Identification and characterization of developmental genes in streptomyces

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The SepF-like proteins SflA and SflB control hyphal branching and are required for proper spore maturation in *Streptomyces coelicolor*

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ABSTRACT

Cell division during the reproductive phase of the *Streptomyces* lifecycle requires tight coordination between synchronous formation of multiple septa and DNA segregation. One remarkable difference with most other bacterial systems is that cell division in *Streptomyces* is positively controlled by the recruitment of FtsZ by SsgB. In *Bacillus subtilis*, SepF tethers FtsZ to the membrane, and this is the only cell division protein from *Bacillus* that is conserved in *Streptomyces*. Uniquely, actinomycetes have multiple SepF paralogs, and we here show that the SepF-like proteins SflA and SflB control branching of the hyphae, with unusual branching spore chains in *sflA* and *sflB* mutants. Additionally, electron microscopy showed that spores of *sflA* and *sflB* mutants were irregular, with variation in sizes and in electron density. Bacterial two-hybrid analysis showed direct interaction of SflB with SepF, SflA, and with itself. SflB localizes to part of the sporulation septa but not close to the hyphal wall, suggesting that perhaps SflB inhibits SepF function. While SflA and SflB were not seen to localize specifically in vegetative hyphae, constitutive expression of SflA and SflB from the *ermE* promoter blocked development and resulted in detachment of the colonies from the agar surface, in line with a role in disturbing hyphal branching. Thus, SflA and SflB are novel sporulation proteins, that affect branching and cell division in aerial hyphae.

INTRODUCTION

The mycelial life style of streptomycetes imposes specific requirements for cell division control. One obvious difference between the mycelial *Streptomyces* and the unicellular *Bacillus subtilis*, the model system for cell division in Gram-positive bacteria, is the absence of a defined midcell position in the long hyphae of streptomycetes. Not surprisingly, the way cell division is controlled is very different between these bacteria. In rod-shaped bacteria, the control of cell division revolves around finding the midcell position. Several proteins are required to assist in septum-site localization, such as FtsA and ZipA (Hale and de Boer, 1997; Pichoff and Lutkenhaus, 2002; RayChaudhuri, 1999) and ZapA (Gueiros-Filho and Losick, 2002). Septum-site localization is negatively controlled, via the action of Min, which prevents Z-ring assembly at the cell poles (Marston *et al.*, 1998; Raskin and de Boer, 1997), and nucleoid occlusion that prevents formation of the Z-ring over non-segregated chromosomes (Bernhardt and de Boer, 2005; Woldringh *et al.*, 1991; Wu and Errington, 2004; Wu and Errington, 2012). Direct homologs of all of these control proteins are missing in streptomycetes. Cell division in *Streptomyces* is positively controlled, via the direct recruitment of FtsZ by the membrane-associated SsgB (Willemse *et al.*, 2011). SsgB is part of the divisome, and hence provides the first example of a divisome component that localizes prior to FtsZ. In turn, the localization of SsgB is mediated through the orthologous SsgA protein, a known activator of cell division (van Wezel *et al.*, 2000b; Kawamoto *et al.*, 1997). This system implies the evolution of an entirely new way of Z-ring control.

In Chapter II we presented evidence that SepG allows docking of SsgB to the membrane. Interestingly, one protein involved in early division control in *B. subtilis* is also conserved in streptomycetes, namely SepF, encoded by a gene that lies immediately upstream of *sepG*. SepF of *B. subtilis* assists in bundling of the FtsZ filaments, and polymerizes *in vitro*, forming large rings of around 50 nm in diameter (Ishikawa *et al.*, 2006; Hamoen *et al.*, 2006). Recently, it was shown that SepF also interacts with the membrane in *B. subtilis*, via its N-terminal domain (Duman *et al.*, 2013). This strongly suggests that besides a direct effect of Z-ring assembly, SepF also acts as a membrane anchor for FtsZ. This makes SepF an important protein required for the localization of FtsZ in bacteria that divide by binary fission, which is also found in *Streptomyces*.

In this chapter, we provide a functional analysis of two new *Streptomyces* developmental genes which are paralogs of *sepF*, and are designated as *sflA* and *sflB* (for *sepF* like). We show that deletion of either *sflA* or *sflB* results in branching of the aerial hyphae, and provide evidence that they may act via

interference with the function of *sepF*.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains used in this work are listed in Table 1. *E. coli* strains JM109 (Sambrook *et al.*, 1989) and ET12567 (MacNeil *et al.*, 1992) were used for routine cloning and for isolation of non-methylated DNA, respectively. *E. coli* transformants were selected on LB agar media containing the relevant antibiotics and grown O/N at 37°C. *Streptomyces coelicolor* A3(2) M145 was used as parental strain to construct mutants. All media and routine *Streptomyces* techniques are described in the *Streptomyces* manual (Kieser *et al.*, 2000). Yeast extract-malt extract (YEME) and tryptone soy broth with 10% sucrose (TSBS) were the liquid media for standard cultivation. Regeneration agar with yeast extract (R2YE) was used for regeneration of protoplasts and with appropriate antibiotics for selection of recombinants (Kieser *et al.*, 2000). Soy flour mannitol (SFM) agar plates were used to grow *Streptomyces* strains for preparing spore suspensions and for morphological characterization and microscopy. *E. coli* strain BTH101, nonreverting adenylate cyclase-deficient (*cya*), was used for BATCH screening assay (Karimova *et al.*, 1998).

Table 1. Bacterial strains

Bacteria strains	Genotype	Reference
E. coli JM109	See reference	(Sambrook *et al.*, 1989)
E.coli ET12567	See reference	(MacNeil *et al.*, 1992)
E. coli BTH101	See reference	(Karimova *et al.*, 1998)
S. coelicolor M145	$SCP1^{-}$ $SCP2^{-}$	(Kieser *et al.*, 2000)
GAL14	M145Δ*sflA*	This work
GAL15	M145Δ*sflB*	This work
GAL16	M145Δ*sflA*Δ*sflB*	This work
GAL17	M145 + pGWS759	This work
GAL18	M145 + pGWS760	This work
GAL19	M145 + pGWS761	This work
GAL44	M145 + pGWS774	This work
GAL45	M145 + pGWS775	This work
GAL46	M145 + pGWS776	This work
GAL70	M145 + pWHM3	This work

