Controlling growth and morphogenesis of the industrial enzyme producer Streptomyces lividans

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Chapter 6

Functional analysis of the SsgA-like proteins in *Streptomyces lividans* 1326

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ABSTRACT

SsgA-like proteins (SALPs) are a family of actinomycete-specific regulatory proteins that control sporulation-specific cell division and spore maturation in actinomycetes. We recently showed that SsgA and SsgB activate cell division by directly controlling the localization of FtsZ, while other SALPs have yet poorly characterized roles in morphogenesis and cell wall synthesis. Here we report the creation of null mutants for the genes encoding SsgA-like proteins SsgA, SsgB, SsgC, SsgD and SsgE, respectively, and show that ssgA and ssgB null mutants had a nonsporulating phenotype. Three alternative translational start sites for ssgA were analyzed, and it was established that of these, changing the first or the third codon into an ATC had a detrimental effect on development, whereby the first ATG is perhaps preferred over the third ATG codon. The ssgB null mutants of *S. lividans* lacked the large colony phenotype seen in *S. coelicolor*, which may reflect differences in the morphogenesis between these sister streptomycetes. Finally, enzyme production by several of the mutants was analyzed using the small laccase SLAC as a reporter.

INTRODUCTION

Streptomycetes have a strong potential to adapt to diverse natural habitats. This is highlighted by the presence of more than 20 clusters specifying secondary metabolites, around 65 sigma factors and an unprecedented number of sugar transporters and polysugar hydrolases on their genomes (Bentley et al. 2002; Ikeda et al. 2003; Ohnishi et al. 2008). The study of *Streptomyces* development is carried out primarily on solid-grown cultures, whereby the vegetative mycelium develops aerial hyphae which then produce chains of spores (Chater 1972). Most developmental genes that control aerial development (the so-called *whi* genes) encode transcription factors (Chater 1972; Flärdh et al. 1999; Ryding et al. 1999). Aerial hyphae are by definition not produced in submerged culture. Nonetheless, several streptomycetes also produce spores in liquid cultures, such as *S. griseus* and *S. venezuelae* (Kendrick and Ensign 1983; Glazebrook et al. 1990). Some *whi* genes also play a role in submerged sporulation. For example, overexpression of the sporulation-specific σ-factor WhiG induces some submerged sporulation in liquid-grown mycelium of *S. coelicolor* (Chater et al. 1989), and deletion of a number of *whi* gene orthologues in *S. venezuelae* (i.e. *whiA*, *whiB*, *whiD*, *whiG*, *whiH* and *whiI*) resulted in a failure to sporulate on agar plates and in liquid-grown cultures (M.J. Buttner and M.J. Bibb, pers. comm.). This suggests significant overlap between the sporulation pathways under both conditions.

The SsgA-like proteins are a family of sporulation control proteins (Traag and van Wezel 2008; Jakimowicz and van Wezel 2012). SsgA was originally identified as a suppressor of a hyper-sporulating *S. griseus* mutant (designated SY1) and was shown to be essential for submerged sporulation (Kawamoto and Ensign 1995; Kawamoto et al. 1997). Overexpression of *S. griseus* SsgA in liquid-grown mycelium of *S. coelicolor* induced mycelial fragmentation and spore formation (van Wezel et al. 2000a). The ability of SsgA to enhance fragmentation and also protein secretion was applied in industrial fermentations, revealing a significant improvement in yield and fermentation time (van Wezel et al. 2006). We recently discovered that SsgA acts by dynamically controlling the localization of its paralogue SsgB, which is a member of

the cell division complex in actinomycetes, and in turn recruits FtsZ to the septum sites to initiate sporulation-specific cell division (Willemse et al. 2011). This rare example of positive control of cell division explains the critical role of SsgAB in the sporulation process. The *ssgA*, *ssgB* and *ssgG* genes directly relate to the control of septum-site localization in *S. coelicolor*, with *ssgA* and *ssgB* essential for sporulation (van Wezel et al. 2000a; Keijser et al. 2003; Sevcikova and Kormanec 2003), while in *ssgG* mutants septa are frequently skipped, resulting in large spores containing multiple well-segregated chromosomes (Noens et al. 2005). The crystal structure of SsgB, which is the archetype of the SALP family and has functional orthologues in all sporulating actinomycetes, revealed a bell-shaped trimer with strong similarity to mitochondrial guide RNA binding proteins, although direct nucleic acid binding by SALPs is unlikely (Xu et al. 2009).

