Function and control of the ssg genes in streptomyces

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Chapter VI

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

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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 syntheny 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. Acidothermus 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 (Flärdh, 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 *S. coelicolor* A3(2)	(Kieser *et al.*, 2000)
GSA3	M145 Δ*ssgA*	(van Wezel *et al.*, 2000a)
GSB1	M145 Δ*ssgB*	(Keijser *et al.*, 2003)
Δ SCO3156	M145 carrying Tn*5062* insertion in SCO3156	This study
Δ SCO3771	M145 carrying Tn*5062* insertion in SCO3771	This study
Δ SCO3772	M145 carrying Tn*5062* insertion in SCO3772	This study
Δ SCO4132	M145 carrying Tn*5062* insertion in SCO4132	This study
Δ SCO5466	M145 carrying Tn*5062* insertion in SCO5466	This study
Plasmids		
pHJL401	*Streptomyces*/*E. coli* shuttle vector (5-10 and around 100 copies per genome, respectively)	(Larson and Hershberger, 1986)
pGWS271	pHJL401 harboring *ssgB*^{SalTr} and its upstream region	This study
pGWS294	pHJL401 harboring *ssgB*^{Sery} and its upstream region	This study
pGWS295	pHJL401 harboring *sacS*^{Sery} and its upstream region	This study
pGWS298	pHJL401 harboring *ssgB*^{AceII} fused to the *S. coelicolor ssgB* promoter	This study
pGWS299	pHJL401 harboring *ssgB*^{Krad} fused to the *S. coelicolor ssgB* 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 *Eco*RI/*Bam*HI-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*^{AcelI}) 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*^{AcelI} or 3147086 for *ssgB*^{Krad} on their respective genomes), based on an alignment with *S. coelicolor* SsgB. *Eco*RI and *Hin*dIII digested inserts were cloned into pHJL401 digested with the same enzymes, resulting in pGWS298 (containing *ssgB*^{AcelI}) and pGWS299 (containing *ssgB*^{Krad}).

Table 2. *ssgB* genes from actinomycetes

Genome tag or contig number	Organism	GeneID	Name in this work
SCO1541	*Streptomyces coelicolor*	1096967	
SAV6810	*Streptomyces avermitilis*	1217088	
scab0975f02.p1k	*Streptomyces scabies*	-	
SGR5997	*Streptomyces griseus*	-	
FRAAL2127	*Frankia alni* ACN14a	4235729	
Francci3_1359	*Frankia* sp. CcI3	3904606	
Franean1_5158	*Frankia* sp. EAN1pec	5675752	
SACE_1961	*Saccharopolyspora erythraea*	4941816	*ssgB*^{Sery}
SACE_5535*	*Saccharopolyspora erythraea*	4945278	*sacS*^{Sery}
Acel_1369	*Acidothermus cellulolyticus*	4486262	*ssgB*^{AcelI}
Krad_3069	*Kineococcus radiotolerans*	5336467	*ssgB*^{Krad}
Noca_2368	*Nocardioides*	4595986	
Tfu_2111	*Thermobifido fusca*	3581046	
Strop_1600	*Salinispora tropica*	5058058	*ssgB*^{SalTr}
Sare_1560	*Salinispora arenicola*	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	CTG*GAATTC*GCGAGCGGGAATTCGATCT	-399
Sery_BR	CTG*AAGCTT*CGGCTGGGCTCTTGATCTC	+563
Sery_2F	CTG*GAATTC*GCAACCTTCTGAACGCCGTG	-408
Sery_2R	CTG*AAGCTT*CGATTCCAGTGCCCGGGAAG	+496
SalTr_BF	CTG*GAATTC*TCCACTGAGGATCGCTCCGTCAC	-296
SalTr_BR	CTG*AAGCTT*CATCACCACCCACGGCTGAGC	+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 Tn*5062* 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*, *Acidothermus* 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*, *Nocardioides* and *Thermobifida* (Figure 1). These observed similarities are unique to the *ssgB* locus, as comparable parallels were not observed for other *ssg* gene homologues.