Plasmids and constructs and oligonucleotides

All plasmids and constructs described in this chapter are summarized in Table

2. The oligonucleotides are listed in Table 3.

Table 2. Plasmids and Constructs

Plasmid and constructs	Description	Reference
pWHM3	*E. coli*/*Streptomyces* shuttle vector, multi-copy and very unstable in *Streptomyces*	(Vara *et al.*, 1989)
pSET152	*E. coli*/*Streptomyces* shuttle vector, high copy number in *E. coli* and integrative in *Streptomyces*	(Bierman *et al.*, 1992)
pHM10a	Conjugative *E. coli-Streptomyces* shuttle vector, harboring P*ermE* and RBS	(Motamedi *et al.*, 1995)
pGWS750	pWHM3 containing flanking regions of *S. coelicolor* SCO1749 with apraloxP-*Xba*I inserted between then in pWHM3 EcoRI-HindIII	This work
pGWS751	pWHM3 containing flanking regions of *S. coelicolor* SCO5967 with apraloxP-*Xba*I inserted between then in pWHM3 EcoRI-HindIII	This work
pUWL-Cre	Cre-recombinase expression plasmid	(Fedoryshyn *et al.*, 2008)
pGWS759	pSET152 harboring *sflA-egfp* under control of *ftsZ* promoter	This work
pGWS760	pSET152 harboring *sepF-egfp* under control of *ftsZ* promoter	This work
pGWS761	pSET152 harboring *sflB-egfp* under control of *ftsZ* promoter	This work
pGWS774	pWHM3 containing P*ermE*, RBS and *sflA*	This work
pGWS775	pWHM3 containing P*ermE,* RBS and *sepF*	This work
pGWS776	pWHM3 containing P*ermE,* RBS and *sflB*	This work
pBTH165	pUT18 harboring +1/+438 region of *sflA* from *S. coelicolor* fused behind the *cya* gene	This work
pBTH109	pUT18 harboring +1/+639 region of *sepF* from *S. coelicolor* fused behind the *cya* gene	This work
pBTH169	pUT18 harboring +1/+408 region of *sflB* from *S. coelicolor* fused behind the *cya* gene	This work
pBTH167	pKT25 harboring +1/+438 region of *sflA* from *S. coelicolor* fused behind the *cya* gene	This work
pBTH111	pKT25 harboring +1/+639 region of *sepF* from *S. coelicolor* fused behind the *cya* gene	This work
pBTH171	pKT25 harboring +1/+408 region of *sflB* from *S. coelicolor* fused behind the *cya* gene	This work

Constructs for creating deletion mutants

Construction for in-frame deletion were based on the instable vector pWHM3

(Vara *et al.*, 1989), essentially as described in Chapter II. For the deletion of *sflA*, its upstream region -1336/+9 (using primers sflA_LF-1339 and sflA_LR+9) and downstream region +427/+1702 (using primers sflA_RF_427 and sflA_RR+1702) were amplified by PCR from *S. coelicolor* M145 genomic DNA and cloned into pWHM3 as EcoRI-HindIII fragments, and the apramycin resistance cassette *aac(3)IV* flanked by *loxP* sites inserted in between. This resulted in plasmid pGWS750 that was used for deletion of *sflA* (SCO1749). The presence of *loxP* sites allows efficient removal of apramycin resistance cassette by Cre-recombinase (Fedoryshyn *et al.*, 2008). The same strategy was used to create construct pGWS751 for the deletion of *sflB* (SCO5967). This plasmid contained the -1258/+9 and +357/+1917 regions relative to *sflB*, and the apramycin resistance cassette inserted in-between. The *sflA* and *sflB* double mutant (GAL16) was constructed in the background of a *sflA* in-frame deletion mutant (GAL14) by deleting *sflB*.

Constructs for the expression of eGFP fusion proteins

The *ftsZ* gene with its own promoter and fused to *egfp* was excised from pKF41 as a BglII-NotI fragment, and cloned into pSET152 digested with BamHI-NotI. Subsequently, the *ftsZ* coding region was removed after digestion with StuI-BamHI and replaced by the coding region of *sflA* (amplified from *S. coelicolor* genomic DNA using primers sflA_F+1 and sflA_R+438), *sepF* (primers sepF_F+1 and sepF_R+639) or *sflB* (primers sflB_F+1 and sflB_R+408). This resulted in constructs pGWS759, pGWS760 and pGWS761, for the expression of SflA-eGFP, SepF-eGFP and SflB-eGFP, respectively.

Constructs for enhanced gene expression

To obtain enhanced expression of *sepF, sflA* and *sflB*, the genes were inserted behind the constitutive *ermE* promoter and an optimized ribosome binding site using plasmid pHM10a (Motamedi *et al.*, 1995). For this, DNA fragments harbouring the entire *sflA*, *sepF* or *sflB* coding region were amplified by PCR from *S. coelicolor* M145 genomic DNA using primer pairs sflA_F+4 and sflA_R+447, sepF_F+4 and sepF_R+648 and sflB_F+4 and sflB_R+417, respectively and cloned into pHM10a. The inserts of the pHM10a-based constructs were subsequently transferred to pWHM3 to generate pGWS774 (for expression of *sflA*), pGWS775 (for *sepF*) and pGWS776 (for *sflB*).

Constructs for BACTH screening

The entire coding region of *sflA* was amplified from *S. coelicolor* M145 genomic DNA using primer pair sflA-fw and sflA-rv, and cloned as an XbaI-KpnI

fragment into pUT18 and pKT25 to generate pBTH165 and pBTH167, respectively. *sepF* was amplified using primers sepF-fw and sepF-rv and cloned into pUT18 and pKT25 as an XbaI-XmaI fragment to generate pBTH109 and pBTH111, respectively. Finally, *sflB* was amplified from *S. coelicolor* M145 genomic DNA using primer pair sflB-fw and sflB-rv, cloned as an XbaI-KpnI fragment into pUT18 and pKT25, so as to generate pBTH169 and pBTH171, respectively.