Here we look into the role of SALPs in *Streptomyces lividans*, studying the effect of deletion of either *ssgA*, *ssgB*, *ssgC*, *ssgD* or *ssgE* on growth and morphogenesis. An initial assessment of the ability of the mutants to produce enzyme is presented.

RESULTS AND DISCUSSION

Functional heterogeneity in SsgA orthologues and the ssgA *translational start*

The differential activity of SsgA orthologues obtained from different *Streptomyces* species is still poorly understood. For example, overexpression of *S. griseus* SsgA in liquid-grown mycelium of *S. coelicolor* induces mycelial fragmentation (van Wezel et al. 2000b), while overexpression of orthologues from *S. coelicolor* or *S. lividans* does not have a major effect on liquid-culture morphology (van Wezel et al. 2006; van Wezel et al. 2009). Apparently, the effect of SsgA is dictated by its amino acid sequence. Indeed, it was recently shown that streptomycetes may be separated phylogenetically on the basis of the SsgA sequence, where six signature amino acid residues allow discrimination between streptomycetes that sporulate in submerged culture (LSp) and those that do not (NLSp) (Girard et al. 2013).

Another controversy relates to the precise translational start site of *ssgA*. Alignment of the promoter regions of *ssgA* orthologues identified three alternative translational start sites, with the first and third possible ATG conserved in all species, including the putative ribosome binding site (RBS) upstream (Figure 1). These are therefore considered as possible alternative translational start sites. In all streptomycetes but *S. ramocissimus*, which contains duplication of the codons for VQA, the first and second ATG are separated by precisely 30 nucleotides, or ten possible codons. To further analyse which of the three alternative ATG start codons of *ssgA* may be used in vivo, clones were prepared with one of the respective ATG start codons mutated to an ATC codon (for isoleucine). For convenience, the three ATG triplets will be referred to as ATG(1), ATG(2) and ATG(3), for the first, second and third of the three candidate start codons, respectively. The *S. griseus ssgA* gene fully complements *ssgA* null mutants of *S. coelicolor* and the proteins have identical N-terminal regions (including the three ATG triplets). The introduction of $ssgA^{Sg}$ in the *S. coelicolor ssgA* null mutant allows us to test not only which start codons are important, but also what the effect is of the introduction of an SsgA with a "submerged sporulation signature" (LSp; (Girard et al. 2013)) on liquid-culture morphology.