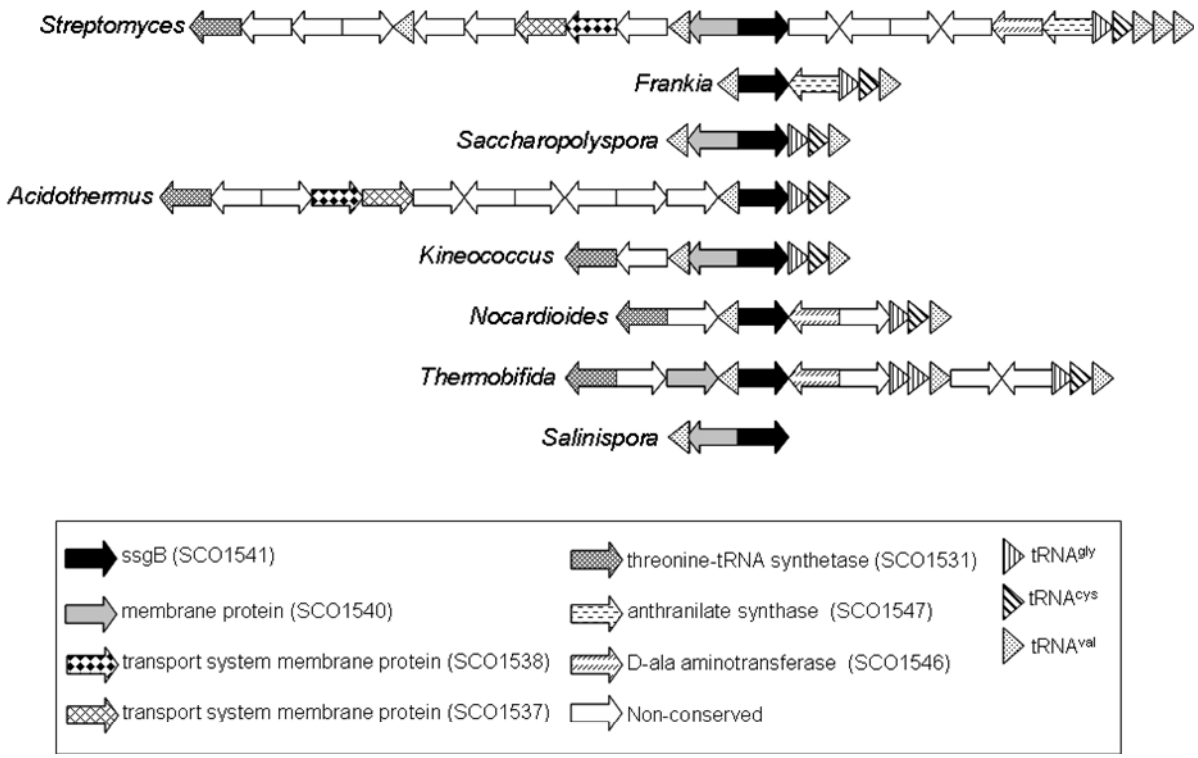

Figure 1. Genetic locus of *ssgB* orthologues from actinomycetes. Genes flanking *Streptomyces ssgB* which are conserved in the flanking regions of *ssgB* orthologues from other SALP-containing actinomycete genera are highlighted. Gene products for conserved genes are given in the box at the bottom.

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 *Acidothermus cellulolyticus* (unicellular) and *Kineococcus radiotolerans* (unicellular or symmetrical multi-cell clusters), the *ssgB* mutant GSB1 was transformed with plasmids pGWS298 (expressing *ssgB*^{AcelI}*)* 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.

