# Control of sporulation-specific cell division in Streptomyces coelicolor

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4

# Loss of the controlled localisation of growth stage-specific cell wall synthesis pleiotropically affects developmental gene expression in an *ssgA* mutant of *Streptomyces coelicolor*

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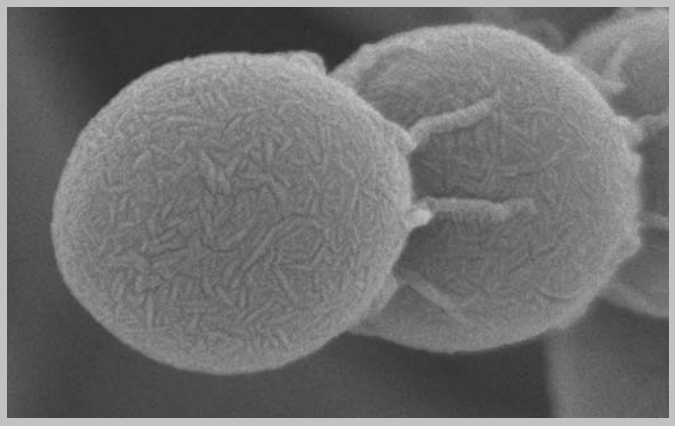
*Mol Microbiol* (2007) 64: 1244-1259



**ABSTRACT**

Members of the family of SsgA-like proteins (SALPs) are found exclusively in sporulating actinomycetes, and SsgA itself activates sporulation-specific cell division. We previously showed that SALPs play a chaperonin-like role in supporting the function of enzymes involved in peptidoglycan maintenance (PBPs and autolysins). Here we show that SsgA localises dynamically during development, and most likely marks the sites where changes in local cell wall morphogenesis are required, in particular septum formation and germination. In sporogenic aerial hyphae, SsgA initially localises as strong foci to the growing tips, followed by distribution as closely spaced foci in a pattern similar to an early stage of FtsZ assembly. Spore septa formed in these hyphae co-localise with single SsgA-GFP foci, and when the maturing spores are separated, these foci are distributed symmetrically, resulting in two foci per mature spore. Evidence is provided that SsgA also controls the correct localisation of germination sites. Transcriptome analysis revealed that expression of around 300 genes was significantly altered in mutants in *ssgA* and its regulatory gene *ssgR*. The list includes surprisingly many known developmental genes, most of which were upregulated, highlighting SsgA as a key player in the control of *Streptomyces* development.

## INTRODUCTION

The correct timing and localisation of cell division, which involves dynamic reorganisation of cell wall synthesis and breakdown, are among the most studied topics in modern microbial cell biology. In most bacteria a single septum forms the cleavage furrow dividing the mother cell into daughter cells, but during growth of the Gram-positive mycelial bacterium *Streptomyces,* vegetative hyphae are divided only occasionally by vegetative septa or cross walls, giving multigenomic compartments, while reproduction involves the simultaneous production of many specialised septa to form long chains of unigenomic spores from multigenomic aerial hyphal compartments (Flärdh *et al.*, 2000; McCormick *et al.*, 1994; Wildermuth and Hopwood, 1970). Correctly organised apical growth and branching of vegetative hyphae depend on the activity of DivIVA (Flärdh, 2003). In contrast to vegetative cross-walls, multiple sporulation septa are produced simultaneously and in a highly coordinated way within a sporogenic aerial hypha. A first step in sporulation-specific cell division is the localisation of FtsZ to many regularly spaced sites along the aerial hyphal wall (Grantcharova *et al.*, 2005). Multiple septa are then simultaneously synthesised in a process in which proteins required for the formation of bacterial divisomes are presumably sequentially directed to the Z ring (Bramhill, 1997; Errington *et al.*, 2003). The segregation of DNA into the prespore compartments is partially dependent on ParA and ParB (Jakimowicz *et al.*, 2002a; Jakimowicz *et al.*, 2005), is accompanied by DNA condensation, and appears to be completed by a process involving septum-located FtsK (Wang et al., 2007). After septum closure, the wall of the prespore compartments thickens and becomes pigmented, and eventually the mature spores are separated by a poorly understood process of autolytic cleavage. Much remains to be learned about the coordinated production of up to 100 sporulation septa in aerial hyphae.

Cell division in mycelial actinomycetes differs from that in other bacteria in lacking *minC* and *minE* homologues for septum-site localisation (Autret and Errington, 2001; Marston *et al.*, 1998), the nucleoid occlusion system Noc, and Z-ring anchoring proteins such as FtsA and ZipA (Errington *et al.*, 2003; Lowe *et al.*, 2004). Instead, several unique protein families have been identified that play a role in the control of cell division (Chater and Chandra, 2006; Flärdh and van Wezel, 2003). One such family is that of the SsgA-like proteins (SALPs), developmental agents that occur exclusively in mycelial actinomycetes, and are unrelated to other known proteins. The model strain *Streptomyces coelicolor* A3(2) harbours seven

paralogues, all involved in specific chaperone-like tasks during development, controlling processes from initiation of sporulation to the autolytic cleavage of spore wall peptidoglycan (PG) (Chapter 2; van Wezel and Vijgenboom, 2004). The most-studied SALP, SsgA itself, was first identified as an effecter of septation in *S. griseus* (Kawamoto and Ensign, 1995). In *S. coelicolor, ssgA* null mutants have an unusual conditional non-sporulating phenotype, the aerial hyphae nearly all being devoid of sporulation septa except on mannitol-containing media, on which some spores are produced (van Wezel *et al.*, 2000a). Overexpression of SsgA results in hyperseptation of both vegetative and aerial hyphae, thus interfering with the control systems that determine the morphological differences between the two cell types. Such overexpression effected the production of extremely thick and irregular septa, often with a gap in the middle, demonstrating disturbance of the concluding stages of the cell division process (van Wezel *et al.*, 2000a). Therefore, SsgA somehow activates the initiation and control of septal peptidoglycan synthesis, probably by influencing the function of Penicillin-Binding Proteins (PBPs) involved in the biogenesis of septal peptidoglycan, which in bacteria is typically carried out by FtsI (Bramhill, 1997).

In this work, we provide an in-depth analysis of *ssgA* using a knockout mutant and an *ssgA-egfp* fusion strain, and employ microarrays to analyse the global changes in gene expression in strains defective in *ssgA*. Clear links between distinct phenotypic anomalies and altered gene expression profiles are provided.

## MATERIALS AND METHODS

### Bacterial strains and media

The bacterial strains described 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 routine cloning and plasmid propagation, and were grown and transformed by standard procedures (Sambrook *et al.*, 1989). *E. coli* ET12567 containing pUZ8002 was used for conjugation to *S. coelicolor* (Kieser *et al.*, 2000). *E. coli* transformants were selected in L-broth containing the appropriate antibiotics. *Streptomyces coelicolor* A3(2) M145 was obtained from the John Innes Centre strain collection, and was the parent for the previously created *ssgA* mutant GSA3 (van Wezel *et al.*, 2000a). All media and routine *Streptomyces* techniques are described in the *Streptomyces* manual (Kieser *et al.*, 2000). SFM agar plates were used for making spore

suspensions and R2YE agar plates for regeneration of protoplasts and, after the addition of the appropriate antibiotic, for selecting recombinants. Pregermination was performed by heat shock at $50^0$C for 10 minutes, followed by shaking at $30^0$C for at least 10 hours. For microscopic analysis, streptomycetes were grown on SFM agar plates. For standard cultivation and plasmid isolation, YEME or TSBS [tryptone soy broth (Difco) containing 10% (w/v) sucrose] were used.