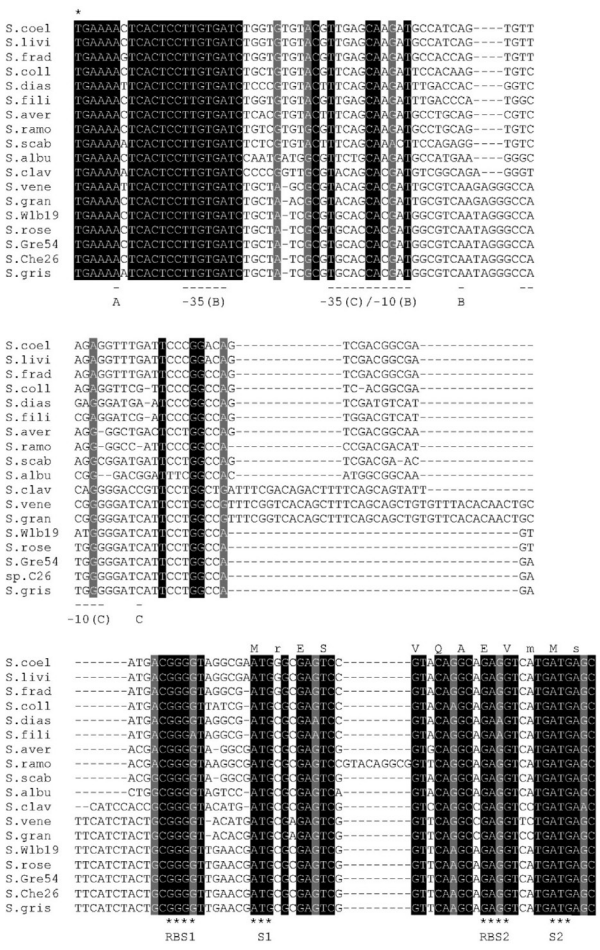

Figure 1. Multiple alignment of *ssgA* promoter regions. Only completely conserved nucleotides are shaded; nucleotides shaded in light grey refer to conserved purines. The two most likely start codons S1 (ATG(1)) and S2 (ATG(3)) for *ssgA* and their respective ribosome binding sites (RBS1 and RBS2) are indicated below the aligned sequence. The ATG immediately upstream of start codon S2 (designated ATG(2) in the text) is most likely not a true translational start codon. The two transcriptional start sites and their respective -35 and -10 recognition sequences from *S. griseus* (Yamazaki et al. 2003) and *S. coelicolor* (Traag et al. 2004) are underlined, where "A" refers to the p1 promoter from *S. griseus*, "B" to p1 from *S. coelicolor* or p2 from *S. griseus*, and "C" to p2 from *S. coelicolor*. The TGA stop codon for *ssgR* is indicated with an asterisk. The consensus amino acid sequence of the N-terminus of SsgA proteins is given above the aligned DNA sequences. Sequences were labeled by their strain of origin and abbreviated as follows: (S.albu) *S. albus*, (S.aver) *S. avermitilis*, (S.clav) *S. clavuligerus*, (S.coel) *S. coelicolor*, (S.coll) *S. collinus*, (S.dias) *S. diastatochromogenes*, (S.fili) *S. filipinensis*, (S.frad) *S. fradiae*, (S.livi) *S. lividans*, (S.gran) *S. granaticolor*, (S.gris) *S. griseus*, (S.rose) *S. roseosporus*, (S.ramo) *S. ramocissimus*, (S.scab) *S scabies*, (S.vene) *S. venezuelae*, (S.Wlb19) *Streptomyces* species Wlb19, (S.Che26) *Streptomyces* species Che26, (S.Gre54) *Streptomyces* species Gre54.

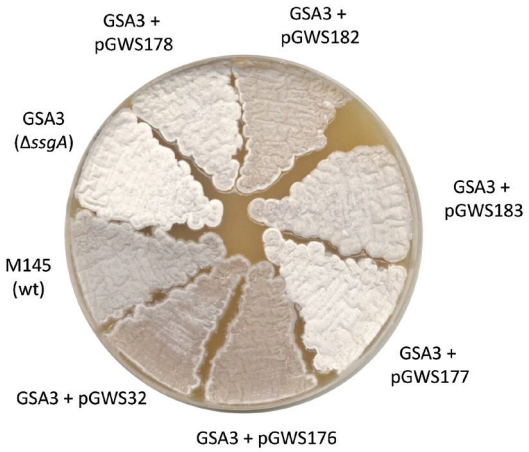

Figure 2. Complementation of the *ssgA* null mutant. Constructs carrying wild-type *ssgRA* or mutations in the first (pGWS177), second (pGWS182) and third (pGWS183) predicted start codons were introduced in the mutant and their ability to restore sporulation assessed on MS medium. M145 and the *ssgA* null mutant GSA3 harbouring pHJL401 as control plasmid are also shown.

To obtain a construct for the ready cloning and expression of *ssgA* variants from the *S. coelicolor* promoter region, around 1.5 kb of the upstream and downstream regions of *S. coelicolor* A3(2) *ssgA* were amplified by PCR and cloned into the low-copy vector pHJL401, resulting in *ssgA* exchange construct pGWS174. The *S. griseus ssgA* variants with mutated translational start codons were then introduced in the *ssgA* exchange construct, resulting in pGWS177 (*ssgA*-ATG(1)→ATC), pGWS182 (*ssgA*-ATG(2)→ATC), and pGWS183 (*ssgA*-ATG(3)→ATC), respectively. In this way, the *ssgA* gene of *S. griseus* (or mutants thereof) is expressed from the natural *S. coelicolor* promoter region. The ssgR gene was included as read-through occurs into *ssgA* from a promoter upstream of *ssgR* (Traag et al. 2004; van Wezel et al. 2000a). pGWS178, which carries a 27 nt in-frame deletion rendering *ssgA* inactive (Kawamoto et al. 1997) was used as negative control, while pGWS176 (carrying wild-type *S. griseus ssgA*) and pGWS32 (wild-type *S. coelicolor ssgA*; (Traag et al. 2007)) were used as positive controls. All constructs were introduced into the *S. coelicolor ssgA* mutant GSA3, and their ability to complement the non-sporulating phenotype of the *ssgA* null mutant was assessed.