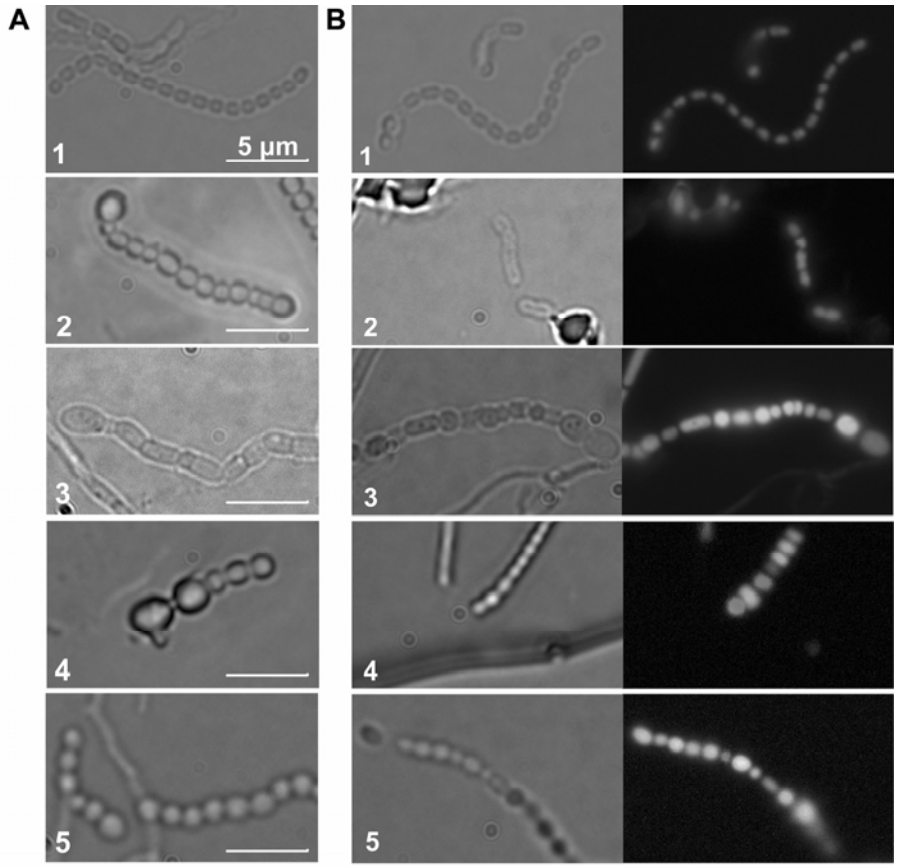

Figure 2. Analysis by phase-contrast and fluorescence microscopy (For colour figure see Appendix A). Samples were prepared from seven days old surface-grown cultures of the parental strain M145 harboring the empty vector (**1**), the *ssgB* mutant harboring pGWS271 (*ssgB*^{SalTr}) (**2**), pGWS294 (*ssgB*^{Sery}) (**3**), pGWS298 (*ssgB*^{AcelI}) (**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 Iodine (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*^{AceII} 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*^{AceII} 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*^{AceII}) 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*^{AcelI}, although some indentations that suggest septum formation were observed (Figure 3G).