**Table 1:** Bacterial strains, Plasmids and constructs.

| **Bacterial strain** | **Genotype** | **Reference** |
|---|---|---|
| *S. coelicolor* A3(2) M145 | SCP1$^-$ SCP2$^-$ | (Kieser *et al.*, 2000) |
| GSA2 | M145 + pGWS4-SD | (van Wezel *et al.*, 2000a) |
| GSA3 | M145 Δ*ssgA* (::*aadA*) | (van Wezel *et al.*, 2000a) |
| GSR1 | M145 Δ*ssgR* | (Traag *et al.*, 2004) |
| K202 | M145 + KF41 | (Grantcharova *et al.*, 2005) |
| *E. coli* JM109 | See reference | (Sambrook *et al.*, 1989) |
| *E. coli* ET12567 | See reference | (MacNeil *et al.*, 1992) |
| *E. coli* ET12567/pUZ8002 | See reference | (Gust *et al.*, 2003) |

| **Plasmids and constructs** | **Description** | **Reference** |
|---|---|---|
| pHJL401 | *Streptomyces/E. coli* shuttle vector (5-10 copies per genome in *Streptomyces*) | (Larson and Hershberger, 1986) |
| pIJ2925 | Derivative of pUC19 (high copy number) with *Bgl*II sites flanking its multiple cloning site. | (Janssen and Bibb, 1993) |
| pGWS116 | pHJL401 with 1.2 kb fragment harbouring a translational fusion of *ssgA* and *egfp* | This chapter |
| pGWS4-SD | Integrative construct over-expressing *ssgA*. | (van Wezel *et al.*, 2000a) |
| KF41 | pSET152-derived integrative vector expressing FtsZ-GFP | (Grantcharova *et al.*, 2005) |
| pFT73 | *mycgfp2*$^+$ cloned into *Bam*HI-*Pst*I of pUWL-SK+ | (Nothaft *et al.,* 2003) |

## Plasmids and constructs

All plasmids and constructs described in this paper are summarised in Table 1. pIJ2925 (Janssen and Bibb, 1993) is a pUC19-derived plasmid used for routine subcloning. The shuttle vector pHJL401 has the pUC19 *ori* for high-copy number replication in *E. coli* and the SCP2* *ori* (around five copies per chromosome) for maintenance in *S. coelicolor* (Larson and Hershberger, 1986). An 1860 bp DNA fragment harbouring *ssgR* and *ssgA* was amplified by PCR from M145 genomic DNA using oligonucleotides Q1 and SsgA-GFP3 (Table 2). In this way, a *Kpn*I restriction site was created replacing the *ssgA* stop codon. The fragment was inserted as an *Eco*RI-*Kpn*I fragment into pIJ2925, creating pGWS113. Subsequently, *egfp* was amplified from pFT73 (Nothaft *et al.*, 2003) using oligonucleotides Har_GFP_For and Har_GFP_Rev (Table 2), replacing the start codon of *egfp* by a *Kpn*I restriction site. This

facilitated in frame fusion of *ssgA* and *egfp*. This 716 bp fragment was inserted as an *Kpn*I-*Hin*dIII fragment into pGWS113, resulting in pGWS114. The complete insert was cloned as an *Eco*RI-*Hin*dIII fragment in pHJL401, resulting in pGWS116. The plasmid expresses *ssgA-egfp* from the natural *ssgA* promoter.

**Preparation of total RNA**

Mycelium used for total RNA isolation was grown on cellophane disks on MM containing 1.5% Hispan agar and 0.5% mannitol as the carbon source. On this medium the strains sporulated well (M145) or poorly (GSA3, GSR1). Mycelium was harvested at times corresponding to vegetative growth (24h), aerial growth (36h, 48h) and sporulation (60h, 72h). RNA was purified using the Kirby-mix protocol (Kieser *et al.*, 2000). RNA purification columns (RNeasy, Qiagen) and DNaseI treatment were used as well as salt precipitation (final concentration 3M sodium acetate pH 4.8) to purify the RNA and fully remove any traces of DNA, respectively. Before use, the RNA preparations were checked for their quality and integrity on the Agilent 2100 Bioanalyzer (Agilent Technologies).

**DNA Microarray analysis**

For microarray hybridization each RNA sample was reverse-transcribed into Cy3-dCTP-labelled cDNA and co-hybridised with Cy5-dCTP-labelled genomic DNA from *S. coelicolor* M145, as common reference (Bucca *et al.*, 2003). The samples were hybridised to in-house (University of Surrey) fabricated oligonucleotide (50mer) microarrays covering essentially all ORFs of the *S. coelicolor* genome (for array coverage and detailed protocols: www.surrey.ac.uk/SBMS/Fgenomics/Microarrays). The hybridised microarrays were scanned with an Affymetrix 428 laser scanner for fluorescent signal acquisition. The 16-bit TIFF microarray images were analysed with BlueFuse (BlueGnome) spot quantification software and further processed using the statistical computing environment R (Version 2.1.1) and the package Limma (R Development Core Team). An ad-hoc R function was written to normalise all $\log_2$ cDNA/gDNA ratios on all arrays via the block-median (dividing the ratio of each of the 48 blocks by its respective median ratio). The $\log_2$ ratios were then scaled to have the same median-absolute-deviation (MAD) across arrays (Smyth and Speed, 2003; Smyth, 2005). Duplicate good spots (with confidence value >0.1 and spot quality of 1 (two parameters calculated by the BlueFuse software)) were averaged. Technical replicates for the M145 wild type strain were averaged. Data were then filtered such that there was at

least one biological replicate with one good spot in each time point/strain, resulting in a gene list of 7,345 genes for differential expression analysis. Data was then imported into GeneSpring (Version GX) and transformed to linear scale. Significantly differentially expressed genes between each of the deletion mutants and the M145 wild type strain were identified by applying one-way ANOVA ($p<0.05$; Benjamini & Hochberg False Discovery Rate) using GeneSpring. Some additional genes were also identified that demonstrated more than a two-fold change in expression level between wild type and mutant strains in at least two time points. The expression of selected genes was independently assessed with RT-PCR analysis (see below).

**Table 2:** Oligonucleotides used in PCR and RT-PCR.

| **Primer** | **Sequence (5' -> 3')** | **Location 5' end** | **Relative to** |
|---|---|---|---|
| **PCR oligo's** | | | |
| Q1 | ctg**gaattc**tagcatcgagggcaggacatcaga | -1450 | *ssgA* |
| ssgA-GFP3 | ctga**ggtacc**cgcgctctgttcctccgccaggatg | +410 | *ssgA* |
| Har_GFP_For | gact**ggtacc**tcgaagggcgaggagctgttcacc | +3 | *egfp* |
| Har_GFP_Rev | gctg**aagctt**ctacttgtacagctcgtccatgccgtg | 716 | *egfp* |
| **RT-PCR oligo's** | | | |
| rRNA-For | tcacggagagtttgatcctggctc | +20 | 16 rRNA |
| rRNA-Rev | cccgaaggccgtcatccctcacgc | +436 | 16 rRNA |
| adpA-F | ctcttcgtggacgacggcgacg | +499 | *adpa* |
| adpA-R | gaaggtgcggcggctcatgtagg | +795 | *adpA* |
| bldN-F | ctacgaccagtacagcgacaccg | +53 | *bldN* |
| bldN-R | gctgtgggttgagccgtcgtacg | +385 | *bldN* |
| divIVA-F | ctatgacgaggacgaggtcgatgc | +86 | *divIVA* |
| divIVA-R | ccatggggccacccatctgctgc | +370 | *divIVA* |
| ftsI-F | gaccatcgaccgcgacatccagtgg | +881 | *ftsI* |
| ftsI-R | gtggcgacgttctcctccagcacg | +1136 | *ftsI* |
| ftsZ-F | catcaccgacctcatcaccacc | +579 | *ftsZ* |
| ftsZ-R | gccgaagatgatgttggcctcg | +876 | *ftsZ* |
| gyrA-F | gttcaggacgaccttggcgaccg | +703 | *gyrA* |
| gyrA-R | gttcaggacgaccttggcgaccg | +978 | *gyrA* |
| parB-F | gtgaaaccgaagagctgacggcacc | +77 | *parB* |
| parB-R | gatggcgtccagctccagctctcg | +345 | *parB* |
| tatA-F | cgtcgtcatcatcctgctgttcgg | +41 | *tatA* |
| tatA-R | cttggtcgtgtccgtcggctcg | +261 | *tatA* |
| SCO0204-F | acgaaccggacatcaccgtggtc | +104 | SCO0204 |
| SCO0204-R | agccgtgccatcagcttggtcg | +446 | SCO0204 |

Restriction sites used for cloning are presented in bold face.

## PCR and RT-PCR analysis

PCRs were performed with *Pfu* polymerase (Stratagene) in the presence of 10% (v/v) DMSO. The programme used was as follows: 2 min at 95°C followed by 35 cycles of 1 min at 95°C (denaturation), 1 min at 58°C (annealing) and 2-3 min elongation at 72°C (time according to the length of the fragment). RT-PCR analysis was carried out using the Superscript III one

step RT-PCR System with Platinum® *Taq* DNA polymerase (Invitrogen) for the verification of gene expression changes observed in microarray experiments. RNA samples (time points 24h, 48h and 72h matching vegetative growth, aerial growth and sporulation, respectively) were the same as those used for microarray analysis. For each RT-PCR reaction 100 ng of RNA was used together with 1μM (final concentration) of each primer. The program used was as follows: 45 min cDNA synthesis at 48°C, followed by 2 min at 95°C and 25, 29, 31 or 35 cycles of: 45 s at 94°C (denaturation), 30 s at 68°C (annealing) and 30 s at 68°C (elongation). The reaction was completed by 5 min incubation at 68°C. 5μl of each sample was visualised by ethidium bromide staining after electrophoresis on a 2% agarose gel in 1xTAE buffer. The oligonucleotides are listed in Table 2. Quantification of the RT-PCRs was done by scanning the gels using the GS-800 imaging densitometer followed by analysis using Quantity One software (Biorad).