The phylogenetic evidence (Figure 1) and its location relative to the upstream RBS, with only three nucleotides spacing (instead of the canonical 6-9 nt), apparently ruled out the second ATG as possible start codon. Indeed, sporulation was fully restored to *ssgA* mutants by the expression of ssgA-ΔATG(2), strongly suggesting that ATG(2) is not used as translational start codon (Figure 2). Mutation of ATG(3) - which in contrast to ATG(2) is completely conserved in all *Streptomyces ssgA* genes - did not dramatically affect sporulation, although less grey spore pigment was observed for GSA3 transformants harbouring this variant than for the transformants expressing wild-type *S. griseus ssgA* or *ssgA*-ATG(2)→ATC. No complementation of sporulation was observed when *ssgA*-ATG(1)→ATC was introduced in the *S. coelicolor ssgA* null mutant, and this should therefore be regarded as the primary start codon for *ssgA*, at least in surface-grown cultures on MS agar plates. Interestingly, when the same *ssgA*-ATG(1)→ATC was introduced in multiple copies, using pWHM3 instead of the low-copy vector pHJL401, sporulation was fully restored to the *ssgA* null mutant (data not shown). Apparently, ATG(3) is a less efficient start codon or full length SsgA (145 aa) is more effective in activating sporulation than the truncated SsgA (135 aa) .

***Construction of null mutants of the cell division genes* ssgA *and* ssgB *in* S. lividans**

We then wondered if indeed the function of the cell division proteins SsgA and SsgB was the same in *S. coelicolor* and *S. lividans*. Firstly, an *ssgA* null mutant was created by replacement of the coding region of the gene by the apramycin resistance cassette *aacC4*, which confers resistance to apramycin, using the instable multi-copy vector pWHM3 as described previously (Colson et al. 2008). *S. lividans* 1326 was transformed with the disruption construct pGWS175 followed by several rounds of nonselective growth to allow loss of the vector. Double recombinants were selected by replication of the colonies to MS agar plates containing either apramycin or thiostrepton as selective markers, whereby double recombinants should be resistant to apramycin (gene replacement) and sensitive to thiostrepton (due to loss of the pWHM3 vector sequences). The putative mutant colonies were verified by PCR amplification with oligonucleotides flanking the *ssgA* gene. This showed that all

correct double recombinants have a white (nonsporulating) phenotype, similar to *ssgA* null mutants of *S. coelicolor* (Figure 3). The mutant phenotype of the *ssgA* null mutant of *S. lividans* could be restored to sporulation by the introduction of a wild-type copy of *ssgA* in the low-copy number vector pHJL401 (Figure 3).

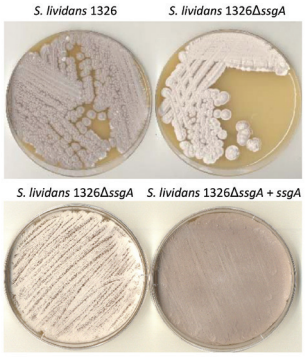

Figure 3. Phenotypic analysis of the *S. lividans ssgA* mutant and complementation with wild-type *ssgA*. The strains were incubated for five days at 30^0C on MS agar plates.

In *S. coelicolor*, deletion of the gene for SsgB result in an unconditionally white (nonsporulating) phenotype on all media, resulting in the formation of long aseptate aerial hyphae (Keijser et al. 2003). Interestingly, the *ssgB* mutant colonies have a large colony phenotype, which grow on to form very large colonies as compared to those formed by the wild-type strain. This suggests that SsgB not only controls cell division, but may also be involved in the correct timing of growth cessation, which is an important checkpoint for the initiation of aerial growth (Chater 2001). To establish the role of *ssgB* in cell division and growth in *S. lividans*, a null mutant was created in similar fashion as for *ssgA*, by replacing the entire coding sequence of the gene (nt positions -6/+469 relative to the translational start of *ssgB*) by the apramycin resistance cassette *aacC4* that was flanked by *loxP* sequences to allow the removal of the cassette after double recombination. Subsequently, the apramycin resistance cassette was excised following introduction of plasmid pUWLcre (Fedoryshyn et al. 2008) expressing the Cre recombinase that recognizes the *loxP* sites, thus leaving an in-frame deletion of the gene except for a scar sequence as a result of the joined *loxP* sites.