Table 3. Oligonucleotides

Name	5'-3' sequence[#]
sflA_LF -1336	GTCA**GAATTC**GTTGAAGGTGCCGCAGCACATCTG
sflA_LR +9	GTCAGAAGTTATCCATCACC**TCTAGA**CGATCCCATGGACGCCTCCTCTCA
sflA_RF+427	GTCAGAAGTTATCGCGCATC**TCTAGA**TTCAACCAGAGCTGAGGCGGGGCC
sflA_RR +1702	GTCA**AAGCTT**CCCATGGCCGCGTCCCCGAAGTT
sflB_LF-1258	GTCA**GAATTC**GAACTGCACCATCAGGTAGGCGT
sflB_LR+9	GTCAGAAGTTATCCATCACC**TCTAGA**CGATTTCACTCGCCTTCATTGCCTGCA
sflB_RF +357	GTCAGAAGTTATCGCGCATC**TCTAGA**ACGTCTTCCTGCTGACCCCGGCG
sflB_RR+1917	GTCA**AAGCTT**CGGTCACGGGCTCAGGTGTACCA
sflA_F +1	GTCAGAATTC**AGGCCT**TCGACATGGGATCGGTACGCAAGGCG
sflA_R +438	GCTA**GGATCC**CTCTGGTTGAAGAATCCGTC
sepF_F +1	GTCAGAATTC**AGGCCT**TCGACATGGCCGGCGCGATGCGCAAG
sepF_R +639	GTCAAAGCTT**GGATCC**CTCTGGTTGAAGAACCCGCC
sflB_F +1	GTCAGAATTC**AGGCCT**TCGACGTGAAATCGGGGGAGCCGGTG
sflB_R +408	GTCA**AGATCT**ACTCCCGGCACCCCCGCCGC
sflA_F+4	GCTAGAATT**CATATG**GGATCGGTACGCAAGGCGAGT
sflA_R+447	GCTA**AAGCTT**CCCGCCTCAGCTCTGGTTGAA
sepF_F+4	GTCAGAATT**CATATG**GCCGGCGCGATGCGCAAGATG
sepF_R+648	GTCAAAGCTT**GGATCC**TAGTGCGTCTCAGCTCTGGTT
sflB_F+4	GTCAGAATT**CATATG**AAATCGGGGGAGCCGGTGAAC
sflB_R+417	GTCA**AAGCTT**GGACCGTCACACTCCCGGCAC
sflA-fw	CGC**TCTAGA**CATGGGATCGGTACGCAAGGC
sflA-rv	CGG**GGTACC**CAGCTCTGGTTGAAGAATCCG
sepF-fw	CG**TCTAGA**CATGGCCGGCGCGATGCGC
sepF-rv	CT**CCCGGG**AGCTCTGGTTGAAGAACCCGCC
sflB-fw	CGC**TCTAGA**AGTGAAATCGGGGGAGCCGGT
sflB-rv	CGG**GGTACC**CACACTCCCGGCACCCCCGCC

[#]Restriction sites used for cloning are underlined and in bold. GGATCC, BamHI; AGATCT, BglII; GAATTC, EcoRI; AAGCTT, HindIII; GGTACC, KpnI; CATATG, NdeI; AGGCCT, StuI; TCTAGA, XbaI; CCCGGG, XmaI.

Microscopy

Light microscopy

Sterile cover slips were inserted at an angle of 45 degrees into SFM agar plates, and spores of *Streptomyces* strains were carefully inoculated at the intersection angle. After incubation at 30°C for 3 to 5 days, cover slips were positioned on a microscope slide prewetted 5 µl of 1x PBS. Fluorescence and corresponding light micrographs were obtained with a Zeiss Axioscope A1 upright fluorescence microscope (with an Axiocam Mrc5 camera at a resolution of 37.5 nm/pixel). The green fluorescent images were created using 470/40 nm band pass (bp) excitation and 525/50 bp detection, for the red channel 550/25 nm bp excitation and 625/70 nm bp detection was used (Willemse and van Wezel, 2009). For membrane staining FM5-95 was used. For stereomicroscopy we used a Zeiss Lumar V12 stereomicroscope. All fluorescence images were background corrected setting the signal outside the hyphae to zero to obtain a sufficiently dark background. These corrections were made using Adobe Photoshop CS4.

Electron microscopy

Morphological studies on surface grown aerial hyphae and/or spores by cryo-scanning electron microscopy were performed using a JEOL JSM6700F scanning electron microscope as described previously (Colson *et al.*, 2008). Transmission electron microscopy (TEM) for the analysis of cross-sections of hyphae and spores was performed with a FEI Tecnai 12 BioTwin transmission electron microscope as described (Noens *et al.*, 2005).

BATCH complementation assay

For BACTH complementation assays, recombinant pKT25 and pUT18 harboring genes of interest were used in various combinations to co-transform *E. coli* BTH101 cells. The trans- formants were plated onto LB-X-Gal-IPTG medium containing ampicillin (100 mg/mL) and kanamycin (50 mg/mL), IPTG (0,5 mM) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 40 µg/mL) and were incubated for 24–36 h at 30°C.

Computer analysis

For DNA and protein searches we used StrepDB (http://strepdb.streptomyces.org.uk/). Phylogenetic analyses and gene synteny analysis were performed by using STRING (http://string.embl.de).

RESULTS

Three *sepF*-like genes in *Streptomyces*

Three genes with homology to *sepF* were found on the *S. coelicolor* genome. The *sepF* gene itself is SCO2079 and lies in the *dcw* cluster in close proximity to *ftsZ* (see also Chapter 1). Two *sepF*-like (*sfl*) genes, *sflA* (SCO1749) and *sflB* (SCO5967), are located elsewhere on the *S. coelicolor* chromosome. SepF is a predicted 213 aa protein, while SflA and SflB are 145 and 136 aa long, respectively. With that, SflA and SflB have lengths very similar to that of SepF of *Bacillus subtilis* (139 aa; accession number KFK80720). An alignment of the three proteins and their comparison to SepF of *B. subtilis* is presented in Figure 1. SepF of *S. coelicolor* has a 66 aa internal extension that runs from aa residues 45-110. The presence of three *sepF*-like genes is typical of streptomycetes, but *sflAB* are absent from many other actinomycetes.

Figure 1. Alignment of SepF proteins. Amino acid sequences of SepF proteins from *B. subtilis* (SepFbs) and *S. coelicolor* (SepFsc), and two SepF paralogs of *S. coelicolor* (SflA and SflB) were aligned using Boxshade program. Identical residues are shaded in black; conservative changes are shaded in grey.

Deletion of *sflA* and *sflB* affects colony morphology

Single mutants of *S. coelicolor* M145 in which either *sflA* or *sflB* was deleted or an *sflAB* double mutant were created using essentially the same strategy as described elsewhere in this thesis (see Chapters II and III; for constructs see

Materials and Methods section). The *sfl* single and double mutants sporulated well on SFM agar plates, developing abundant aerial mycelium and grey-pigmented spores (Fig. 2). However, when single colonies were analyzed in more detail, differences in colony morphology were observed. *S. coelicolor* M145 formed colonies with a smooth edge, while those of *sflA* or *sflB* mutants had a more 'fluffy' phenotype, and this was even more obvious in the *sflAB* double mutant (Fig. 2). First visual assessment of the vegetative hyphae showed that the hyphae of the *sfl* mutants had enhanced (around 50% increased) branching frequency as compared to wild-type hyphae.

