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The SepF-like proteins SflA and SflB prevent ectopic localization of FtsZ and DivIVA during sporulation of *Streptomyces coelicolor*

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ABSTRACT

Bacterial cytokinesis starts with the polymerization of the tubulin-like FtsZ, which forms the cell division scaffold. SepF aligns FtsZ polymers and also acts as a membrane anchor for the Z-ring. While in most bacteria cell division takes place at midcell, during sporulation of *Streptomyces* many septa are laid down almost simultaneously in multinucleoid aerial hyphae. The genomes of streptomycetes encode two additional SepF paralogs, SflA and SflB, which can interact with SepF. Here we show that the sporogenic aerial hyphae of *sflA* and *sflB* mutants of *Streptomyces coelicolor* frequently branch, a phenomenon never seen in the wild-type strain. The branching coincided with ectopic localization of DivIVA along the lateral wall of sporulating aerial hyphae. Constitutive expression of SflA and SflB largely inhibited hyphal growth, further correlating SflAB activity to that of DivIVA. SflAB localized in foci prior to and after the time of sporulation-specific cell division, while SepF co-localized with active septum synthesis. Foci of FtsZ and DivIVA frequently persisted between adjacent spores in spore chains of *sflA* and *sflB* mutants, at sites occupied by SflAB in wild-type cells. Taken together, our data show that SflA and SflB play an important role in the control of growth and cell division during *Streptomyces* development.

1. Introduction

Streptomycetes are multicellular mycelial bacteria that reproduce via sporulation [1,2]. As producers of half of all known antibiotics as well as many anticancer, antifungal and immunosuppressant compounds, streptomycetes are of great medical and biotechnological importance [3,4]. The mycelial life style of streptomycetes imposes specific requirements for the control of growth and cell division [5,6], and they have an unusually complex cytoskeleton [7,8].

The so-called *dcw* gene cluster contains various genes required for division and cell wall synthesis [9,10]. Some genes in this cluster have gained species-specific functions. An obvious example is DivIVA, which in *Bacillus subtilis* is involved in division-site localization by preventing accumulation of the cell division scaffold protein FtsZ [11], while in Actinobacteria DivIVA is required for apical growth [12]. As a consequence, *divIVA* is dispensable in *B. subtilis* but essential for growth in Actinobacteria [12,13]. Conversely, *ftsZ* is essential in *B. subtilis*, but is no required for normal growth of Actinobacteria [14].

Streptomycetes have two different mechanisms of cell division. During vegetative growth, divisome-independent cell division occurs, whereby occasional cross-walls separate the vegetative hyphae into connected multicellular compartments. The cross-walls depend on FtsZ, but not on other canonical divisome proteins such as FtsI, FtsL and FtsW [5,6,15]. Interestingly, mutants lacking *ftsZ* are viable, forming long hyphae devoid of septa [14]. Intricate membrane assemblies ensure that chromosome-free zones are created during septum formation in vegetative hyphae, apparently protecting the DNA from damage during division [16,17]. The control of cross-wall formation is still poorly understood, though mutants that lack the novel cell division gene *sepX* hardly produce any cross-walls [18]. Sporulation-specific cell division in *Streptomyces* may be regarded as canonical cell division as it requires all components of the divisome. At the onset of sporulation, up to 100 septa are formed more or less simultaneously, seen as spirals of FtsZ in the aerial hyphae [19]. Cell division is positively controlled, via the direct recruitment of FtsZ by the membrane-associated SsgB [20].

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SsgB is a member of the SsgA-like proteins, which only occur in morphologically complex actinomycetes [5,21]. The localization of SsgB depends on the homologous SsgA protein, which activates sporulation-specific cell division [22,23]. The small transmembrane protein SepG acts as an anchor for SsgB to the membrane and also controls nucleoid organization [24]. SepF is involved in early division control by stimulating the polymerization of FtsZ. In *B. subtilis*, SepF forms large rings of around 50 nm in diameter *in vitro*, and assists in bundling of the FtsZ filaments [25,26]. SepF interacts with the membrane via its N-terminal domain [27], strongly suggesting that SepF plays a role in Z-ring assembly and anchoring. In the actinomycete *Mycobacterium* SepF also interacts with FtsZ, and is essential for division [28,29]. Thus, SepF is a rare example of a cell division control protein that is shared between firmicutes and by actinobacteria.