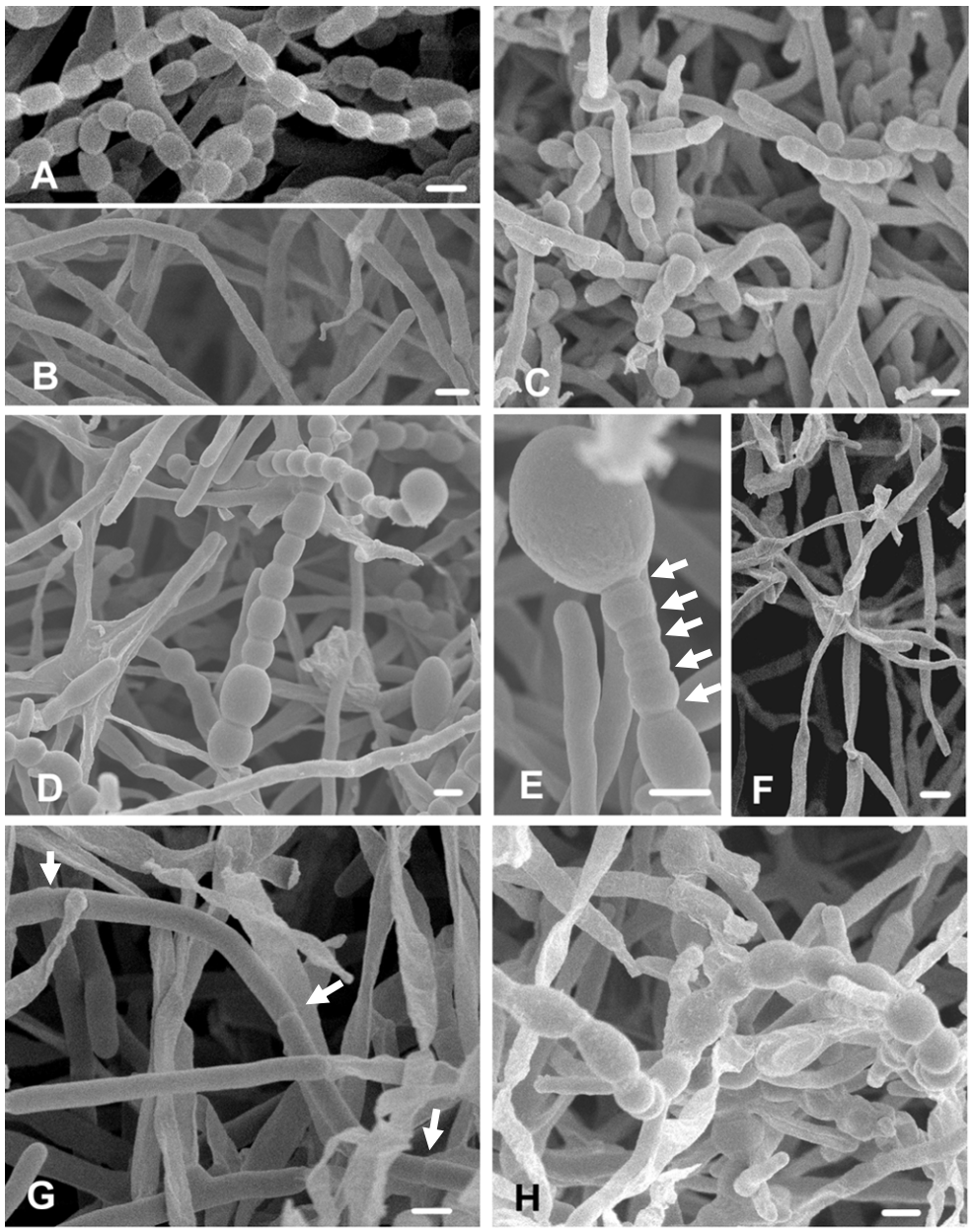

Figure 3. Analysis by cryo-scanning electron microscopy. Seven days old surface-grown colonies of the wild type M145 (A), the *ssgB* mutant (B), and *ssgB* mutant transformants containing pGWS271 (*ssgB*^{SalTr}) (C), pGWS294 (*ssgB*^{Sery}) (DE), pGWS295 (*sacS*^{Sery}) (F), pGWS298 (*ssgB*^{AcelI}) (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 iodine (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*^{AcelI} 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 *et al.*, 2005)
SCO5466	cell-wall hydrolase, implicated in the control of spore-wall thickness (Piette *et al.*, 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.

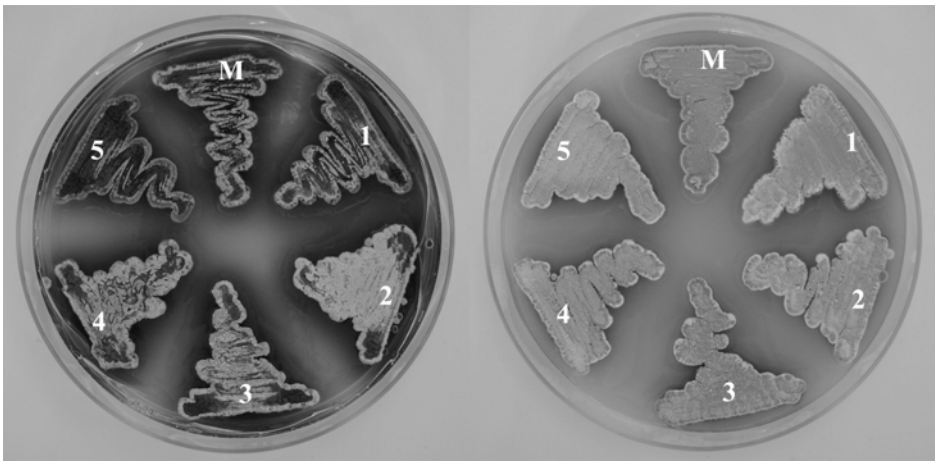

Figure 4. Mutational analysis of developmentally controlled PBP- and autolysin-encoding genes. The parental strain *S. coelicolor* M145 (M), and its mutant derivatives ΔSCO3156 (1), ΔSCO3771 (2), ΔSCO3772 (3), ΔSCO4132 (4) and ΔSCO5466 (5) (Table 1), grown for five days on R2YE (left) and SFM (right) 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).