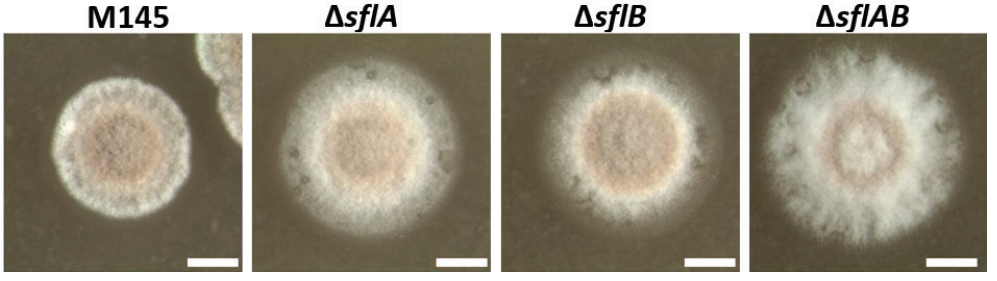


Figure 2. Phenotypic analysis of *sfl* mutants. Stereomicrographs show representative colonies of *S. coelicolor* M145 and its *sflA* and *sflB* null mutants. Strains were grown on SFM agar plates for three days at 30^{0}C. Colonies of *sfl* mutants were 'fluffier' than those of the parental strain M145. Bar, 2 mm.

sflA and *sflB* mutants have branching aerial hyphae

Surface-grown *S. coelicolor* M145, Δ*sflA*, Δ*sflB* and Δ*sflA*Δ*sflB* were analyzed in more detail by scanning electron microscopy (SEM). After three days of growth, *S. coelicolor* M145 produced abundant and regular spore chains (Fig. 3A). However, strains lacking *sflA* (*i.e.* both Δ*sflA* and Δ*sflA*Δ*sflB*) produced significantly fewer spore chains (Fig. 3B & 3D), while the *sflB* mutant produced a comparable amount of spore chains as the parental strain M145 (Fig. 3C). After prolonged incubation, sporulation of strains lacking *sflA* sporulated more abundantly, which suggests that sporulation was delayed in the mutants (data not shown). A more striking phenotype of *sflA* and *sflB* null mutants was that they frequently produced branching spore chains, which is never observed in the wild-type strain (Fig. 3E-G).

Transmission electron microscopy (TEM) allowed imaging of thin sections and this again revealed branching spore chains in *sflA* and *sflB* mutants (Fig. 4, arrows). Furthermore, variation in sizes within spore chains was frequently observed. Another obvious difference was that while wild-type spores had a

typical dark spore wall and well-condensed DNA, the spores of the mutants typically had lighter spore walls as well as less clearly visible DNA in many of the spores (Fig. 4). This suggests pleiotropic changes in spore maturation in the *sflA* and *sflB* mutants.

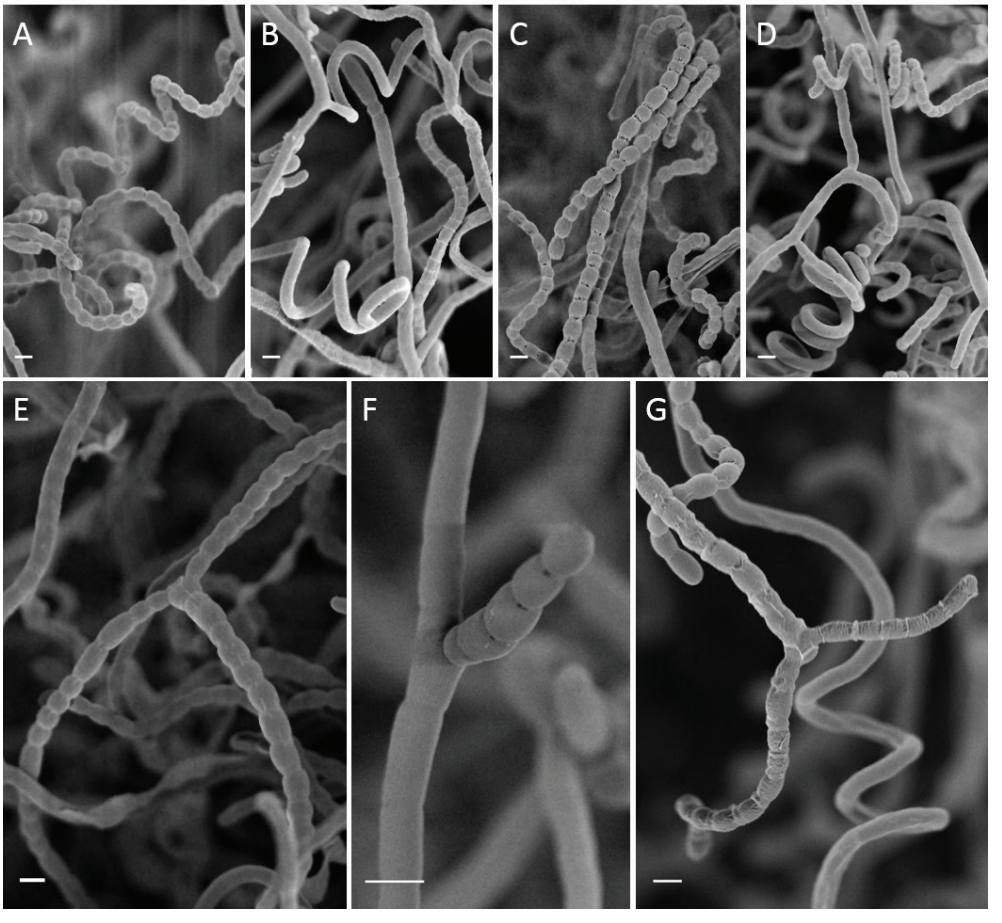


Figure 3. Cryo-scanning electron micrographs of spore chains of *S. coelicolor* M145 and its *sfl* mutants. Wild-type *S. coelicolor* M145 **(A)** sporulated abundantly after three days of incubation, while mutants lacking either *sflA* **(B & E)** or *sflAB* **(D & G)** showed reduced sporulation; the *sflB* null mutant **(C & F)** produced comparable amount of spores as the parental strain. Most notable change in all mutants was that the spore chains frequently branched. Bar, 1 μm.