In this work, we analyzed the role of two paralogs of SepF in development and sporulation-specific cell division *Streptomyces coelicolor*. These are encoded by SCO1749 and SCO5967, which we designated *sflA* and *sflB* (for *sepF*-like), respectively. SflA and SflB play an important role in the control of branching of aerial hyphae, whereby branching spore chains were frequently seen in *sflA* and *sflB* mutants, coinciding with the unusual localization of DivIVA along the lateral wall. Conversely, overexpression of *sflA* or *sflB* resulted in reduced extension of the hyphal apex. FtsZ foci persisted during spore maturation in *sflA* and *sflB* mutants. These data suggest that SflAB help to prevent the ectopic assembly of DivIVA and FtsZ during sporulation of *Streptomyces*.

2. Materials and methods

2.1. Bacterial strains and media

The bacterial strains used in this work are listed in Table S1. *E. coli* strains JM109 [30] and ET12567 [31] were used for routine cloning and for isolation of non-methylated DNA, respectively. All media and routine *Streptomyces* techniques are described in the *Streptomyces* manual [32]. Yeast extract-malt extract (YEME) and tryptic 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 [32]. Soy flour mannitol (SFM) agar plates were used to grow *Streptomyces* strains for preparing spore suspensions and for morphological characterization and microscopy.

2.2. Plasmids and constructs and oligonucleotides

All plasmids and constructs are summarized in Table S2. The oligonucleotides are listed in Table S3. The constructs are described in the Supplemental Methods.

2.3. Microscopy

2.3.1. Light microscopy

Sterile cover slips were inserted at an angle of 45° into SFM agar plates, and spores of *Streptomyces* strains were carefully inoculated at the intersection angle. After incubation at 30 °C for 3–5 days, cover slips were positioned on a microscope slide prewetted 5 μl of 1xPBS. 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 [33]. DAPI was detected using 370/40 nm excitation with 445/50 nm emission band filter. For staining of the cell wall (peptidoglycan) we used FITC-WGA, for membrane staining FM5-95 and for DNA staining DAPI (all obtained from Molecular Probes). For stereomicroscopy we used a Zeiss Lumar V12 stereomicroscope. All 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.

2.3.2. 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 [34]. 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 [35].

3. Results

3.1. Three sepF-like genes in Streptomyces

Three genes with homology to *sepF* occur in the *S. coelicolor* genome. The canonical *sepF* gene (SCO2079) lies within the *dcw* cluster in close proximity to *ftsZ*, an arrangement that is conserved in all Gram-positive bacteria [9,10]. 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 (146 aa) and SflB (136 aa) are significantly smaller. Thus, SflA and SflB have lengths very similar to that of SepF of *Bacillus subtilis* (139 aa; accession number KFK80720). Alignment of the three proteins and their comparison to SepF of *B. subtilis* and *Mycobacterium smegmatis* is presented in Fig. S1; predicted α-helices and β-strands are boxed with dotted and solid lines, respectively. Compared to SflA and SflB, SepF proteins of *S. coelicolor* and *M. smegmatis* have an approximately 60 aa internal extension at the N-terminal half. The presence of three *sepF*-like genes is common in Actinobacteria, except for *Coriobacteriaceae*, which only have *sepF*. The N-terminal α-helix (aa 1–12) of *Bacillus* SepF is essential for lipid binding to support cell division [27]. Based on the predicted secondary structure of the protein (using JPRED), this α-helix is absent in SflB [36], suggesting that this protein may not bind to the membrane. Conversely, the C-terminal domain of SepF, which is involved in the interaction with FtsZ [27–29,37], is conserved in SflA and SflB.

3.2. Deletion of sflA and sflB affects colony morphology

Deletion mutants were created for the two genes, separately and in combination, using a strategy based on the instable multi-copy plasmid pWHM3 [38] (see Materials and Methods). The *sfl* single and double mutants sporulated well on SFM agar plates, developing abundant grey-pigmented spores after 3 days of growth, suggesting that these proteins are dispensable for sporulation (Fig. 1A). Nonetheless, deletion of *sflA* accelerated aerial growth and sporulation, while deletion of *sflB* delayed sporulation. In *sflAB* double mutants, aerial hyphae formation was accelerated while sporulation was delayed (Fig. S2).