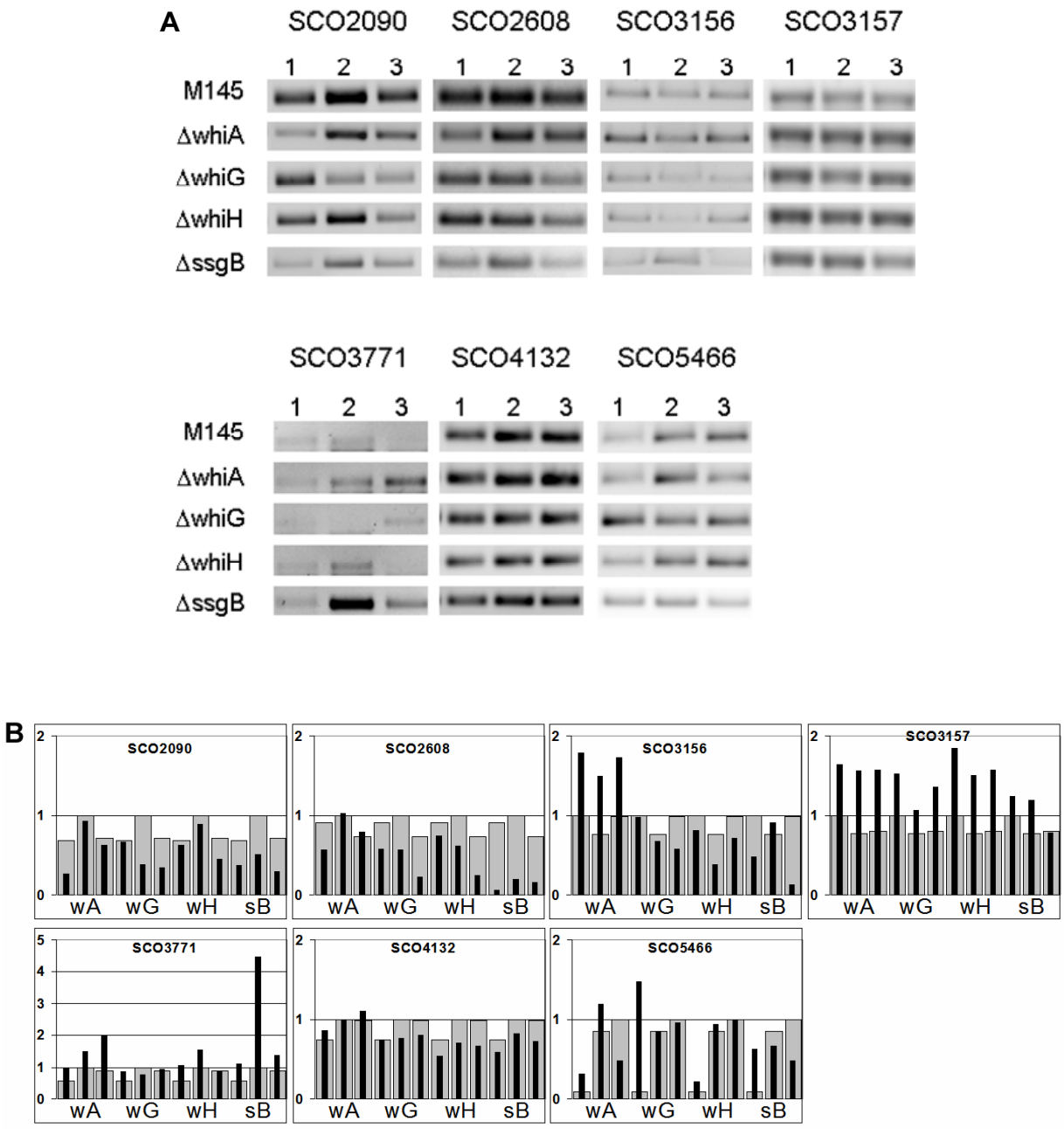

Figure 5. Transcriptional analysis of *pbp* and autolysin genes. A. Representative picture of semi-quantitative RT-PCR results of *pbp* and autolysin genes (top) in RNA purified from the parental strain *S. coelicolor* M145 and its sporulation-mutant derivatives Δ*whiA*, Δ*whiG*, Δ*whiH* and Δ*ssgB* (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** (Δ*whiA*), **wG** (Δ*whiG*), **wH** (Δ*whiH*), and **sB** (Δ*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 Willemse 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 orthologous 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. Acidothermus*, *Kineococcus* and *Nocardioides*).

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