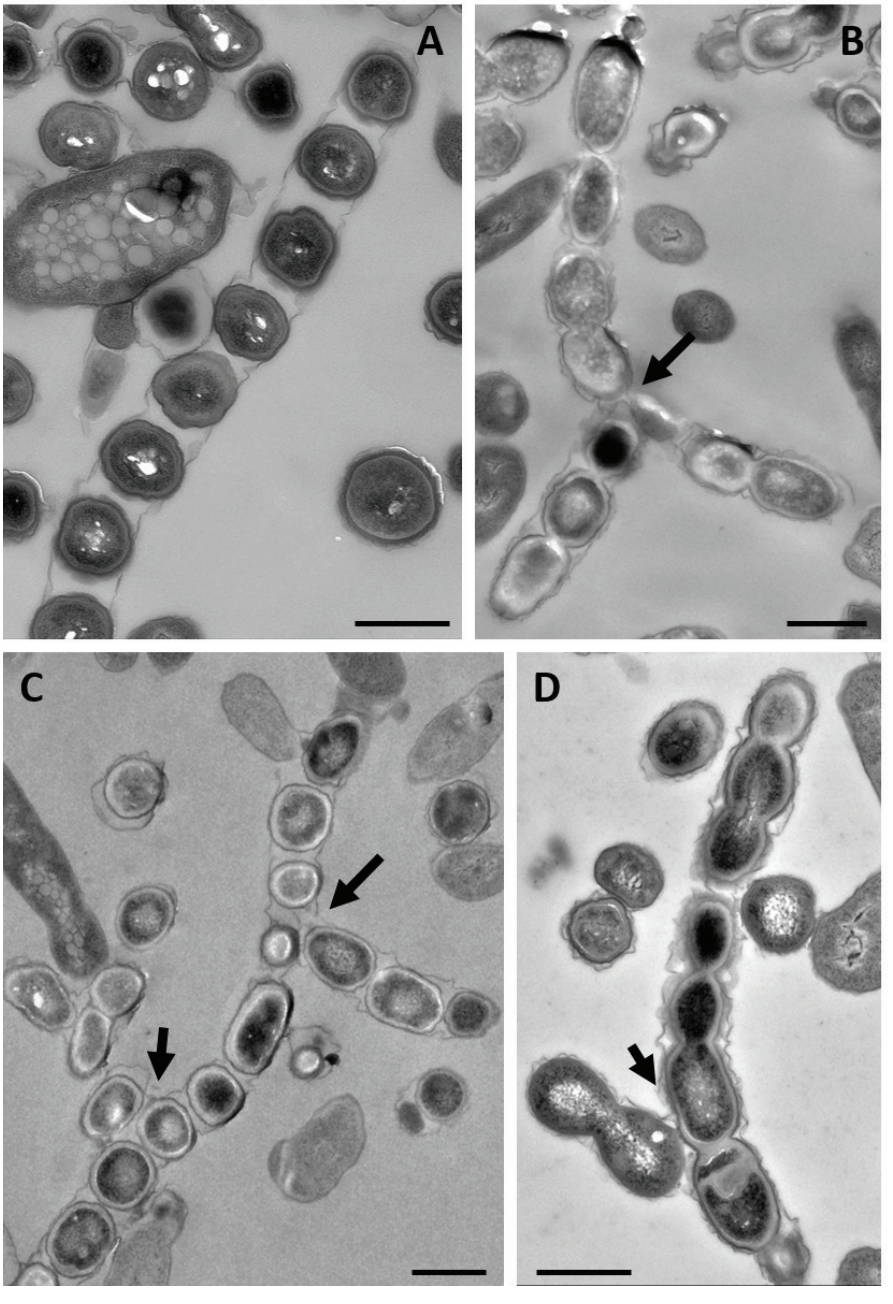


Figure 4. Transmission electron micrographs of spore chains of *S. coelicolor* M145 and its *sfl* mutants. While spore chains of wild type M145 **(A)** do not branch and contain regularly sized spores, mutant lacking either *sflA* **(B)**, *sflB* **(C)** or *sflAB* **(D)** produce irregular spores and spores chains frequently branch, in line with the SEM images (Figure 3). Cultures were grown on SFM agar plates for 5 days at 30^{0}C. Bar, 1 μm.

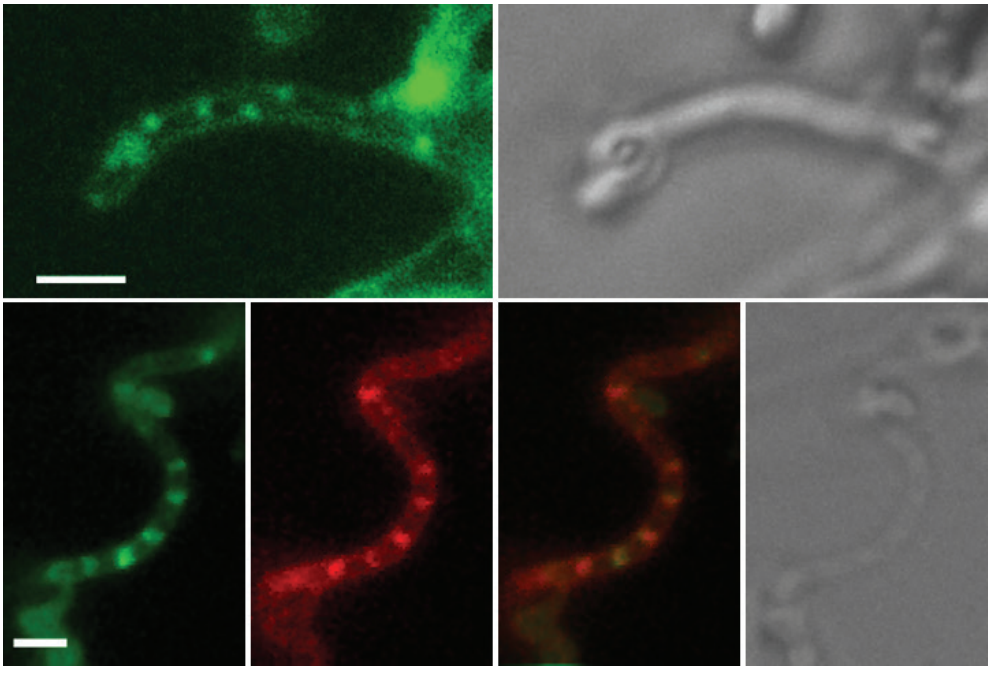
Localization of SflA and SflB *in S. coelicolor*

To analyze the localization of the SepF paralogs, constructs based on the integrative vector pSET152 were made containing in-frame fusion of *sepF* with *egfp* (for details see Materials and Methods section). These allow expression of *sflA-egfp*, *sepF-egfp* and *sflB-egfp* from the *ftsZ* promoter and were called pGWS759, pGWS760 and pGWS761 respectively. The constructs integrated at the ΦC31 attachment site on the *S. coelicolor* M145 chromosome.