Interestingly, while *S. coelicolor* M145 formed colonies with a smooth edge, those of *sflA* or *sflB* mutants had a 'fluffier' phenotype, a difference that was more pronounced in *sflAB* double mutants (Fig. 1A). Genetic complementation of *sflA* and *sflB* null mutants by the introduction of plasmids pGWS1005 (expressing *sflA* from the *ftsZ* promoter) and pGWS1006 (expressing *sflB* from the *ftsZ* promoter), respectively, restored the wild-type colony morphology. To investigate the change in colony morphology in *sfl* mutants, the tip-to-branch distance and side branch length were measured in young

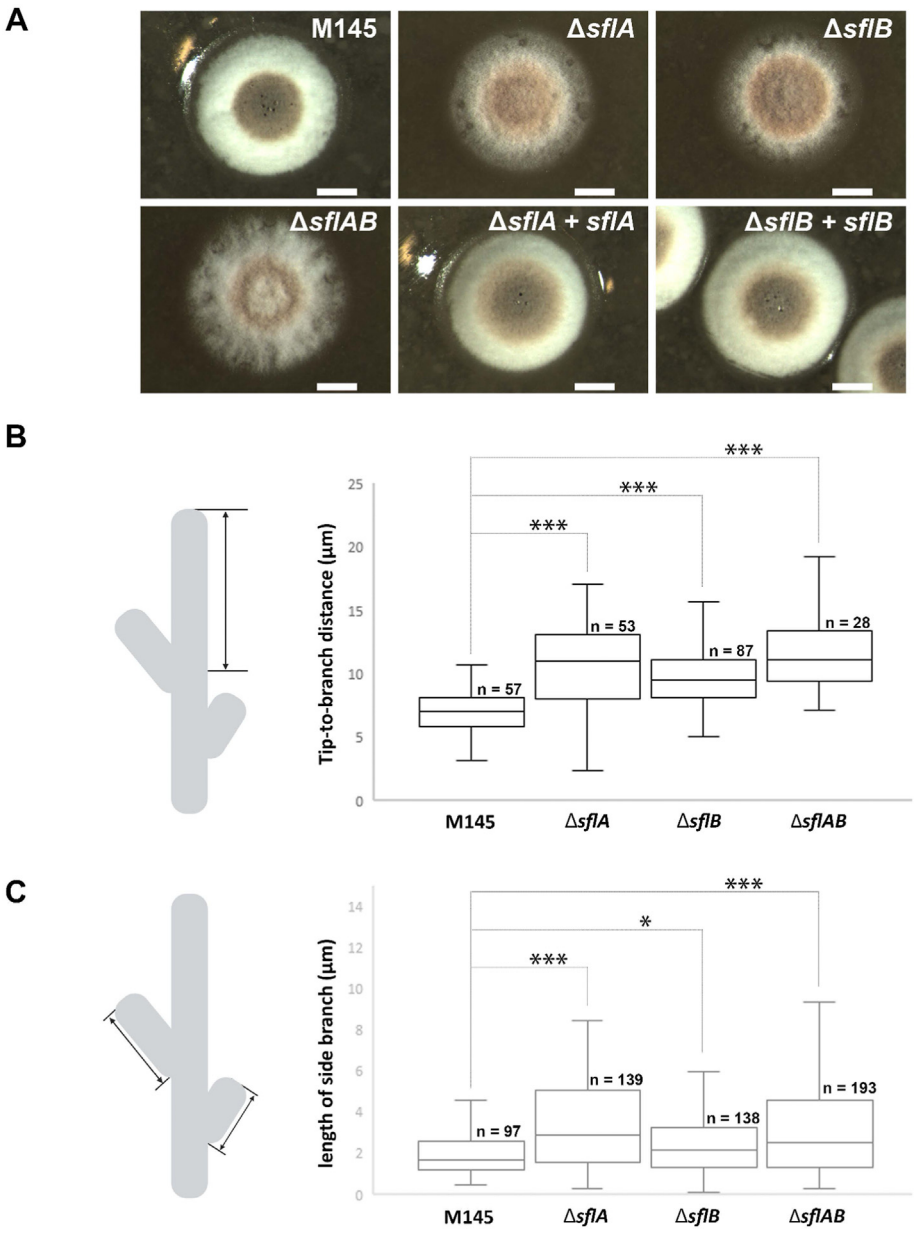

Fig. 1. Phenotypic analysis of *sfl* mutants. (A) Stereomicrographs of representative colonies of *S. coelicolor* M145, its *sflA* and *sflB* null mutants and the genetically complemented strains. Strains were grown on SFM agar plates for three days at 30 °C. Note that colonies of *sfl* mutants were 'fluffier' than those of the parental strain M145, and expression of wild-type SflA or SflB restored smooth colony edge to the corresponding mutants. Bar, 1 mm. (B, C) Boxplots showing tip-to-branch distance **(B)** and side branch length **(C)** of wild-type hyphae and those of *sflA*, *sflB* and *sflAB* mutants after 10 h of growth. Data are presented as median and interquartile range in the boxplots, with whiskers spread to the maximal and minimal values. The number (n) of hyphae measured for each strain are indicated. The two-tailed P values were calculated using a student's *t*-test. The two-tailed P values (*, $p < 0.05$; ****, $p < 0.001$) show that the tip-to-branch distance and side branch length of the mutant hyphae deviated significantly from those of wild-type hyphae.