Expectedly, colonies had a *whi* mutant phenotype, failing to sporulate in surface-grown culture, and regardless of what media were used (Figure 4). However, in *S. lividans* a large colony phenotype (LCP) was not observed. This suggests that these two functions - sporulation and LCP - are not functionally coupled. However, this cannot be due to intrinsic functional differences between the *ssgB* orthologues of *S. coelicolor* and *S. lividans*, as the genes are identical at the nucleotide level.

***Deletion of* ssgC, ssgD *and* ssgE *in* S. lividans**

In similar fashion as described for *ssgB*, deletion mutants were also prepared for *ssgC*, *ssgD* and *ssgE*. For *ssgC*, the +1 to +425 region was removed, for *ssgD* +1/+408, and for *ssgE* -7/+367. The precise function of SsgC, SsgD and SsgE is less clear. SsgC was suggested to be involved in septum formation and peptidoglycan maintenance and the mutant in *S. coelicolor* has a phenotype that is more or less the inverse of that of *ssgA* null mutants, with increased septation for null mutants, while overexpression results in inhibition of cell division. Therefore, it was proposed that SsgC may function as an antagonist of SsgA (Noens et al. 2005). In addition, *S. coelicolor ssgC* null mutants produced long spore chains with spores of variable length. Direct orthologues of *ssgC* have only been found in streptomycetes of the NLSp branch (species that do not sporulate in submerged cultures), which invariably have a low SsgA expression level (Girard et al. 2013). As published previously for *S. coelicolor*, colonies of deletion mutants of *ssgC* in *S. lividans* also gave a dark grey phenotype on MS agar plates, and showed enhanced production of the antibiotic actinorhodin on R5 agar plates with added copper (Figure 4). No major effect on septum formation was observed in *S. lividans*, although closer examination is required, for example by high resolution transmission electron microscopy.

SsgD is the only SALP that is highly expressed during vegetative growth, and *ssgD* null mutants of *S. coelicolor* show major defects in lateral cell wall synthesis; it was therefore suggested to play a role in the synthesis of the lateral cell wall, perhaps by enabling the proper function of one or more penicillin binding proteins (PBPs), such as PBP2 (Den Blaauwen et al. 2003; Errington et al. 2003). The *ssgD* mutant of

S. coelicolor shows defects in integrity of the hyphae and spores, whereby the spores have a significantly thinner peptidoglycan layer than those of the wild-type strain. Deletion of *ssgD* in *S. lividans* resulted in mutants with reduced sporulation on R5 agar plates and normal sporulation on MS agar plates (Figure 4). SsgE was previously reported as being involved in spore maturation in *S. coelicolor*, with hypersporulation and in particular the formation of single spores rather than spore chains, suggesting accelerated autolytic spore separation. However, major differences between *ssgE* null mutants and the parental strain were not observed (Figure 4). The phenotypes of the mutants are currently being investigated in more detail and should reveal the extent of the morphological changes. Furthermore, growth curves should be carried out under different conditions to check for the phenotype of the mutants in liquid-grown cultures.

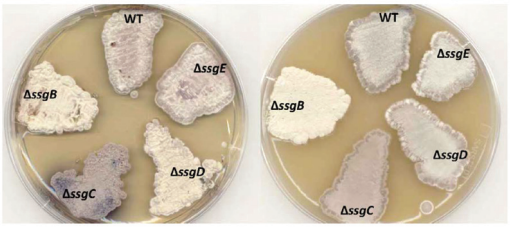

Figure 4. Phenotypic analysis of *S. lividans* mutants Δ*ssgB*, Δ*ssgC*, Δ*ssgD* and Δ*ssgE*. wt, *S. lividans* 1326. The strains were incubated for five days at 30^0C on R5 agar (left) or MS agar (right).

Enzyme production in selected mutants

The *ssgB*, *ssgC*, *ssgD* and *ssgE* mutants, together with the *cslA* and *glxA* mutants (Chapter 3), were tested for productivity using the small laccase SLAC as a reporter enzyme. Laccases are multicopper oxidases with a wide variety of substrates in nature. A small laccase (SLAC) has been characterized in *S. coelicolor* (Machczynski et al. 2004) and the gene was inserted in the low copy number vector pHJL401 under the control of the strong constitutive promoter P_{ermE} to generate P_{ermE}*slac* (see Chapter 6 of this Thesis). The reproducibility of the assay using a gel-based system makes it a suitable reporter for this study. The assays were performed as described in Materials and Methods.