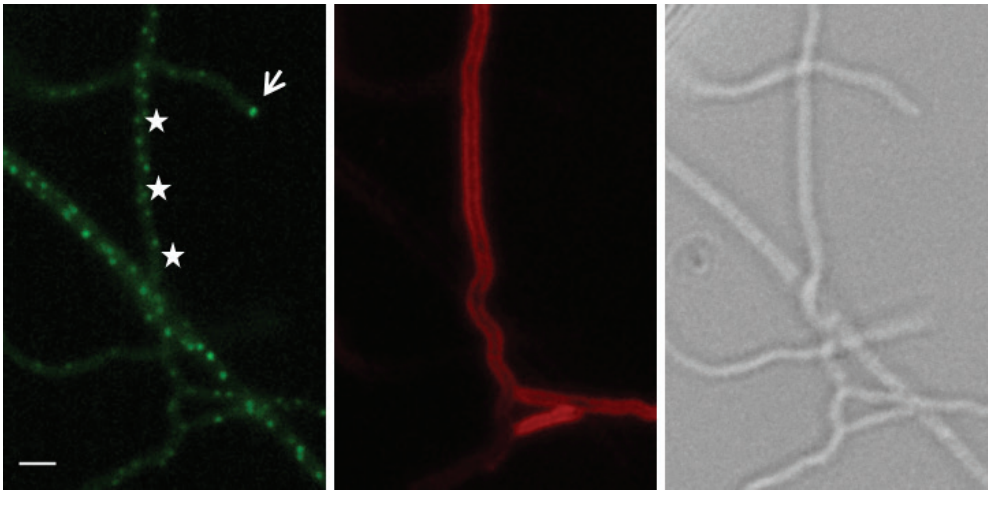
In *B. subtilis*, $SepF^{Bs}$ localizes to division sites (Ishikawa *et al.*, 2006; Hamoen *et al.*, 2006). During vegetative growth of *S. coelicolor*, we failed to detect specific localization for SepF or SflAB (data not shown). In aerial hyphae, SepF localize to septum sites as $SepF^{Bs}$. It formed foci at either side of the hyphae, which most likely represented septum sites (Fig. 5A). And eventually, SepF-eGFP localized in a lader-like pattern, which co-stained with the septa as seen by membrane staining (Fig. 5A). The similar localization profiles of the SepF proteins from *B. subtilis* and *S. coelicolor* suggests that *Streptomyces* SepF plays a very similar role in cell division as *Bacillus* SepF.

SflA-eGFP formed foci at many positions along the entire lateral wall of the aerial hyphae, and often formed bright foci at apical sites (Fig. 5B, arrow). The pattern of SflA-eGFP localization varied, with foci that were widely spaced, distributed more symmetrically or clustered closely together (Fig. 5B, star). SflA-eGFP foci were only seen in aerial hyphae in which septum synthesis had not yet initiated (Fig. 5B). SflA-eGFP was not detected in mature spores. In early aerial hyphae, SflB-eGFP showed a similar localization pattern as SflA-eGFP, forming foci at hyphal tip and along the lateral wall of the aerial hyphae (Fig. 5C, **α**). SflB-eGFP localized in a ladder-like pattern prior to the initiation of septum synthesis (Fig. 5C, **β**). Interestingly, the 'sports' of the ladders formed by SflB were shorter than those formed by SepF or FtsZ, and did not span the entire width of the hyphae. Instead, they were biased towards the central part. This suggests that SflB stays away from the sites where cell division is initiated. At the time when septa were seen (with FM5-95 staining of membranes), ladder-like structure of SflB-eGFP largely disappeared (Fig. 5C, **γ**). This suggests that in contrast to SepF, SflB only localizes to the central part of the septum, and only during the onset of sporulation-specific cell division.

5A



5B



5C

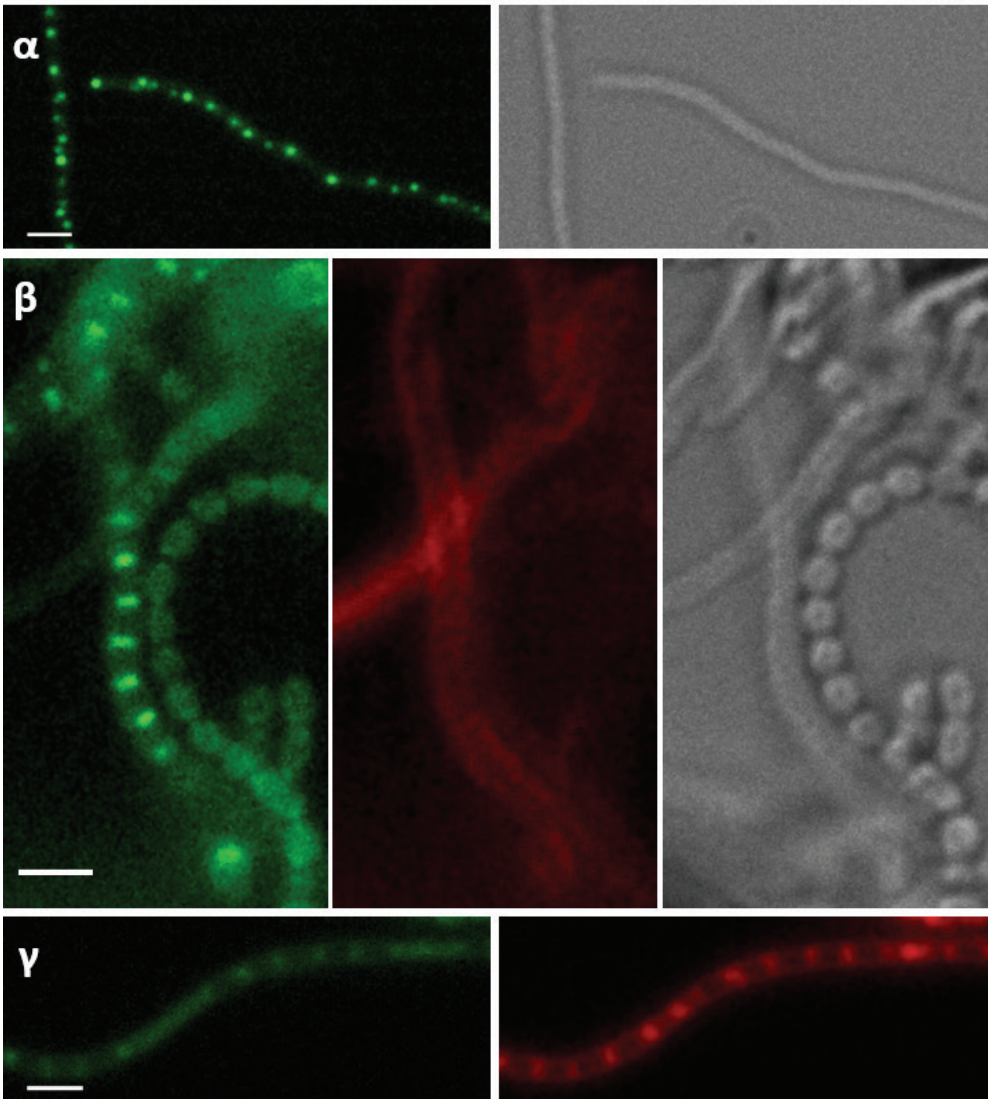


Figure 5. Localization of SepF-eGFP, SflA-eGFP and SflB-eGFP in *S. coelicolor*. Sporogenic aerial hyphae of *S. coelicolor* M145 were imaged by fluorescence microscopy visualizing the eGFP fusion proteins, membrane (stained with FM5-95; red), the merged image and corresponding light micrograph. **A.** SepF-eGFP localized in a ladder-like pattern that overlapped the sporulation septa. **B.** SflA-eGFP formed foci along aerial hyphae (star) and at apical tip (arrow) prior to septum synthesis. **C.** SflB-eGFP had similar localization pattern as SflA-eGFP in early aerial hyphae (α), while in sporogenic hyphae SflB-eGFP localized in a ladder-like pattern prior to septum synthesis (β), and SflB-eGFP ladders vanished septal membranes formed (γ). Bar, 2 μm.