vegetative hyphae that had been grown for 10 h (Fig. 1B &C). The average tip-to-branch distance was 7.12 ± 2.00 μm in the parental strain M145, while it had increased significantly in *sflA*, *sflB* and *sflAB* mutants, to 10.43 ± 3.46 μm, 9.80 ± 3.04 μm and 10.53 ± 3.27 μm, respectively (Fig. 1B). The average side branch length was 1.99 ± 1.09 μm in wild-type hyphae, which increased to 3.45 ± 2.41 μm, 2.41 ± 1.45 μm and 3.32 ± 2.56 μm for *sflA*, *sflB* and *sflAB* mutants, respectively (Fig. 1C). The longer tip-to-branch distance and side branch in *sfl* mutants - and thus reduced compactness of the mycelia - may explain the altered colony morphology of *sfl* mutants.

Conversely, knockdown of *sepF* resulted in severe developmental defects, overproduction of actinorhodin and largely reduced cross wall formation (Fig. S3), which was very similar to that reported for *ftsZ* null mutants [14], in line with the expected crucial role of SepF in Z-ring formation in *S. coelicolor*. The severe phenotype of the *sepF* knock-down mutants suggests that *sflA* and *sflB* cannot functionally compensate for the lack of *sepF*.

3.3. Aerial hyphae of sflA and sflB null mutants show unusual branching

Surface-grown *S. coelicolor* M145 and its *sflA*, *sflB* and *sflAB* mutants were analyzed in more detail by cryo-scanning electron microscopy (SEM). After three days of growth, *S. coelicolor* M145 produced abundant and regular spore chains (Fig. 2A). However,