**Microscopy**

*Confocal fluorescence microscopy*

The visualisation of DNA (with propidium iodide, Sigma) and cell wall material (FITC-WGA, Biomedica) by confocal fluorescence microscopy was performed as described previously (Chapter 2). For the visualisation of strains containing proteins translationally fused with EGFP, sterile cover slips were inserted at a 45° angle into SFM plates and spores were inoculated in the acute angle. After incubation at 30°C for 2-5 days, coverslips were removed and samples positioned in a drop of water on a microscope slide.

*Electron microscopy*

Morphological studies of surface-grown aerial hyphae and spores of *S. coelicolor* M145 and mutant derivatives by cryo-scanning electron microscopy (cryo-SEM) were performed as described previously, using a JEOL JSM6700F scanning electron microscope (Keijser *et al.*, 2003).

## RESULTS

### The *ssgA* mutant has branching aerial hyphae

We showed previously that *ssgA* mutants produce aerial mycelium but have a conditional non-sporulating phenotype, failing to sporulate on glucose-containing solid media, while some spores are produced on mannitol-containing media (van Wezel *et al.*, 2000a). To get a more detailed view of the developing aerial hyphae, we subjected the *ssgA* mutant to detailed cryo- SEM. Interestingly, a significant number (approximately 40%) of the *ssgA* mutant aerial hyphae branched, and some showed multiple branches, with an average branch spacing of 5-10 μm. Despite extensive searches we never observed this phenomenon for aerial hyphae of the parental strain M145 (Fig. 1). Branching was also apparent in double mutants where besides *ssgA* also *ssgB, ssgC, ssgD, ssgE* or *ssgF* and *ssgG* was disrupted, and the branching was significantly enhanced in *ssgAC* double mutants, which had up to five branches at one single point of the aerial hyphae (Noens *et al.,* 2007, Fig. S1; Chapter 3, Fig. 5).

### SsgA localises in a development-dependent and dynamic manner

To analyse the localisation of SsgA, we constructed plasmid pGWS116 expressing an *ssgA-egfp* in frame fusion from the natural *ssgA* promoter and introduced it into *S. coelicolor* M145 (see Materials and Methods for details). This plasmid fully complemented *ssgA* mutant GSA3, both in terms of sporulation and of mycelial morphology, indicating that the SsgA-EGFP fusion product was indeed functional and could replace the wild type SsgA.

Confocal fluorescence microscopy revealed a highly dynamic localisation for SsgA-GFP during development (Fig. 2). Discriminating the various stages of aerial development, the following observations were made. Initially, foci were relatively distantly spaced, namely 0.84 ± 0.19 foci per μm (average of 386 foci, measured along the hyphal wall) in the younger aerial hyphae (Fig. 2A, stage 1), which probably correspond to the recently designated and developmentally distinct sub-apical stem compartments (Dalton *et al.*, 2007). At this stage, the aerial hyphae still have the width of vegetative hyphae (approximately 400-500 nm). The hyphae later widen to about 800 nm, which is the width of spores (see *e.g.* Fig. 2D, which demonstrates both types of aerial hyphae). Early stage sporogenic hyphae carried a large amount of SsgA-GFP in the tips, sometimes showing very bright foci (Fig. 2A,C; stage 2). As growth progressed, the foci became less intense and were replaced by foci at alternating sides of the hyphae and around twice as frequent as in young aerial hyphae, namely 1.59 ± 0.34 foci

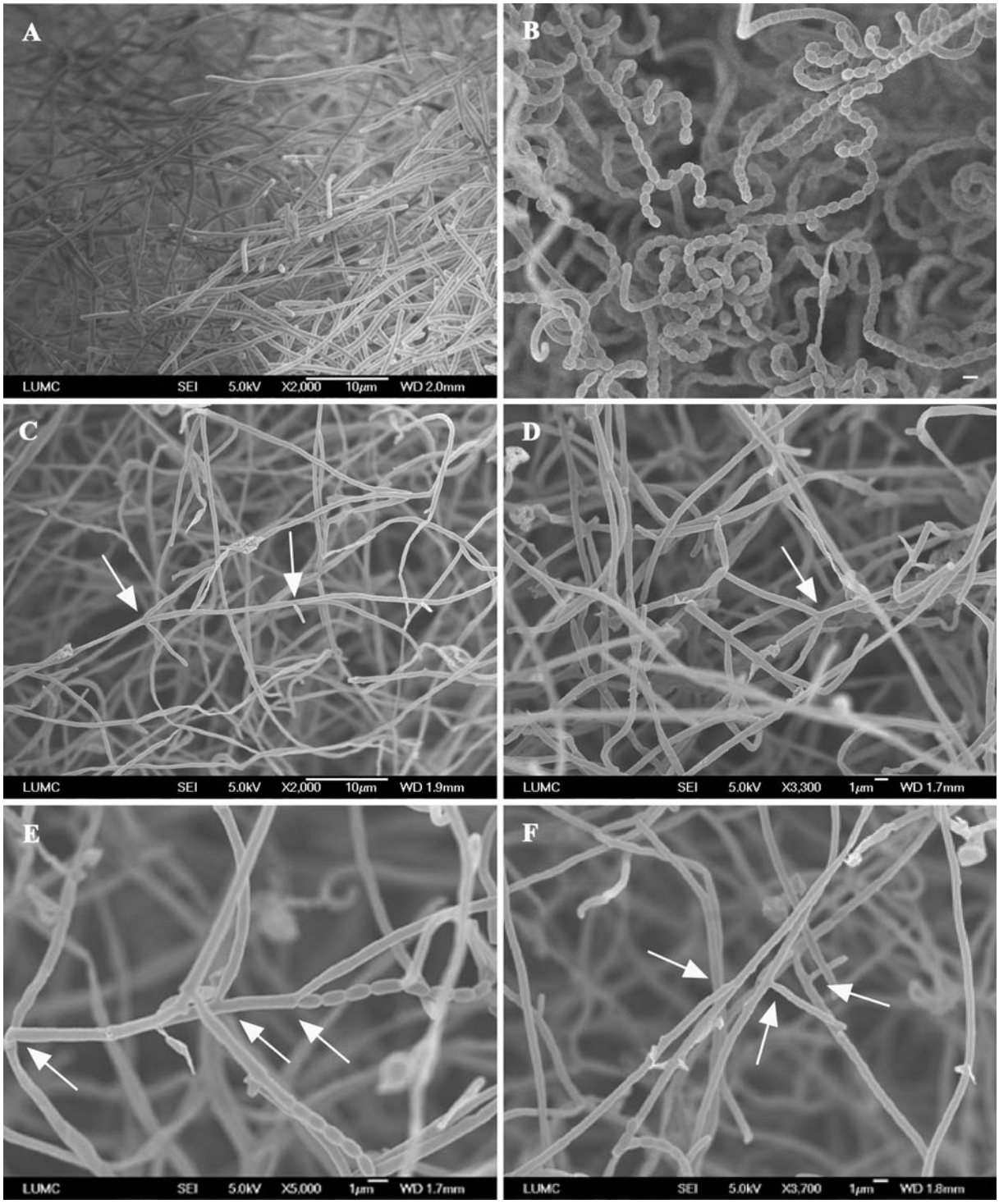



**Figure 1: Cryo-scanning electron micrographs of *S. coelicolor* M145 and its *ssgA* mutant GSA3**. Cultures were grown on SFM for 2 days (**A-C**) and 5 days h (**B-D-E-F**) at 30°C. **A.** Young aerial hyphae of M145. **B.** Wild type spore chains. **C.** Young, unseptated, aerial hyphae of the *ssgA* mutant that often branched (white arrows). **D-E-F.** Late aerial hyphae of the *ssgA* mutant that often branched and occasionally produced spore chains. A higher proportion of the aerial hyphae in the *ssgA* mutant was lysed, compared with the wild type. Bar = 10 μm.

per μm (average of 870 foci and measured along the hyphal wall) (Fig. 2A, stage 3). Eventually, at sporulation septa we found one spot per septum (Fig. 2A and Fig. 3 (stage 4)). The position of the foci relative to the septa, namely a single spot and immediately adjacent to the hyphal wall, strongly suggests that SsgA is not a part of the divisome, as this is by definition localised in a ring-like fashion. Most of the mature spores (97 out of 127; Table 2) had two foci (average 2.0 ± 0.1 foci per spore: stage 5 in Fig. 3). This suggests splitting and segregation of the initial focus, or alternatively its disassembly and reassembly as two foci. To our knowledge this is the first subcellular localisation of a protein in *Streptomyces* spores.

During vegetative growth, SsgA-GFP foci were observed primarily in (very) young hyphae, including the tips of germinating spores (see below), but no foci were observed in older vegetative mycelium, until differentiation occurred from vegetative to aerial growth (not shown). The previous observation that vegetative cross-wall formation is normal in *ssgA* mutants (van Wezel et al., 2000a) suggests that SsgA does not play a major role in septum synthesis during early growth, so in what follows we focus on the role of SsgA during aerial development.