Effect of enhanced expression of the *sepF* and *sfl* genes

To study the effect of overexpression of SepF paralogs in *S. coelicolor*, the *sflA*, *sepF* and *sflB* genes were all cloned individually behind the *ermE* promoter region with an optimized ribosome binding site (see Materials and Methods for details), and the expression cassettes were then inserted in the multi-copy shuttle vector pWHM3. The expression constructs were designated pGWS774, pGWS775 and pGWS776, respectively. Plasmid pWHM3 is unstable in *Streptomyces* and this is not only useful for gene disruption but also for varying the expression level. Indeed, the copy number of pWHM3 is largely dependent on the level of thiostrepton, and increasing the thiostrepton concentration allows the proportional increase of its copy number. Strains GAL44, GAL45, GAL46 and GAL70 (*S. coelicolor* M145 transformed with pGWS774, pGWS775, pGWS776 or control pWHM3) were plated onto R5 agar plates with different concentration of thiostrepton and the colony morphology was investigated after 5 days of incubation (Fig. 6).

On R5 agar plates without thiostrepton, all colonies had normal development and comparable colony size (Fig. 6). However, in the presence of thiostrepton (20 μg/ml), the colonies harboring the SflA and SflB expression constructs were blocked in development, while those expressing SepF produced aerial hyphae but formed significantly smaller colonies indicative of growth inhibition (Fig. 6). Interestingly, colonies of either GAL44 (expressing SflA) or GAL46 (expressing SflB) did not attach to the agar surface. This is shown clearly by touching colonies with a toothpick, which resulted in mashed up but fixed colonies for control plasmid (GAL70) as well as for colonies expressing SepF (GAL45), while colonies expressing SflA or SflB were moved easily over the plate. When the thiostrepton concentration was increased to 50 μg/mL, colonies of GAL44 and GAL46 more or less floated on the agar surface, while those of GAL70 and GAL45 were still firmly attached to the agar surface (not shown). Furthermore, in particular GAL44 produced very small colonies (Fig. 6). Taken together, this suggests that forced expression of SflA and SflB in the vegetative mycelium alters colony morphology and blocks development, while enhanced expression of SepF does not affect development, but also reduces colony size.

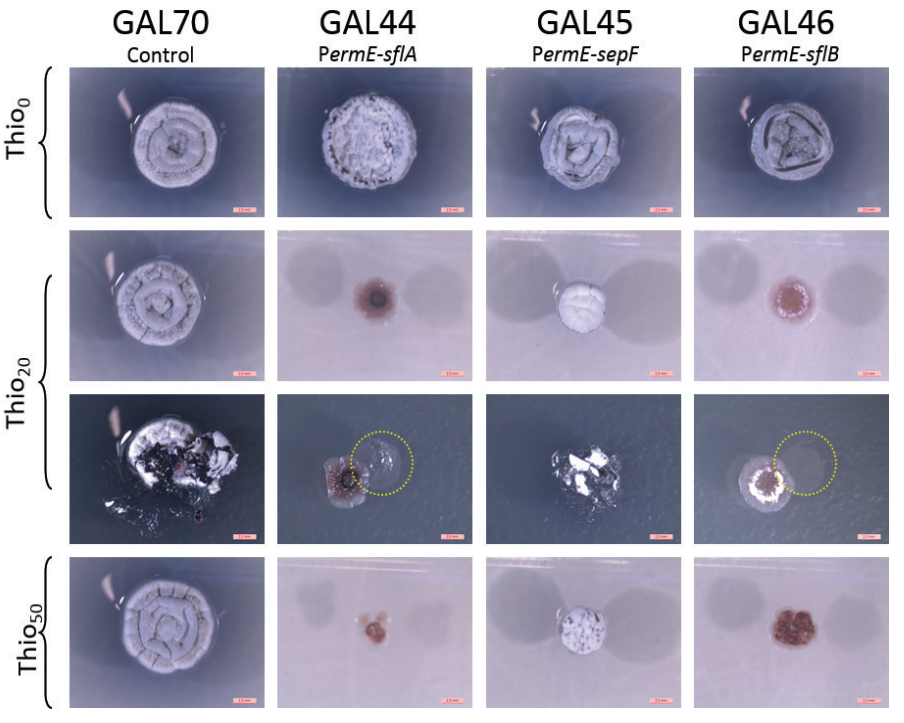


Figure 6. Effect of enhanced expression of *sepF* and *sfl* genes on colony morphology. Stereomicrographs showing the phenotype of GAL70 (*S. coelicolor* M145 + empty plasmid pWHM3 control), GAL44 (M145 + pGWS774, expressing *sflA*), GAL45 (M145 + pGWS775, expressing *sepF*) and GAL46 (M145 + pGWS776, expressing *sflB*) were grown on R5 agar plates containing different concentrations of thiostrepton (0-50 mg/ml). Plates were incubated for 5 days at 30^0C. Note that colonies overexpressing either *sflA* or *sflB* were blocked in development and had lost the ability to adhere to the agar surface, while overexpression of *sepF* primarily reduced colonial size while colonies adhered normally. Bar, 2 mm.

Direct interactions between the *sepF* paralogs

To examine possible interaction between the SepF and Sfl proteins, we performed two-hybrid screening using the bacterial two-hybrid system (see the Materials and Methods section for details). The result is displayed in Figure 7. The data showed that SflB directly interacts with SflA, SepF and also with itself, while no interaction was observed between SepF and SflA. Surprisingly, no self-interaction was seen for SepF. This indicates that the SepF-Cya fusion protein may not be entirely functional. Further biochemical and two-hybrid interaction studies are required to get more detailed insight into the way the SepF-like proteins interact with each other and with other (cell division) proteins.

