Function and control of the ssg genes in streptomyces

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Chapter II

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

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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 succesfully 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 streptomycetes 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 streptomycetes 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 streptomycetes 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). Streptomycetes 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.

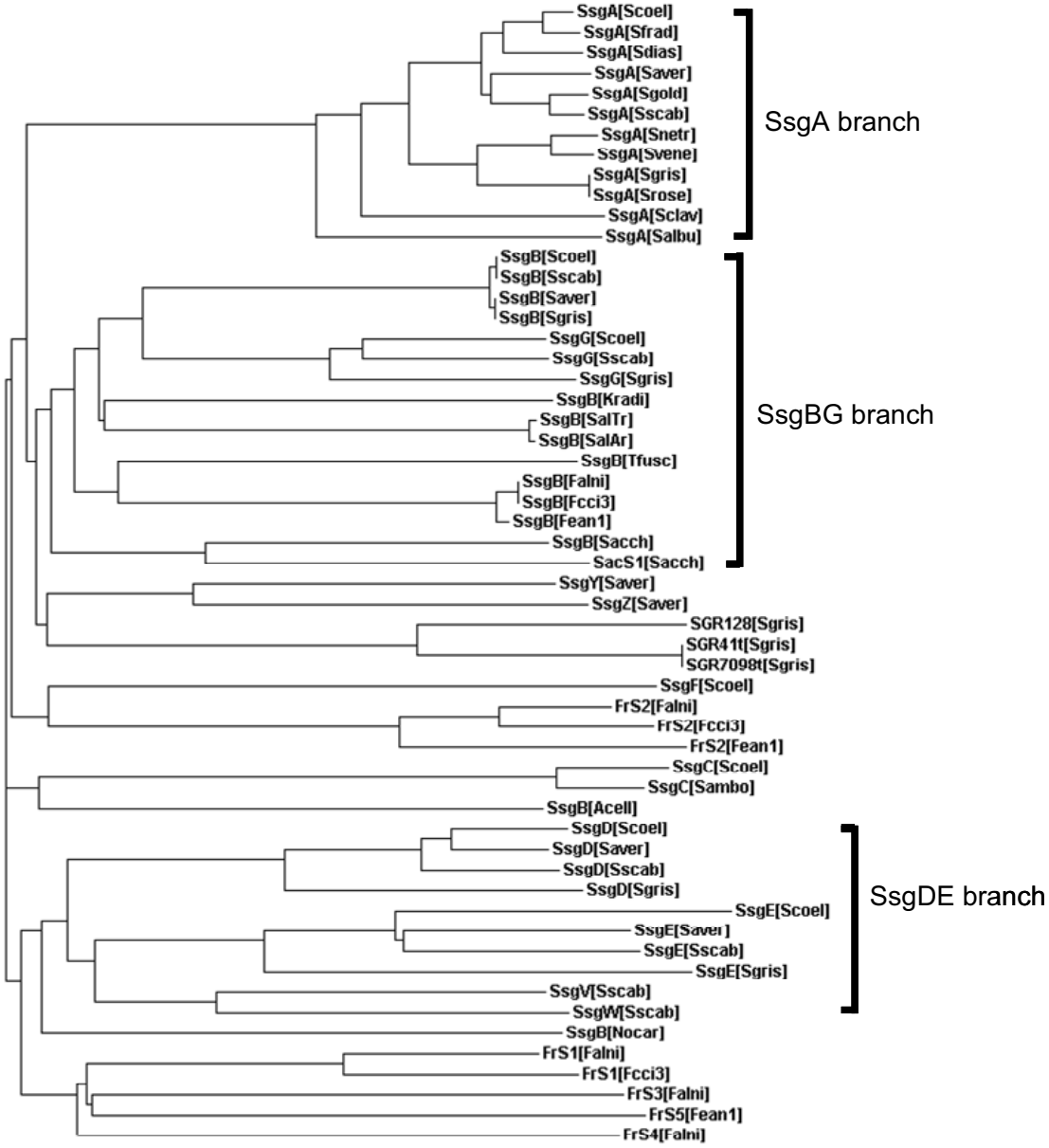

Figure 1. Phylogenetic tree of the SALP proteins listed in Table 1. Phylogenetic analysis of all SsgA-like proteins (SALPs) from different actinomycetes was performed using the CLUSTALX program (Thompson et al., 1997). Accession numbers and other information on the proteins in the tree are listed in 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]	*S. coelicolor*	SCO3926
SsgB[Scoel]	*S. coelicolor*	SCO1541
SsgC[Scoel]	*S. coelicolor*	SCO7289
SsgD[Scoel]	*S. coelicolor*	SCO6722
SsgE[Scoel]	*S. coelicolor*	SCO3158
SsgF[Scoel]	*S. coelicolor*	SCO7175
SsgG[Scoel]	*S. coelicolor*	SCO2924
SsgA[Saver]	*S. avermitilis*	SAV4267
SsgB[Saver]	*S. avermitilis*	SAV6810
SsgD[Saver]	*S. avermitilis*	SAV1687
SsgE[Saver]	*S. avermitilis*	SAV3605
SsgY[Saver]	*S. avermitilis*	SAV570
SsgZ[Saver]	*S. avermitilis*	SAV580
SsgA[Sscab]	*S. scabies*	contig: scab0274d04.q1k
SsgB[Sscab]	*S. scabies*	contig: scab0975f02.p1k
SsgD[Sscab]	*S. scabies*	contig: scab0372d01.q1k
SsgE[Sscab]	*S. scabies*	contig: scab0136f09.q1k
SsgG[Sscab]	*S. scabies*	contig: sab0313c04.q1k
SsgV[Sscab]	*S. scabies*	contig: scab0162c08.q1k
SsgW[Sscab]	*S. scabies*	contig: scab0039b09.q1k
SsgA[Sgris]	*S. griseus*	SGR3655
SsgB[Sgris]	*S. griseus*	SGR5997
SsgD[Sgris]	*S. griseus*	SGR1004
SsgE[Sgris]	*S. griseus*	SGR4320
SsgG[Sgris]	*S. griseus*	SGR4615
SGR128[Sgris]	*S. griseus*	SGR128
SGR41t[Sgris]	*S. griseus*	SGR41t
SGR7098t[Sgris]	*S. griseus*	SGR7098t