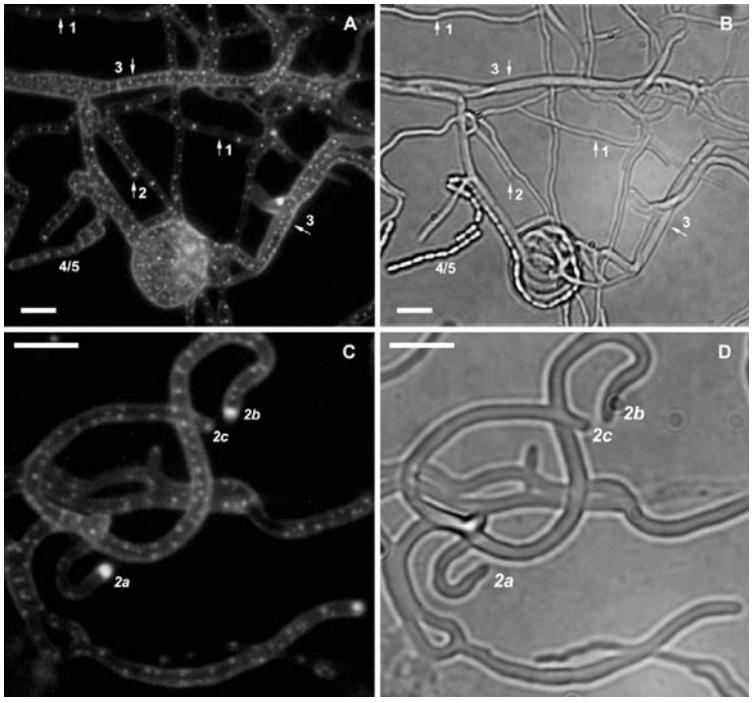



**Figure 2: Fluorescence micrographs showing localisation of SsgA-GFP in *S. coelicolor*.** Fluorescence microscopy (**A-C**) and phase contrast microscopy (**B-D**) of aerial hyphae expressing SsgA-GFP. Cultures were grown on SFM for 5 days at 30°C. **A-B.** Overview of aerial hyphae showing all stages of development except mature spores. SsgA was localised in several distinguishable patterns in aerial hyphae. Stages (indicated by arrows with a number; see also Fig. 3 and the scheme in Fig. 7): (1) early aerial hyphae; (2) tips of early sporogenic hyphae; (3) sporogenic hyphae; (4) septum site-initiation; (5) septa. The last stages - namely (6) spore separation and (G) germination - are not visible in this image, but are highlighted in Fig. 3, p97. **C-D.** Images showing close up of aerial hyphae with SsgA-GFP foci in the tips. Arrows indicate foci in the tips (stages 2a, 2b and 2c refer to Fig. 7). Bar = 2 μm.

**SsgA and the selection of germination sites**

The presence of the SsgA-GFP foci in the spores suggested a possible link with the selection of germination sites. To test this, we allowed spores from *S. coelicolor* M145, its *ssgA* mutant and a strain overproducing SsgA (GSA2; (van Wezel *et al.*, 2000a)) to germinate, and counted the number of germ tubes. There was a clear correlation between the average number of germ tubes per spore and the copy number of *ssgA*, with on average 1.7 (± 0.1) germ tubes per spores for the *ssgA* mutant, 2.0 (± 0.1) germ tubes per spore in M145 and 2.5 (± 0.1) germ tubes per spore in GSA2 (Table 2). The number of on average two germ tubes per spore in *S. coelicolor* M145 or in GSA3 complemented with SsgA-GFP corresponded very well to the average number of SsgA-GFP foci per spore (also 2.0 per spore, see above). Furthermore, more of the SsgA-overproducing spores carried four germ tubes (8 out of 115) than the parent M145 (2 out of 99) or the *ssgA* mutant (3 out of 89). In GSA2 we occasionally even identified spores with five emerging germ tubes (one among those that were counted), which was never seen in the parental strain. Generally, in older spores fluorescence was lost, suggesting that SsgA-GFP does not physically support the initiation of germ tube formation, but rather marks the future sites. However, in fresh spores the SsgA-GFP foci co-localised with the emerging germ tubes (labelled 'G' in Fig. 3). Thus, besides its role in the localisation of septa, these data suggest that SsgA is also involved in germination site-localisation. Interestingly, as mentioned above, SsgA-GFP was produced soon after the emergence of vegetative hyphae, and in particular at the tips, suggesting a possible role in (the control of) aerial apical growth (Fig. 3).

**Table 3:** Statistical analysis of germination frequencies.

| **Germ tubes per spore** | M145 | GSA3 | GSA3 + *ssgA*-GFP | GSA2 | **Foci per spore** | GSA3 + *ssgA*-GFP |
|---|---|---|---|---|---|---|
| **1** | 20 | 42 | 14 | 8 | 1 | 14 |
| **2** | 61 | 34 | 56 | 56 | 2 | 97 |
| **3** | 16 | 8 | 16 | 42 | 3 | 12 |
| **4** | 2 | 3 | 1 | 8 | 4 | 4 |
| **5** | 0 | 0 | 0 | 1 | 5 | 0 |
| **6** | 0 | 0 | 0 | 0 | 6 | 0 |
| **Total** | **99** | **87** | **87** | **115** | **Total** | **127** |
| **Average** | **2.0 ± 0.1** | **1.7 ± 0.1** | **2.0 ± 0.1** | **2.5 ± 0.1** | **Average** | **2.0 ± 0.1** |

The table shows the number of spores observed with a certain number of germ tubes (one to six, see left column). Strains analysed were wild type *S. coelicolor* M145, its *ssgA* mutant GSA3, the same GSA3 but complemented with SsgA-GFP, or an SsgA over-expressing strain (GSA2). *Foci per spore* refers to the number of SsgA-GFP foci observed in spores of the *ssgA* mutant GSA3 complemented with a construct expressing SsgA-GFP.

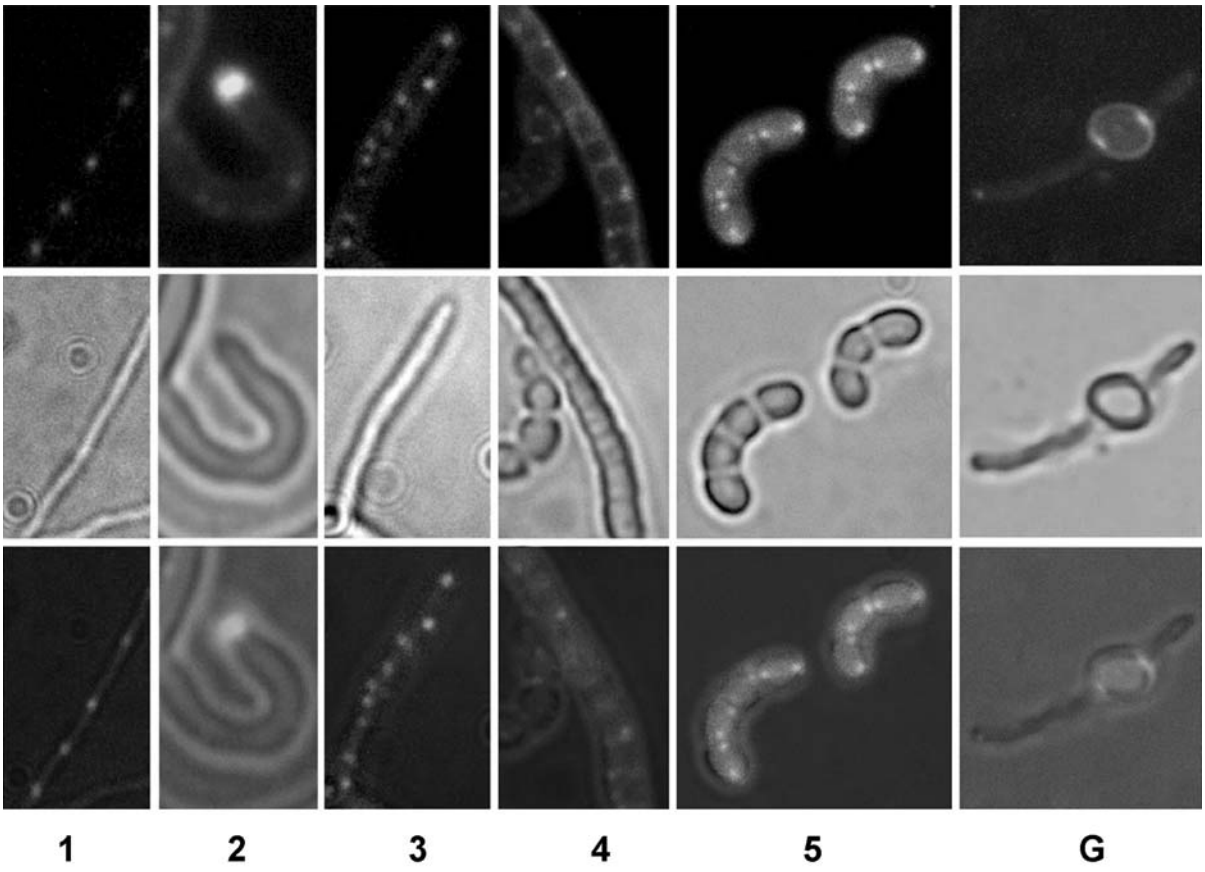



**Figure 3: Fluorescence micrographs showing representative localisation of SsgA-GFP in all stages of aerial development.** Developmental stages are sequentially labelled **1-5** and **G**, to facilitate comparison to the scheme in Fig. 7. The putative 'sub-apical stem' was postulated recently (Dalton *et al.*, 2007). For strains and growth conditions see Fig. 2, p95.