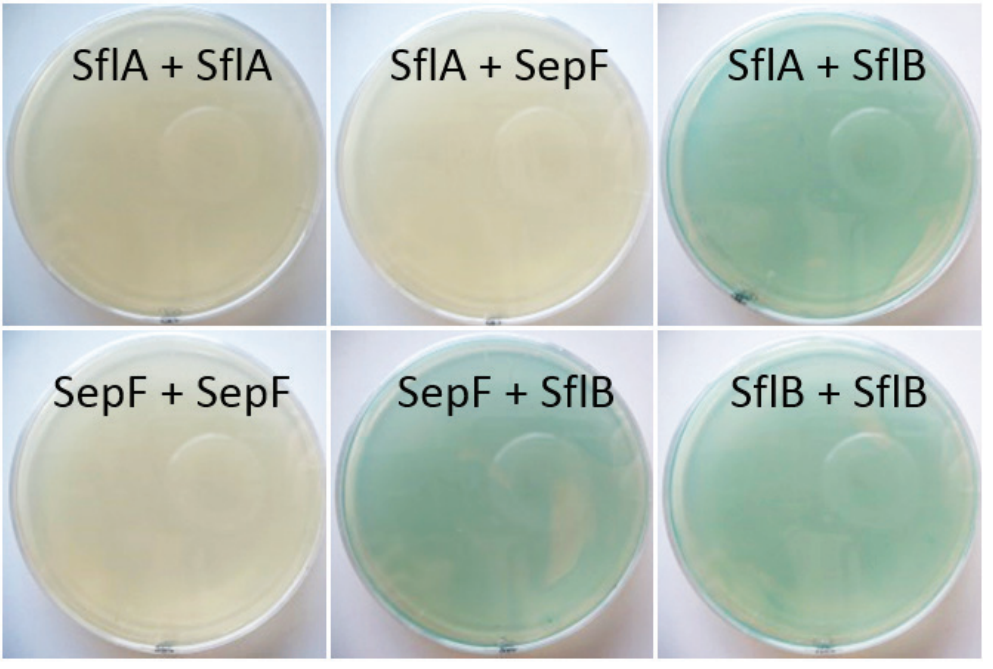


Figure 7. Analysis of protein interaction using the bacterial two-hybrid system. Possible interactions between SflA, SflB and SepF from *S. coelicolor* were assayed on LB agar plates containing X-Gal and IPTG. Blue pigmentation indicates interaction between the partners. SflB interacts with SlfA, SepF and itself. The lack of self-interaction of SepF is surprising and indicates that perhaps the protein is not fully functional.

DISCUSSION

A major question in the developmental biology of *Streptomyces* that we seek to address is, how do *Streptomyces* ensure that septa are controlled properly in time and space in the long and multinucleoid hyphae? We have shown previously that in streptomycetes the correct localization of FtsZ is governed by a rather unique system of positive control, whereby the actinomycete-specific SsgA and SsgB proteins recruit FtsZ to the septum sites to initiate sporulation-specific cell division (Willemse *et al.*, 2011). As a consequence, deletion of either *ssgA* or *ssgB* blocks sporulation (van Wezel *et al.*, 2000b; Keijser *et al.*, 2003). In Chapter 2 we added a new chapter to this story by providing evidence that SepG (YlmG in *B. subtilis*) is the protein that allows SsgB to dock to the membrane. Still, the question remains how the perfect spacing of the Z-rings in the aerial hyphae is governed, resulting in up to a 100 septa that are formed simultaneously, visualized as a spectacular ladder of FtsZ-GFP.

In this work we present another piece of the jigsaw, which points at the possible existence of a layer of negative control, which revolves around the SepF-like proteins SflA and SflB. Typical ladders of Z-rings were produced in both wild type *S. coelicolor* and in *sflA* or *sflB* null mutants, while the average distance between adjacent Z-rings in mutants was shorter compared to that of in wild-type *S. coelicolor*. The difference was most noteworthy in *slfA* null mutants. In wild-type hyphae, the average distance between neighboring Z-rings was 734 ± 146 nm, while it was reduced to 598 ± 134 nm and 672 ± 111 nm in *sflA*- and *sflB*-null mutants respectively, and 618 ± 135 nm in the *sflAB*-null mutant. It highlights the possibility that SflA plays a role in determining the (distance between the) septum sites.

In aerial hyphae, SflA and SflB localize primarily in young sporogenic aerial hyphae at the stage prior to septum formation, suggesting they start functioning in early aerial hyphae stage, possibly before FtsZ polymerization. An important observation is that SflB interacts with SepF as evidenced by two-hybrid studies, suggesting the protein may compete directly with SepF, *e.g.* by forming hetero-multimers. SflB also interacts with SflA and with itself. It should be noted that SflA and SepF share significant homology in particular in their C-terminal parts, with 74% aa identity (88% similarity) between residues 135-192 of SepF and residues 62-119 of SflA. The rest of the protein shows significantly lower homology. The C-terminal part of SepF is known to interact with FtsZ (Duman *et al.*, 2013), and the high degree of conservation suggests that SflA may also interact with FtsZ, although this remains to be proven. However, the fact that this domain is the only region that is well con-

served between SepF and SflA suggests that it also candidates for interaction with SflB.

Interestingly, SflA localized in many foci along the hyphal wall, while SflB localized at the middle of the septum, but away from the hyphal wall. While more extensive studies are required, these initial data suggest that as a net result, SflB leaves a gap at the intersection between the future septum and the lateral wall of the aerial hyphae. If indeed SflB interferes with SepF function by direct interaction, SepF will therefore be present precisely where it should be active, namely at the site where Z-ring formation is initiated following the recruitment by SsgB. We therefore hypothesize that SflB may function to block premature SepF polymerization away from the lateral wall. While localization of SflA-eGFP and SflB-eGFP fusion proteins was primarily seen in the aerial hyphae, it should be noted that many vegetative hyphae will have stopped growing, and only occasional septa occur in vegetative hyphae. Furthermore, enhanced expression of SflA and SflB has a distinct effect on colony morphology and surface attachment by the vegetative hyphae. Therefore, more extensive studies are required to establish what role SflAB play in branching of vegetative hyphae.

The most striking phenotype of *sflA* and *sflB* mutants is the extensive branching of the aerial hyphae. Furthermore, forced expression of SflA and SflB from a constitutive promoter largely blocked aerial growth and reduced adhesion of the colonies to the agar surface, indicating that branching is impaired in the vegetative mycelium. Conversely, deletion of *sflA* or *sflB* results in hyper-branching and in particular branching at non-canonical sites, as evidence by the branching aerial hyphae. These data strongly suggest an inverse correlation between the expression level of SflA and SflB and branching frequency. How can these data be explained if SepF controls cell division? The most obvious link is *divIVA*, which lies two genes downstream of *sepF* and next to the septum-localizing *sepG*. DivIVA is required for driving apical growth, and hence branching, and a high branching frequency is typical of strains over-expressing DivIVA (Flärdh, 2003a). Interestingly, initial experiments using two-hybrid studies revealed weak but significant interaction between SflB and DivIVA, which suggests that SflB may act via direct interaction with (and inhibition of) DivIVA which would indeed explain the inverse correlation between SflB expression and branching frequency (data not shown). SflA may compete indirectly with DivIVA through SflB, since clear interaction was observed between SflA and SflB in bacterial two-hybrid studies. The competition between SflA/SflB and DivIVA may serve to ensure that tip extension and branching are stopped at the right time to initiate development of the

sporogenic aerial hyphae into spore chains, but this speculative idea requires further substantiation by experiments.