And that's what I will remember the most. Seba, you claim we met years ago during a meeting in Münster, I thought it was when you started as a postdoc years later. But the important thing is we did meet, and we have a friendship to proof it. By the way, Marco van Basten could run circles around Maradona. Luisa, I remember very well the first day you were in the lab, and how you asked if it would be ok if you could have lunch with us. Now a few years later, you are one of my closest and dearest friends. I'm proud you will be by my side as my paranimf. *Ti volgio bene.*

Finally, I would like to thank my family and friends outside of the lab, for all their support and generally for being the way they are.

Curriculum vitae

Bjørn Traag was born on 11 January 1980 in The Hague (the Netherlands). In 1997 he obtained the diploma for secondary education at the Veurs College in Leidschendam. Thereafter he attended the Hogeschool Leiden and in 2001 obtained a Bachelor equivalent degree in Biochemistry. Between 2001 and 2003 he studied at Leiden University, where in 2003 he obtained a Master's degree (drs./M.Sc.) in Chemistry. During this time he completed internships under the supervision of Dr. Gilles van Wezel at Leiden University and Dr. Gabriella Kelemen at the University of East Anglia (Norwich, United Kingdom). After obtaining his M.Sc. he participated in a project supported by the Netherlands Technology Foundation (STW) under the supervision of Dr. Gilles van Wezel studying effects of SsgA overproduction on growth behaviour and product formation of streptomyces. In December 2003 he started as a Ph.D. student in Dr. Gilles van Wezel's research group Microbial Development (formerly Genexpress) with Prof. C.W.A. Pleij as his promotor. The work done as a Ph.D. student between 2003 and 2008 is presented in this thesis. During this time he participated in five international meetings and presented his work at two of these. After his Ph.D. he will join the laboratory of Prof. Richard Losick at Harvard University (Cambridge, USA), supported by a Rubicon grant for talented researchers from the Netherlands Organisation for Scientific Research (NWO), to work on nutrient sensing and the onset of sporulation in *Bacillus subtilis*.

List of publications

- Traag BA** and van Wezel GP (2008). Review: The SsgA-like proteins in actinomycetes: small proteins up to a big task. *Antonie van Leeuwenhoek* (94): 85-97.
- Noens EE, Mersinias V, Willemse J, **Traag BA**, Laing E, Chater KF, Smith CP, Koerten HK, van Wezel GP (2007). Loss of the controlled localization of growth stage-specific cell-wall synthesis pleiotropically affects developmental gene expression in an *ssgA* mutant of *Streptomyces coelicolor*. *Mol Microbiol.* 64(5):1244-59.
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