### Global gene expression profiles in M145 and its *ssgA* and *ssgR* deletion mutants

To study the cellular response to the absence of either *ssgA* or *ssgR* and in this way discover more about the function of the respective gene products, we compared the development-dependent gene expression profiles of *S. coelicolor* M145 and its mutants GSA3 (M145 Δ*ssgA*) and GSR1 (M145 Δ*ssgR*) by DNA microarray analysis. Strains were cultivated on MM agar plates with mannitol as the sole carbon source and RNA was isolated from mycelium harvested at five time points, corresponding to vegetative growth (24h), aerial growth (36h, 48h) and sporulation (60h, 72h). Under these conditions, which have been employed for a variety of microarray experiments with *S. coelicolor*, the mutants showed a similar timing of development as its parent, although only a limited number of spores was produced. We used oligonucleotide-based microarrays of *S. coelicolor* (see Materials and Methods for details). The microarray experiments were carried out as independent duplicates. After data normalisation, genes were selected that were differentially expressed between *ssgA*

and *ssgR* deletion mutants compared to M145. We were surprised to find over 300 differentially expressed genes (Noens *et al*., 2007, Table S2).

We previously demonstrated that the readily observable *ssgR* mutant phenotype is primarily caused by the absence of *ssgA* (Traag *et al.*, 2004). However this does not completely rule out that SsgR may regulate other genes. To investigate this, we compared the global expression patterns of both mutants. Strikingly, after normalisation, the overall gene expression profiles in GSA3 and GSR1 were almost identical (Fig. 4, p180), again providing strong evidence that SsgR is the specific activator of *ssgA*.

**Effect on transcription of developmental genes**

Surprisingly, the genes that were up- or downregulated in the *ssgA* (and *ssgR*) mutant as compared to the parental strain M145 included no less than 35 well-known developmental genes (*e.g. bld, whi, chp, rdl, ssg, par*; see Fig. 5A-B, p180; Noens *et al.,* 2007, Table S2). Both the total number of genes affected by *ssgA* and the proportion that have recognizable developmental relevance were markedly higher than we have found in similar studies of other developmental mutants (*bldA*: A. Hesketh, G.B., E.L., F.F., G.H., C.P.S. and K.F.C., manuscript submitted; *whiI* (Tian *et al.*, 2007); *ssgC*, E.E.N., V.M., K.F.C., C.P.S. and G.P.v.W., unpublished). Thus, SsgA had a stronger impact on the control of the sporulation process than was expected on the basis of data obtained for other developmental mutants. Transcripts of *ftsI,* involved in septum synthesis and encoding the transpeptidase specifically required for septal PG synthesis (Errington, 2001; Holtje, 1998) were among the most over-represented mRNA species in the *ssgA* and *ssgR* mutants (more than three-fold during early aerial growth). Of the SALPs, mRNA of *ssgD* (whose function is still unclear) was more abundant in the mutants from the onset of aerial growth onwards, while the spore maturation *ssgE* and *ssgF* mRNAs were less abundant in the *ssgA* mutant, although the difference for *ssgE* was below the set threshold (two-fold). The mRNA for the major developmental regulatory gene *adpA* (*bldH*) was more than three-fold more abundant at all time points. AdpA is required for *ssgA* transcription in *S. griseus* and affects its expression in *S. coelicolor* (Traag *et al.*, 2004; Yamazaki *et al.*, 2003). *bldN* mRNA, encoding an ECF σ-factor whose transcription is dependent on AdpA, was more abundant in the *ssgA* mutant from early aerial phase onwards (Bibb *et al.*, 2000). Supportive evidence for the increased *adpA* and *bldN* mRNA levels is the increased representation of mRNAs for several components of the BldN regulatory cascade (*bldM, chpA-H;* (Bibb *et al.*, 2000; Elliot *et al.*, 2003)) in the *ssgA* mutant.

The mRNAs for all the chaplin genes *chpACDEFGH* (*chpB* was filtered from the analysis as it failed to pass the quality control) and rodlin (*rdlAB*) genes were very strongly increased. Many *bld* gene transcripts were affected as well, particularly those known to be active throughout the life cycle, which are therefore likely to play an important role during sporulation as well as during earlier stages of the *Streptomyces* life cycle. The mRNAs of the sporulation genes *whiA* and *whiH* were increased in the *ssgA* mutant from early aerial phase onwards, while mRNAs of the late sporulation gene *whiD* and the poorly defined sporulation gene *whiJ* were decreased; *whiB* and *whiG* were not significantly affected in the *ssgA* mutant (Noens *et al.,* 2007, Table S2). Finally, *divIVA* transcript was more than three-fold over-represented in the *ssgA* mutant at a time corresponding to late aerial growth and sporulation, an observation that is possibly linked to the branching of aerial hyphae observed in GSA3 (see Discussion). Despite the strongly enhanced septum formation that follows the over-expression of SsgA, the only differentially abundant cell division-related transcripts were those of of *ftsL* (which is co-transcribed with *ftsI*) and *ftsQ,* both of which were over-represented in the *ssgA* mutant.

**Effect of *ssgA* deletion on transcription of secretion genes**

The transcripts of five out of thirteen known secretion genes were more abundant in the *ssgA* mutant (Fig. 5C, p180; Noens *et al.,* 2007, Table S2), most of them, namely *tatA*, *tatC, secD, yidC1* (SCO3883)*,* and a homologue of *tatA* (SCO3768 or *tatA2*), by more than two-fold. In contrast, mRNA of *secDF* (SCO6160), encoding the SecDF protein, which is a natural fusion gene of *secD* and *secF* and is required to maintain a high capacity of protein secretion (Bolhuis *et al.*, 1998), was less abundant in all samples of the *ssgA* mutant.

**Genes involved in DNA segregation and topology**

The transcript abundance for several genes involved in DNA replication and segregation was also disturbed in the *ssgA* mutant (Fig. 5D, p180; Noens *et al.,* 2007, Table S2). More abundant transcripts included those of *parA* and *parB*, encoding chromosome partitioning proteins (Jakimowicz *et al.*, 2002b) and of *gyrA* and *gyrB*, for the two subunits of DNA gyrase (Calcutt, 1994) The *parAB* operon was upregulated during sporulation, while the *gyrAB* operon was upregulated throughout development. Conversely, transcripts of the DNA translocase gene *ftsK* (SCO5750: *spoIIIE* in *Bacillus*)*,* which is recruited to the FtsZ-ring in *E. coli* and *Bacillus subtilis* (Bath *et al.*, 2000; Liu *et al.*, 1998); reviewed in (Errington *et al.*,

2001)), and to developing sporulation septa in *S. coelicolor* (Wang et al., 2007) was less abundant in GSA3 at all time points. Besides the changed abundance of these five mRNAs, transcript abundance was also affected for genes close to the chromosomal origin of replication, *oriC*: from SCO3826 until SCO3923, mRNAs for 30% of the genes in this segment are differentially abundant, perhaps as a result of the enhanced expression of parB and/or DNA gyrase in the mutants (see Discussion).

**Verification of microarray data by RT PCR**

To independently verify the expression patterns of the microarray experiment, semi-quantitative RT-PCR was performed on RNA from the parental strain M145 and the *ssgA* mutant, using oligonucleotide pairs for a selection of targets (*ftsZ, divIVA, ftsI, parB, adpA, bldN*, *tatA,* and the *luxR*-like SCO0204) with 16S rRNA as the control (Fig. 6A). We used RNA samples corresponding to 24h (vegetative growth), 48h (aerial growth) and 72h (spores). The relative transcript abundance of *divIVA, ftsI, parB, ftsZ, adpA, bldN* and *tatA* (all up in GSA3 in comparison to in M145) and SCO0204 (strongly down in GSA3) corresponded well to the results obtained from the microarray analysis. We could not reproduce the microarray patterns for *ssgF* and *ftsK* (not shown). The RT-PCR data were quantified and presented after correction for 16S rRNA (Fig. 6B).