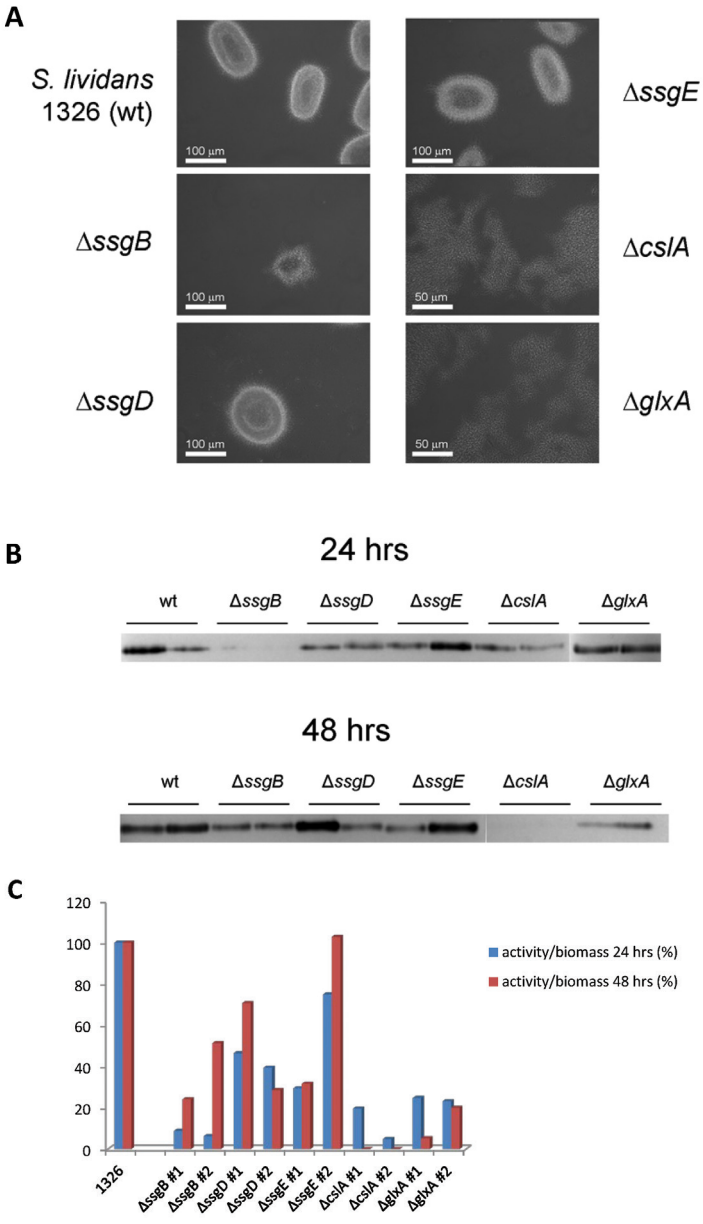

Figure 5. (A) Phenotype of SLAC-producing strains after 48 hrs growth in liquid TSBS. All strains harboured plasmid P_{ermE}*slac*. *S. lividans* 1326 was the parent for all mutants. (B) In-gel laccase activity assay performed on samples obtained from 24 hrs (top) and 48 hrs (bottom). Activity in the *ssgC* mutants was zero (data not shown). (C) Quantification of the signals corresponding to active SLAC as determined by the in-gel assay. Band intensities were normalized for biomass and *S. lividans* 1326 harbouring P_{ermE} construct was set at 100%.

The experiments were carried out in duplicate, with samples taken at 24 and 48 hrs, analyzed on gel for their activity and normalized for dry weight. The phenotypes of the transformants were analyzed after 48 hrs of growth in TSBS cultures (containing 5 µg/ml thiostrepton for plasmid maintenance). This showed that *ssgC, ssgD* and *ssgE* null mutants had a phenotype similar to that of the parental strain 1326, while *ssgB* mutants harbouring the plasmid expressing laccase grew poorly and produced small pellets. In accordance with the experiments presented in Chapter 3, transformants of *cslA* and *glxA* mutants grew fast, forming open mycelial structures, often referred to as mycelial mats (Figure 5A).