SsgA[Salbu]	*S. albus*	AF195771
SsgA[Sgold]	*S. goldeniensis*	AF195773
SsgA[Snetr]	*S. netropsis*	AF195772
SsgB[Tfusc]	*Thermobifido fusca*	Tfu_2111
SsgB[Kradi]	*Kineococcus radiotolerans*	Krad_3069
SsgB[Nocar]	*Nocardioides*	Noca_2368
SsgB[Acell]	*Acidothermus cellulolyticus*	Acel_1369
SsgB[SalTr]	*Salinispora tropica*	Strop_1600
SsgB[SalAr]	*Salinispora arenicola*	Sare_1560
SsgB[Falni]	*Frankia alni* ACN14a	FRAAL2127
FrS1[Falni]	*Frankia alni* ACN14a	FRAAL5533
FrS2[Falni]	*Frankia alni* ACN14a	FRAAL4594
FrS3[Falni]	*Frankia alni* ACN14a	FRAAL5373
FrS4[Falni]	*Frankia alni* ACN14a	FRAAL6494
SsgB[Fcci3]	*Frankia* sp. CcI3	Francci3_1359
FrS1[Fcci3]	*Frankia* sp. CcI3	Francci3_3418
FrS2[Fcci3]	*Frankia* sp. CcI3	Francci3_1632
SsgB[Fean1]	*Frankia* sp. EAN1pec	Franean1_5158
FrS2[Fean1]	*Frankia* sp. EAN1pec	Franean1_2058
FrS5[Fean1]	*Frankia* sp. EAN1pec	Franean1_4060
SsgB[Sacch]	*Saccharopolyspora erythraea*	SACE_1961
SacS1[Sacch]	*Saccharopolyspora erythraea*	SACE_5535

Table 2. Distribution of SALPs across actinomycetes.

Strain	Genome accession number	Genome size (Mbp)	Number of SALPs	Cell morphology; development
Streptomyces avermitilis	BA000030	9	6	Filamentous growth, spore chains on aerial hyphae
Streptomyces coelicolor	AL645882	8.7	7	
Streptomyces griseus	(project ID: 20085)	8.5	5	
Streptomyces scabies	(project ID:12985)	10.1	7	
Frankia alni	CT573213	7.5	5	Filamentous growth, multilocular sporangia either terminally or intercalary
Frankia sp. CcI3	CP000249	5.4	3	
Frankia sp. EAN1pec	AAII00000000	9	3	
Saccharopolyspora erythraea	AM420293	8.2	2	Filamentous growth, short spore chains on fragmented aerial hyphae
Acidothermus cellulolyticus	CP000481	2.4	1	Slender floccules, non-spore forming
Kineococcus radiotolerans	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.
Nocardioides sp. JS614	CP000509	4.9	1	Single rods/cocci, non-spore forming.
Salinispora arenicola	AAWA00000000	5.7	1	Filamentous growth, single spores borne on substrate mycelium.
Salinispora tropica	AATJ00000000	5.2	1	
Thermobifida fusca	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).