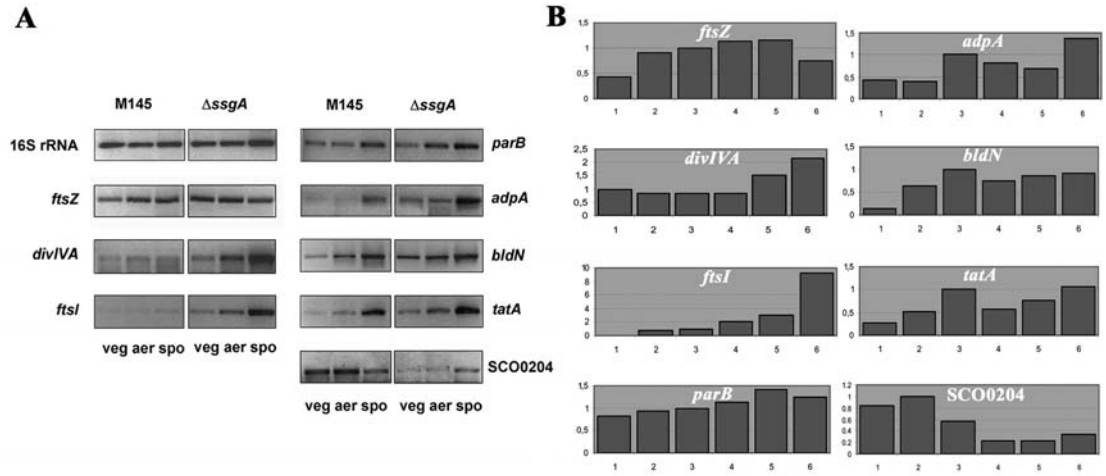



**Figure 6: A. Transcriptional analysis of important SsgA-responsive genes by RT-PCR.** Transcriptional analysis of *ftsZ, divIVA, ftsI, parB, adpA, bldN*, *tatA,* SCO0204 and 16S rRNA by semi-quantitative RT-PCR in M145 and the *ssgA* mutant. RNA samples at time points 24h, 48h and 72h were chosen corresponding to vegetative growth (veg), aerial growth (aer) and sporulation (spo) respectively. 16S rRNA and *ftsZ* were used as a control. These expression patterns conform well to the data obtained by transcriptomic analysis. **B. Quantification of RT-PCR results.** After the intensity of the bands was measured, the background value was subtracted from the total value, which was than corrected for 16S rRNA. For each gene, the highest value of the wild type was set to one. The RNA samples of M145 (1-3 on the X-axis) and of GSA3 (4-6) correspond to 24h (1, 4), 48h (2, 5) and 72h (3, 6). To allow easy comparison the highest value obtained for any of the three time points for the parental strain M145 was set to the arbitrary value of 1. The data were quantified and analysed using a GS-800 imaging densitometer and Quantity One software (BioRad).

**The *ssgA* mutant shows disturbed DNA segregation and/or condensation**

Considering the unexpectedly strong effect of the absence of *ssgA* on the expression of genes involved in DNA topology and segregation/condensation, we analysed the DNA distribution in aerial hyphae and (pre)spores by confocal fluorescence microscopy of samples stained with propidium iodide (PI). When aerial hyphae are actively growing, the DNA is distributed evenly in the hyphae (*i.e.* DNA segregation follows septation); while in mature spores the DNA is completely segregated, with one chromosome per spore (Jakimowicz *et al.*, 2005; Chapter 2). Interestingly, distribution of chromosomes was rather irregular in the *ssgA* mutant spore compartments (Fig. 7A, for a full colour version of Fig. 7, see p181), with many spores showing either very strong staining of nucleoids or no staining at all, while the wild type strain showed normal DNA segregation. This suggests disturbed segregation and/or condensation of chromosomes. In an *S. coelicolor* transformant with enhanced expression of SsgA, DNA segregation was normal.

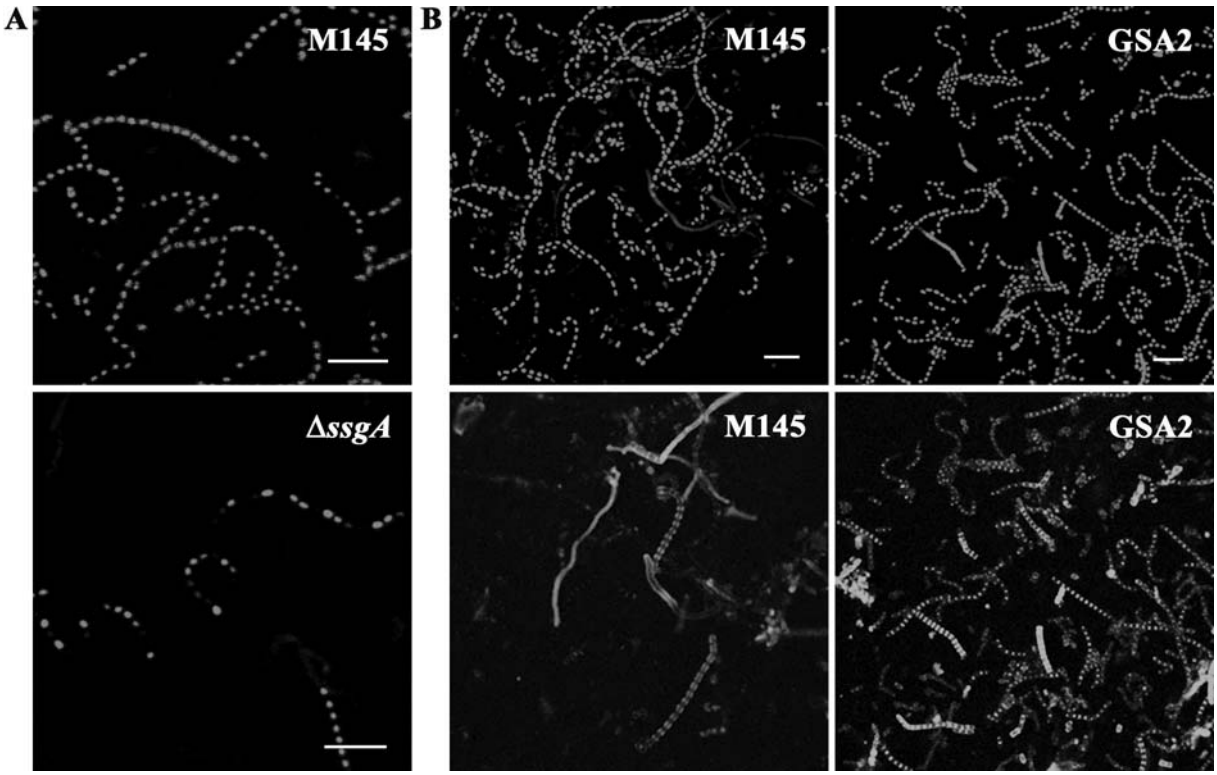



**Figure 7: Visualisation of DNA and peptidoglycan subunits by fluorescence microscopy.** Cultures were grown on SFM for 5 days at 30 °C**. A.** DNA content of *S. coelicolor* and the *ssgA* mutant revealed by propidium iodide (PI). The *ssgA* mutant is disturbed in DNA segregation. **B.** *S. coelicolor* M145 and its SsgA-overexpressing derivative GSA2. Left column shows DNA visualised with PI; right column shows peptidoglycan subunits visualised with f-WGA. GSA2 shows strongly enhanced septation in young aerial hyphae. f-WGA-stained foci also were observed between spores in the mature spore chains, most likely indicative of autolysis. Bar = 5 μm. (Full colour version, see p181).

To visualise *de novo* septal peptidoglycan synthesis, samples were also stained with fluorescein-conjugated wheat germ agglutinin (f-WGA), which stains unincorporated peptidoglycan subunits. Young aerial hyphae of surface-grown cultures of a strain with enhanced expression of SsgA showed strongly enhanced production of septal peptidoglycan with many ladders typical of sporulation-specific cell division (Fig. 7B), in line with our earlier observation that SsgA activates the synthesis of septa (van Wezel *et al.*, 2000b). This phenotype is similar to the phenotype of a mutant in *ssgC*, which may antagonise SsgA function (Chapter 2). Additionally, f-WGA-stained foci were observed between spores in the mature spore chains, most likely indicative of the autolytic activity needed for spores to separate.