In terms of laccase production, there was strong variability between the two independent transformants, for wild-type (in particular after 24 hrs of growth) and in particular also for the mutants. The *ssgB* mutant not only grew slowly but also produced only small amounts of the laccase per gram of biomass, while *ssgD* and *ssgE* mutants on average produced similar amounts of laccase as the parent but with strong variability in production between transformants (Figure 5B and C). Enzyme activity in the *ssgC* mutants was zero and will need to be confirmed further (data not shown). Transformants of the *cslA* and *glxA* mutants, which produced open mycelial mats and grew fast (Figure 5A) produced only small amounts of laccase, and in particular *cslA* mutants had completely lost their ability to produce laccase after 48 hrs of growth. This is most likely due to poor stability of the plasmid in the mutants at times when the thiostrepton pressure drops. This is supported by the fact that following sporulation on MS plates, more than 80% of the spores do not contain plasmid (unpublished results). This loss of plasmid may tentatively be explained by lack of a protective layer of the CslA-dependent polysaccharide at apical sites, resulting in plasmid leakage. This interesting phenomenon needs to be analyzed in more detail. Finally, the high variation in productivity between individual transformants is typical of streptomycetes and underlines the need to analyze a large amount of transformants, for example by prescreening using high throughput systems, such as using 96 well microtitre plates.

In conclusion, mutants deleted for the genes encoding SsgA-SsgE were created, whereby *ssgA* and *ssgB* null mutants had the same nonsporulating phenotype

as seen for *S. coelicolor*. The alternative translational start sites for *ssgA* were analyzed, which showed that two alternative start sites are used, whereby the first ATG may be preferred, with less frequent use of the third ATG. Changing the second ATG codon (annotated as the start codon in *S. coelicolor*) into an ATC had no effect on morphogenesis, and therefore most likely does not function as a start codon for translation. The *ssgB* null mutants lacked the large colony phenotype seen in *S. coelicolor*, which was unexpected and also unfortunate as an 'immortal' phenotype of *ssgB* null mutants seems like a promising phenotype from the perspective of growth during fermentations. More extensive study of the mutants, both phenotypically by various microscopy methods and in terms of growth and enzyme production, is required to assess the role of the SALPs in morphogenesis and productivity of S. *lividans*.

MATERIAL AND METHODS

Strains and media

The parent of all strains described in this study was *S. lividans* 1326 (also known as *S. lividans* 66; stock number 1326 from the John Innes Centre (Hopwood et al. 1985). *Escherichia coli* JM109 was used for routine cloning and plasmid amplification (Messing et al. 1981). For growth and phenotypic characterization, soy flour mannitol (Ms) agar plates, R5 agar plates supplemented with 10μM copper or minimal medium (MM) agar plates with 1% (w/v) mannitol were used. For liquid-grown cultures, liquid minimal medium (NMMP) supplemented with 1% mannitol or tryptic soy broth with 10% sucrose (TSBS) were used (Kieser et al. 2000). *E. coli* strains were routinely grown on Luria-Bertani medium (LB).

Table 1. Constructs used in this study.

Constructs	Description
pWHM3	Multi-copy shuttle vector, colE1 replicon, pSG5 replicon, Tsr^R, Amp^R (Vara et al. 1989)
pHJL401	Low-copy shuttle vector, SCP2*, pUC19 replicon, Tsr^R, Amp^R (Larson and Hershberger 1986)
pGWS174	pHJL401 with 1.5 kb of the upstream (including ssg^R) and 1.5 kb of the downstream region of *S. coelicolor ssgA*
pGWS175	pWHM3 harbouring the insert of pGWS174 and an apramycin resistance cassette cloned between the 1.5 kb flanking regions
pGWS176	pGWS174 containing *ssgAsg*-WT
pGWS177	pGWS174 containing *ssgAsg*- ATG(1)→ATC
pGWS178	pGWS174 containing *ssgAsg* with a 27 bp SacI in-frame deletion (Kawamoto et al. 1997)
pGWS182	pGWS174 containing *ssgAsg*- ATG(2)→ATC
pGWS183	pGWS174 containing *ssgAsg*- ATG(3)→ATC
pGWS32	pHJL401 containing 2kb *ssgRA* region of *S. coelicolor* (Traag 2004)

Plasmids and constructs

All constructs used in this study are presented in Table 1. The gene replacement mutants were obtained by cloning the flanking regions of the genes with the apramycin resistance cassette *aacC4* flanked by *loxP* sites in the unstable multi-copy vector pWHM3 (Vara et al. 1989) as described previously (van Wezel et al. 2005).