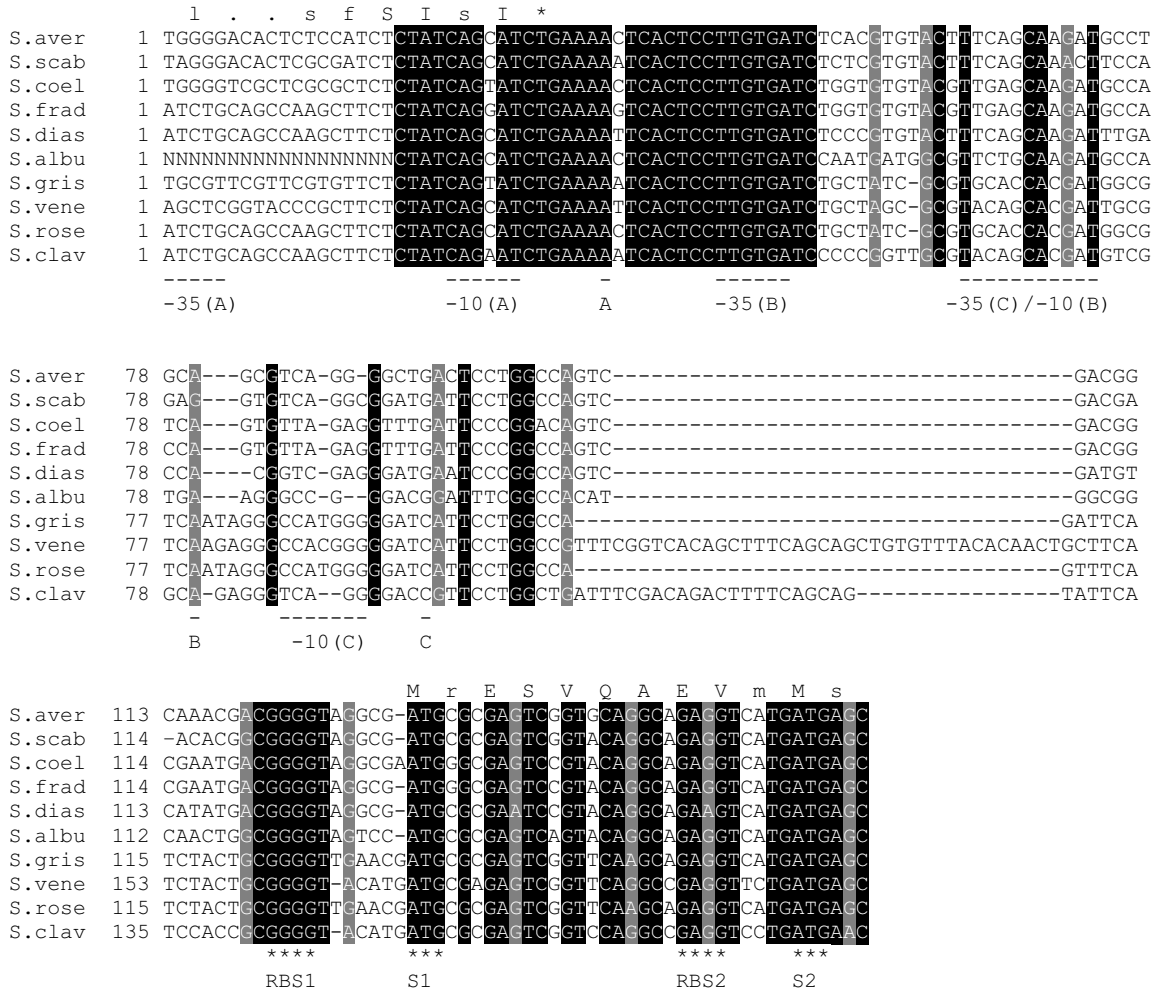

Figure 2. Alignment of *ssgA* promoter regions. Alignment was produced using the Boxshade program (www.ch.embnet.org/software/BOX_form.html). Only completely conserved nucleotides are shaded; nucleotides shaded in light grey refer to conserved purines. The two alternative start codons (S) for *ssgA* 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).