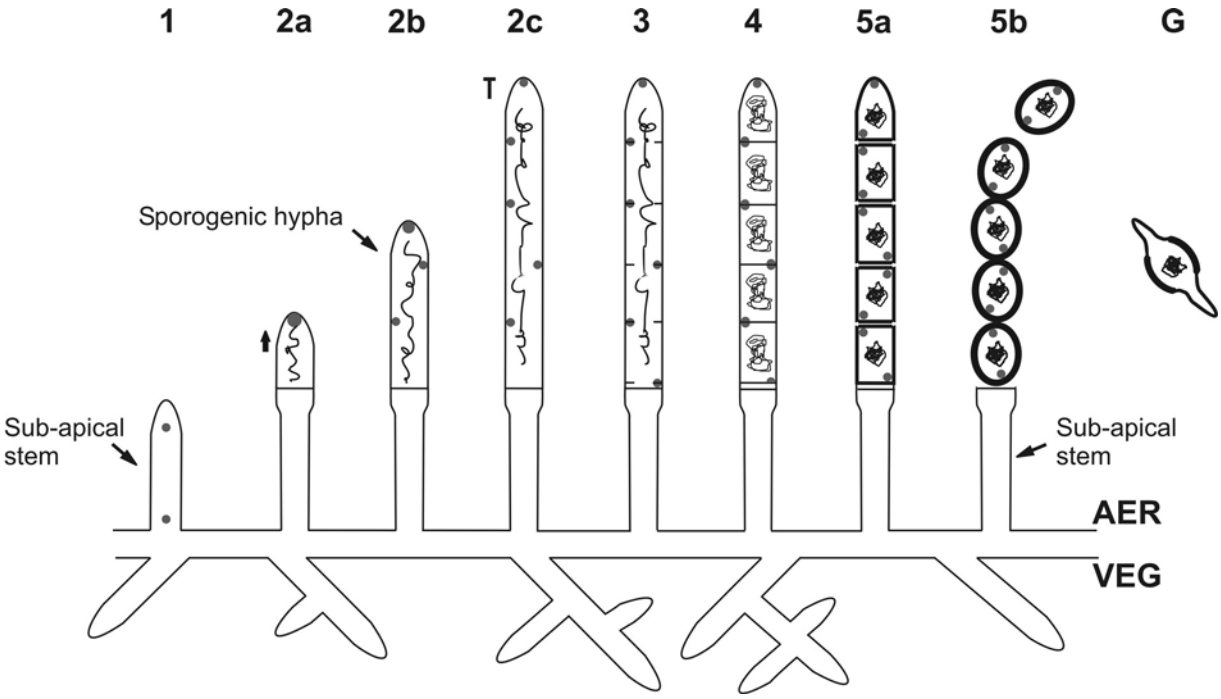



**Figure 8: Scheme showing the localisation of SsgA-GFP during development of *S. coelicolor* M145.** Grey spots refer to the observed localisation of SsgA-GFP foci. **Developmental stages:** (**1**) early aerial growth; (**2**) growth of aerial hyphae destined to be converted into spores ('sporogenic hyphae'); (**3**) in-growth of septa and DNA segregation/condensation; (**4**) completion of septa; (**5**) spore maturation (**5a**, completion of spore-wall synthesis; **5b**, autolytic spore separation); (**G**) germination of spores. Note that DNA is segregated after the initation of septum synthesis (between stages 3 and 4). For close-up images of SsgA-GFP localisations see Fig. 2, p95 and Fig. 3, p97.

## DISCUSSION

SsgA is an activator of sporulation-specific cell division: *ssgA* mutants have almost no sporulation septa, while over-expression of the protein results in hyperseptation, which has been exploited to reduce mycelial clump size in industrial fermentations (van Wezel *et al.*, 2006). As shown previously, overexpression of SsgA alone can change morphology such that not only massive septa (up to 10 times the thickness of regular vegetative cross-walls) are produced, but the width of vegetative hyphae is approximately doubled to match that of sporogenic aerial hyphae (around 800 nm (van Wezel *et al.*, 2000a)). Hence, SsgA directly activates the synthesis of septal PG. Since SALPs lack a DNA binding motif, their effects may well be mediated via a direct interplay with components of the cell-division apparatus, and in particular with PBPs (in the cases of SsgA, SsgB and SsgG) and autolysins (in the cases of SsgE and SsgF) (Chapter 2). Two major issues concerning SsgA have been addressed here: its cellular localisation and likely function at different developmental stages; and the global transcriptional response to its absence.

### SsgA controls events that require major changes in local cell wall morphogenesis at several developmental stages

Confocal fluorescence microscopy showed that most relatively fresh spores have two well-separated SsgA foci that co-localise with emerging germ tubes, implicating SsgA in determining where germination is initiated. SsgA is not essential for germination, but in its absence the average number of germ tubes per spore is reduced (<1.7 germ tubes per spore). Overproduction of SsgA enhances the number of germ tubes (2.5 per spore, against 2.0 in the parent M145).

Although SsgA is not important for vegetative cross-wall formation, we did observe SsgA foci at the tips of emerging germ tubes and young vegetative hyphae. Over-expression results in submerged sporulation, suggesting that ectopic SsgA expression disturbs the mechanisms that discriminate vegetative hyphae from aerial hyphae (van Wezel *et al.*, 2000a). Thus, SsgA-mediated submerged sporulation can now be reinterpreted as a response of the sporulation regulatory cascade to an ectopically realised checkpoint, the initiation of multiple septation. During sporulation of aerial hyphae, SsgA occurs at several distinct localisations (Fig. 2 and 3). The data are summarised in Fig. 8. Initially, SsgA-GFP formed relatively widely-spaced foci in the centre of young aerial hyphae; this may either represent an early

stage of SsgA assembly, or a yet unresolved compartment-specific function of SsgA in the so-called sub-apical stem (Dalton *et al.*, 2007). At the next stage, sporogenic hyphae emerged. These were distinguished by their increased diameter to around 600 nm, from around 400 nm for vegetative and young aerial hyphae. As sporogenic hyphae extended, bright foci were visible in the tips at first. These then faded while an increasing number of closely spaced foci formed behind the tip, resulting in a spacing similar to the foci of FtsZ-GFP (Grantcharova *et al.*, 2005) and the localisation of ParB complexes before septation (Jakimowicz *et al.*, 2005), and in line with the earlier observed upregulation of *ssgA* from the onset of sporulation (Traag *et al.*, 2004). This suggests that SsgA carried by the growing aerial tip may be distributed at regular intervals in aerial hyphae to mark future sites of septum formation. One speculative possibility is that the initial function of such SsgA foci is to establish new sites for peptidoglycan synthesis, associated with the accelerating elongation of aerial hyphal apical compartments, as the numbers of chromosomes increase exponentially (see (Chater and Losick, 1997), for a discussion). In turn, this could underpin much of the spatial regularity associated with sporulation. When hyphal elongation stops, the SsgA-associated peptidoglycan biosynthetic capacity could then instead be used for the formation of septum-associated wall material, via the acquisition of FtsI. Transcripts of *ftsI* were over-represented in the *ssgA* mutant, and FtsI is the only PBP known to be recruited by the divisome (after Z-ring formation has completed) and in other bacteria is responsible for the production of septal peptidoglycan (Botta and Park, 1981). The enhanced expression of *ftsI* may be a compensatory response to the absence of SsgA and hence of septal PG synthesis.

As sporulation septation progresses, SsgA localises on one peripheral edge of the sporulation septa, and finally appears as two foci, one at each end of each spore, which, by marking the sites of future germ tube emergence, complete the sequence of functions that SsgA mediates during the life cycle. The surprisingly regular pattern of septation presumably requires proteins that help mark the wall of aerial hyphae where in the future the (up to 100) septa must be localised (Flärdh and van Wezel, 2003; Schwedock *et al.*, 1997). Otherwise it is difficult to see how the highly regular spacing of around 1 μm can be achieved (almost) simultaneously. Interestingly, such cell wall marking occurs in filamentous fungi by septins, such as SepA in *Aspergillus nidulans* (Harris *et al.*, 1999). Like SsgA, SepA belongs to an entirely new family of cell division-related proteins with little similarity to other protein families (Longtine *et al.*, 1996; Longtine and Bi, 2003).

Comparison of the SsgA-GFP localisation data with the observed morphological changes due to either deletion or over-expression of *ssgA* shows an obvious relationship of SsgA with the processes that require major changes to the cell wall, and in particualr with germination, tip growth and septum synthesis. The enhanced expression of *divIVA* in the *ssgA* mutant, together with the localisation of SsgA in tips of germ tubes and growing (aerial) hyphae suggested a possible functional relationship with DivIVA, which determines the polarity of mycelial growth and is located at the tips of germ tubes and hyphae. SsgA disappeared as vegetative growth progressed, suggesting a role for SsgA during the initial stages of vegetative growth. The upregulation of *divIVA* in *ssgA* mutants was particularly apparent at later time points correlating to aerial growth and when branching of mutant aerial hyphae was observed by cryo-SEM. Hyphae grow by tip extension controlled among others by DivIVA, and enhanced expression of DivIVA gave rise to several new sites of cell wall synthesis, with hyperbranching as a result (Flärdh, 2003). A role of SsgA in the control of branching frequency and/or branch-site selection is supported by the large number of small branches produced when SsgA is overproduced. However, many of these small branches fail to extend beyond 1-2 μm, suggesting that the machinery required to extend apical growth is not sufficiently abundant to support growth of most of the branches. Although SsgA-GFP was abundant in the tips of all aerial hyphae, the localisation of DivIVA in aerial hyphae is unknown, but perhaps similar during hyphal extension (K. Flärdh, personal communication). Upregulation of *divIVA* during later development in the *ssgA* mutant may well be responsible for the branching observed in the *ssgA* mutant aerial hyphae, which is in line with recent experiments that established a function for DivIVA in aerial growth (K. Flärdh, personal communication).