The flanking regions were (nucleotide positions are relative to the translational start of the genes): *ssgB* -1500/-7 and +470/+1968; for *ssgC* -1497/-1 and +426/+1920; for *ssgD* -1494/-1 and +409/+1917; for *ssgE* -1500/-8 and +368/+1881. To obtain the deletion mutants, the antibiotic cassettes were removed by the Cre-lox recombinase using plasmid pUWLcre (Fedoryshyn et al. 2008), leaving a scar *loxP* site flanked by XbaI restriction sites. All the primers are presented in Table 2.

Laccase assay

The SLAC gene from *S. coelicolor* (Machczynski et al. 2004) was cloned in the low copy number vector pHJL401 (Larson and Hershberger 1986; van Wezel et al. 2000c) under the control of the strong constitutive promoter P_{ermE} as described in Chapter 5 of this Thesis. Each mutant was transformed with the construct and the spores harvested. 10^7 spores were inoculated in 10 ml TSBS supplemented with 25μM copper and 2 μg/ml thiostrepton and grown at 30 ºC for 48 hrs. Alternatively, mycelium was harvested from two MS plates and inoculated in the case of the non sporulating strain Δ*ssgB*. The experiment was carried out in duplicate, with 1 ml sample per transformant taken at 24 and 48 hrs and centrifuged for 10 min at 14000 rpm. The pellet was incubated overnight at 100 ºC for dry weight determination, while 10 μl of the supernatant were mixed with an equal volume of SDS-PAGE loading buffer and loaded on a 10% acrylamide gel. The gel was incubated for 1 hr in 100mM phosphate buffer pH 6.8 at room temperature on a rocking platform refreshing the buffer every 20 min and for an additional hr at 30 ºC. The activity assay is based on the conversion of N-N-Dimethyl-p-phenylenediamine (DMPPDA) into a dark blue precipitate. The gel is therefore incubated with 0.125 mg/ml DMPPDA and 0.125 mg/ml 1-naphtol until the appearance of the signal. The intensity of the bands is directly proportional to the amount of protein and can be quantified with the software ImageJ.

ACKNOWLEDGMENTS

The authors would like to acknowledge Bjorn Traag for discussion.

SUPPLEMENTAL MATERIAL

Table S1. Oligonucleotides used in this study.

Name	5'-3'sequence	Restriction sites
ssgB-1500F	CGCAGATCTCCCGCATC ACCTG CCGC	BglII
ssgB-7RV	GCGTCTAGAACATGCCACCTACGGTGCCG	XbaI
ssgB+470F	CGCTCTAGAGAAAGCTAGGGCGGGGCCTC	XbaI
ssgB+1968RV	GCGAAGCTTGGTGGCACCCGCAAGCAGCG	HindIII
ssgC-1497F	GCGGAATTCCGCGTACCGGGTGGTCTTCGG	EcoRI
ssgC-1RV	CGCTCTAGAGGGGGCCTCCAGCAGGAC	XbaI
ssgC+426	GCGTCTAGATGAACCGCCCGGGCCGGC	XbaI
ssgC+1920RV	CGCAAGCTTGTCCGGCCTGCTGACCGG	HindIII
ssgD-1494F	CGCGAATTCTGATGTGCTCGTACTGCCGC	EcoRI
ssgD-1RV	GCGTCTAGACGCCTTGCTCCCTCGTGAC	XbaI
ssgD+409F	CGCTCTAGAGCTGCTGACCGGCTCCCG	XbaI
ssgD+1917	GCGAAGCTTGCCAGCCGCAGCAGCACC	HindIII
ssgE-1500F	CGCGAATTCGAGCACCGCGAAGGCGAC	EcoRI
ssgE-8RV	GCGTCTAGACCCTTACGCTCTGCCACCTG	XbaI
ssgE+368F	CGCTCTAGAGGTGGCCCACTGAGCCGC	XbaI
ssgE+1881RV	GCGAAGCTTAGCGGTTCCTGACCGCCTG	HindIII