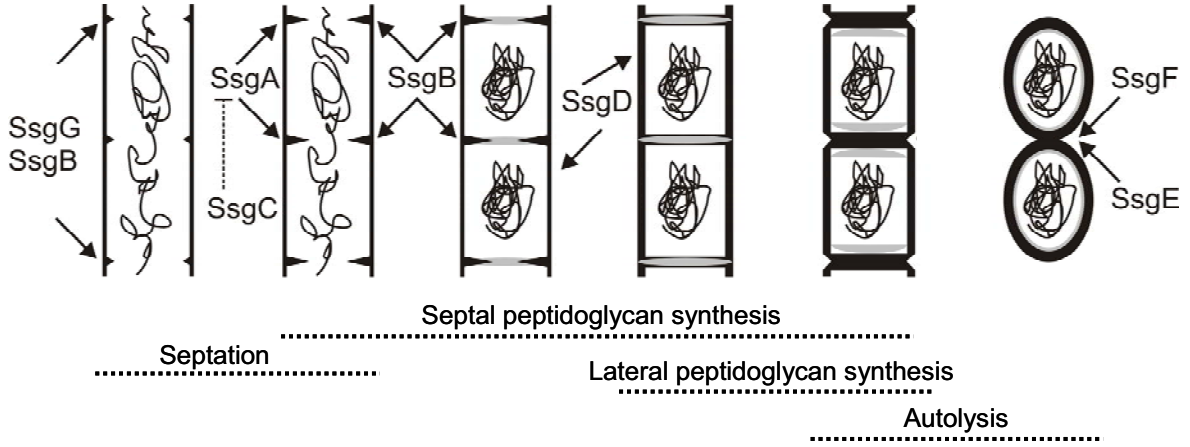

Figure 3. Model for the proposed functions of the SALPs in the control of morphogenesis of *S. coelicolor*. Arrows indicate the approximate time and place of action for the SALPs, namely septum-site localization (SsgABG), septum growth (SsgB), spore wall synthesis (SsgD) and autolytic spore separation (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.

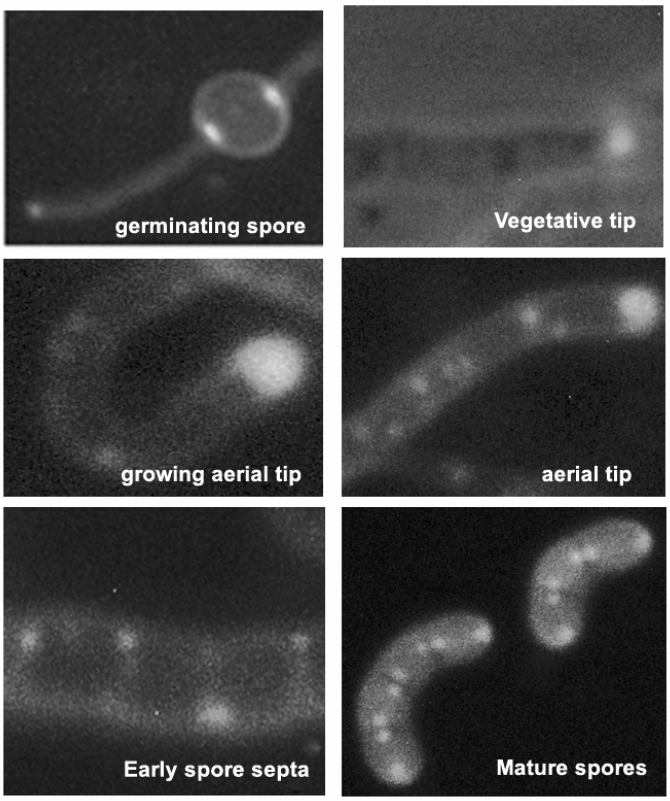

Figure 4. Dynamic localization of SsgA-GFP (For colour figure see Appendix A). Fluorescence micrographs of SsgA-GFP localization during the several stages of development of *S. coelicolor*. The width of the vegetative hyphae is around 400 nm (A, B), that of aerial hyphae and spores is around 800 nm (C-F).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

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