What model can we distill from these seemingly diverse data to explain the function of SsgA? As argued above, in each case (septation, branching and germination) a significant change in the local cell wall morphogenesis is effected, requiring opening of the cell wall (and peptidoglycan), followed by *de novo* peptidoglycan and membrane synthesis and - certainly in the case of sporulation-specific cell division – coordination along the hypha and probably previously determined site selection. Taking into account the observed relationship between SALPs and peptidoglycan-related enzymes (PBPs and autolysins; (Chapter 2)) we propose that SsgA is a chaperonin-like protein that plays an important role in marking the cell wall for major changes at a later moment in time, and in particular in determining the sites for

septation and germination. Such a function is comparable to that of SepA in the morphologically very similar filamentous fungi.

**Disturbance of SsgA-mediated changes in cell wall biosynthesis causes many changes in the expression of developmentally relevant genes, suggesting that SsgA contributes to an important developmental checkpoint.**

Changes in apparent gene expression in the *ssgA* mutant are likely to be the combined result of two major factors: feedback to the genome of information from specific changes in the cellular state caused by *ssgA* mutation; and changes in the relative abundance of cell types. Remarkably, the transcripts of around 300 genes were increased or decreased in relative abundance by at least two-fold in the mutant, at two or more time points. This is much more than observed for other developmental mutants: the highest number previously reported is the 125 transcripts affected in a *whiI* mutant (Tian et al, 2007), and only 17 transcripts were reported to be affected by a *bldN* mutation (Elliott et al., 2003). Overall, these considerations make it likely that the changes in the transcriptome caused by mutation of *ssgA* are largely specific effects on gene expression, rather than simply reflecting changes in the proportions of different cell types.

Strikingly, the products of genes affected by *ssgA* included close to 40 well-known cell-cycle control proteins (see below). These do not include genes that encode the components of the divisome scaffold (*i.e.* the Z-ring and supporting proteins), with *ftsQ* and the *ftsI-ftsL* operon as exceptions. This, and the fact that the *ssgA* mutant produces occasional spore chains on mannitol-containing media, and that FtsZ-GFP rings were formed under these conditions, indicates that indeed SsgA is not essential *per se* for Z-ring formation in aerial hyphae (Fig. 1, Chapter 3).

The mRNA profiles in the *ssgA* and *ssgR* mutants were very similar, in line with our earlier experiments showing that SsgR is the specific transcriptional activator of *ssgA* (Traag *et al.*, 2004). It is therefore highly likely that SsgR regulates only the downstream-located *ssgA*. In fact, the observed (high) similarity in the mRNA profiles provides strong support for the validity of the microarray data obtained for the *ssgA* mutant (Fig. 5, p180).

**Effects on chromosome segregation and secretion**

We observed a highly irregular distribution of chromosomes in the spore compartments when *ssgA* mutants were grown under conditions that allow full development. Our microarray

analysis suggests that this disturbed DNA distribution is at least partially due to the altered expression of *parAB* and *gyrAB*. The development-specific expression of *gyrA* and *gyrB* and of *parA* and *parB* in the wild type was further enhanced in the *ssgA* mutant. *gyrA* and *gyrB* are the two genes for DNA gyrase, a type II DNA topoisomerase responsible for the catalysis of the negative supercoiling of the DNA in replication, while ParA and ParB contribute to correct partitioning of the chromosomes into the unigenomic spore compartments ((Kim *et al.*, 2000); Jakimowicz et al., manuscript submitted). Changes in the expression of *gyrAB, parAB* and also *ftsK* (although the latter could not be corroborated by RT-PCR analysis) might well have effects on the correct segregation, condensation and topology of the chromosomes. Surprisingly, besides the *gyr* and *par* genes, many other *ori*-proximal genes showed deregulated expression in the *ssgA* mutant. Genome-wide, the expression of >4 % of the genes is affected significantly in the *ssgA* mutant, while 30 out of 98 *ori*-proximal genes, namely ORFs SCO3826 – SCO3923, are affected. Suggestively, *ssgRA* (SCO3925-3926) lie immediately next to this cluster of genes. Many of these genes are essential genes involved in DNA synthesis and maintenance.

Finally, the expression of genes that encode proteins involved in the Sec and Tat secretion pathways is also significantly affected by *ssgA* mutation. Most of these genes show increased transcription in the mutant relative to its parent M145, except for the *secDF* gene (decreased). We recently showed that enhanced *ssgA* expression has a strongly positive effect on secretion (and production) of enzymes in *Streptomyces lividans* (van Wezel *et al.*, 2006). Thus, the secretion genes respond similarly as *ftsI*: SsgA is known to enhance the associated processes (septum formation, secretion), but the relevant genes are upregulated in an *ssgA* mutant, perhaps as a compensatory effect. Comparison of microarray data on the *ssgA* mutant with those of a strain over-expressing *ssgA* (not yet available) could shed more light on this puzzling phenomenon.

**The position of *ssgA* in the developmental hierarchy**

What is the position of SsgA in the developmental control hierarchy? Our experiments and previous observations strongly suggest a function for SsgA in the coordination of septal peptidoglycan synthesis. The prime candidate for the latter is *ftsI*, one of the more strongly over-expressed genes in the *ssgA* mutant (see below). The timing for SsgA function is also highlighted by the response of the *whi* genes: the earlier-acting aerial hyphal developmental genes *whiA*, *whiH, whiI* and *whiJ* were over-expressed in the *ssgA* mutant, while spore

maturation genes, including *ssgE* and *ssgF* (both important for correct autolytic spore separation), *sigF, whiD,* and the spore pigment cluster *whiE* (Kelemen *et al.*, 1998; Molle *et al.*, 2000; Chapter 2)), were under-expressed. This remarkable tendency suggests that the irregularities of the *ssgA* mutant in developmental gene expression are most likely a checkpoint effect, *i.e.* the septum formation/DNA partitioning checkpoints have not been passed. We also observed significant over-expression of several *bld* genes, including *bldB, bldC, bldD, bldG* and the *adpA* (*bldH*) responsive genes, including *bldN*, in the *ssgA* mutant. While *bld* genes are by definition essential for early developmental events, their mRNA levels remain high and in some cases strongly increase late on (*e.g. bldN* (Bibb *et al.*, 2000)) and they were particularly abundant in the *ssgA* mutant at late time points. The *chp* genes for the chaplin proteins, which are part of the spore coat (Claessen *et al.*, 2003; Elliot *et al.*, 2003), as well as the functionally related *rdl* genes, were all markedly over-expressed in the *ssgA* mutant (Fig. 5B, p180; Noens *et al.,* 2007, Table S2). Thus, the phenotypic and transcriptional analyses together suggest that SsgA plays a crucial role in the control of morphogenetic processes leading to the formation of septal peptidoglycan in aerial hyphae, at a time relating to the activities of WhiA and WhiB, and prior to or at the onset of Z-ring formation, and that the failure of the septation checkpoint prevents the expression of genes for post-septation functions. A similar effect was implied in a previous report (Flardh *et al.*, 2000) showing that at least some late functions were not carried out when sporulation septum formation was prevented by a developmental stage-specific deficiency of FtsZ.

**Conclusion**

SsgA is an important cell-cycle control protein that most likely functions by (facilitating the) marking of the cell wall for future major changes in cell wall morphogenesis, with a clear involvement in and stimulatory effect on coordinated sporulation-specific cell division and spore germination, and perhaps also on branching. This, together with the localisation data, supports the idea that SsgA fulfils a chaperone-like function, aiding the cell wall synthesising machineries to find the exact positions for future peptidoglycan synthesis. In this way, SsgA may be one of the proteins that compensate for the absence of septum-localising proteins such as FtsA, ZipA, Noc and MinCE in unicellular bacteria. The search for direct interaction partners for SsgA should better our understanding as to how SsgA functions at the molecular level.

**ACKNOWLEDGEMENTS**

We thank Gerda Lamers for help with fluorescence microscopy, Marie Elliot and Sebastien Rigali for discussions and Klas Flärdh for plasmid KF41 and for sharing unpublished data. The work was supported by EU Marie Curie training grant HPMF-CT-2002-01676 to KFC and Jay Hinton (Institute of Food Research, Norwich), by BBSRC grants G18886 to CPS and G18887 to KFC, and by grants of the Royal Dutch Academy for Arts and Sciences (KNAW) and the Netherlands Organisation for Scientific Research (NWO) to GPvW.