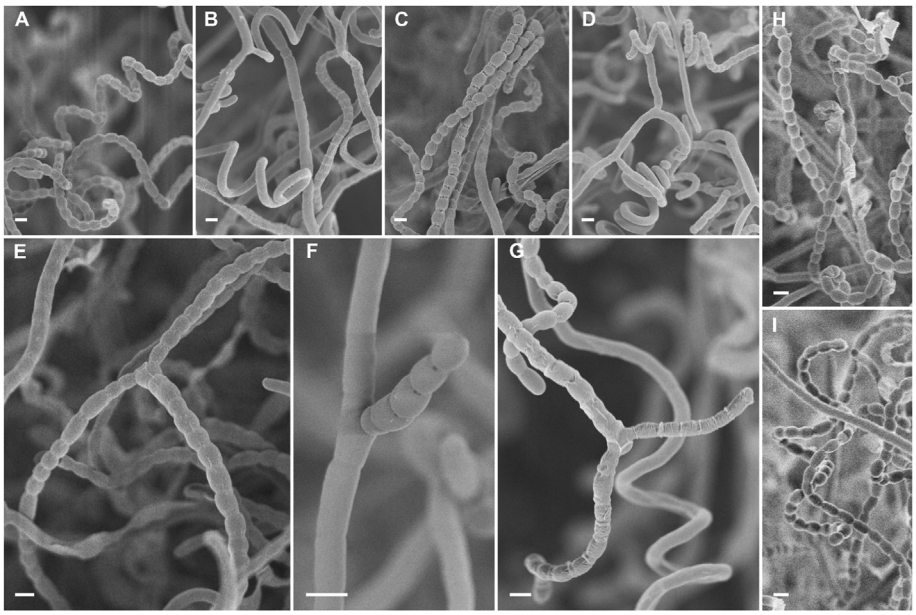

Fig. 2. Cryo-scanning electron micrographs of spore chains of *S. coelicolor* M145, its *sfl* mutants and complemented *sfl* mutants. Wild-type *S. coelicolor* M145 **(A)** sporulated abundantly after three days of incubation, while mutants lacking either *sflA* **(B & E)** or *sflAB* **(D & G)** showed 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, while spore chains in genetically complemented *sflA* **(H)** and complemented *sflB* **(I)** did not show any branching. Cultures were grown on SFM agar plates for 5 days at 30 °C. Bar, 1 μm.

strains lacking *sflA* (Δ*sflA* and Δ*sflAB*) produced fewer spore chains (Fig. 2B & D), while deletion of only *sflB* did not significantly affect sporulation (Fig. 2C). Strikingly, sporogenic aerial hyphae of *sflA*, *sflB* and *sflAB* null mutants branched frequently (Fig. 2E–G), a phenotype that was never seen in the wild-type strain. Introduction of wild-type *sflA* or *sflB* largely complemented the mutant

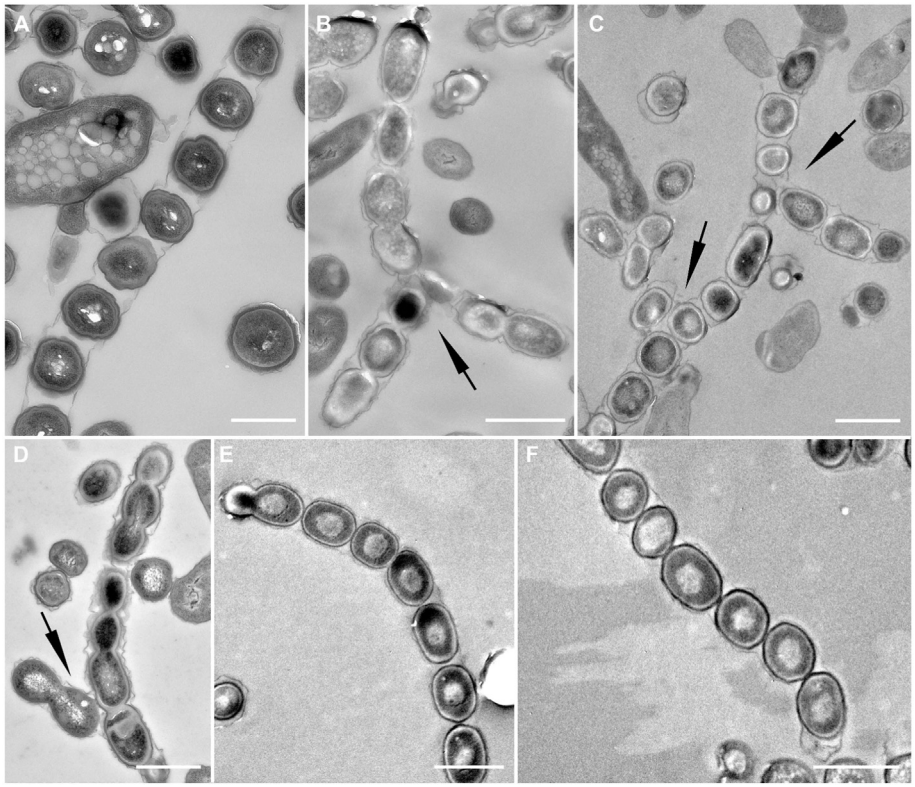

Fig. 3. Transmission electron micrographs of spores chains of *S. coelicolor* M145, its *sfl* mutants and complemented *sfl* mutants. Spore chains of the parental strain M145 **(A)** did not branch and contained regularly sized spores. Mutants lacking either *sflA* **(B)**, *sflB* **(C)** or both **(D)** produced irregular spores and spore chains frequently branched, which corresponds well to the SEM images (Fig. 3). Genetically complemented *sflA* **(E)** or *sflB* **(F)** mutants produced unbranched spore chains similar to the parental strain. Cultures were grown on SFM agar plates for 5 days at 30 °C. Arrows indicate branching points of the spore chains. Bar, 1 μm.

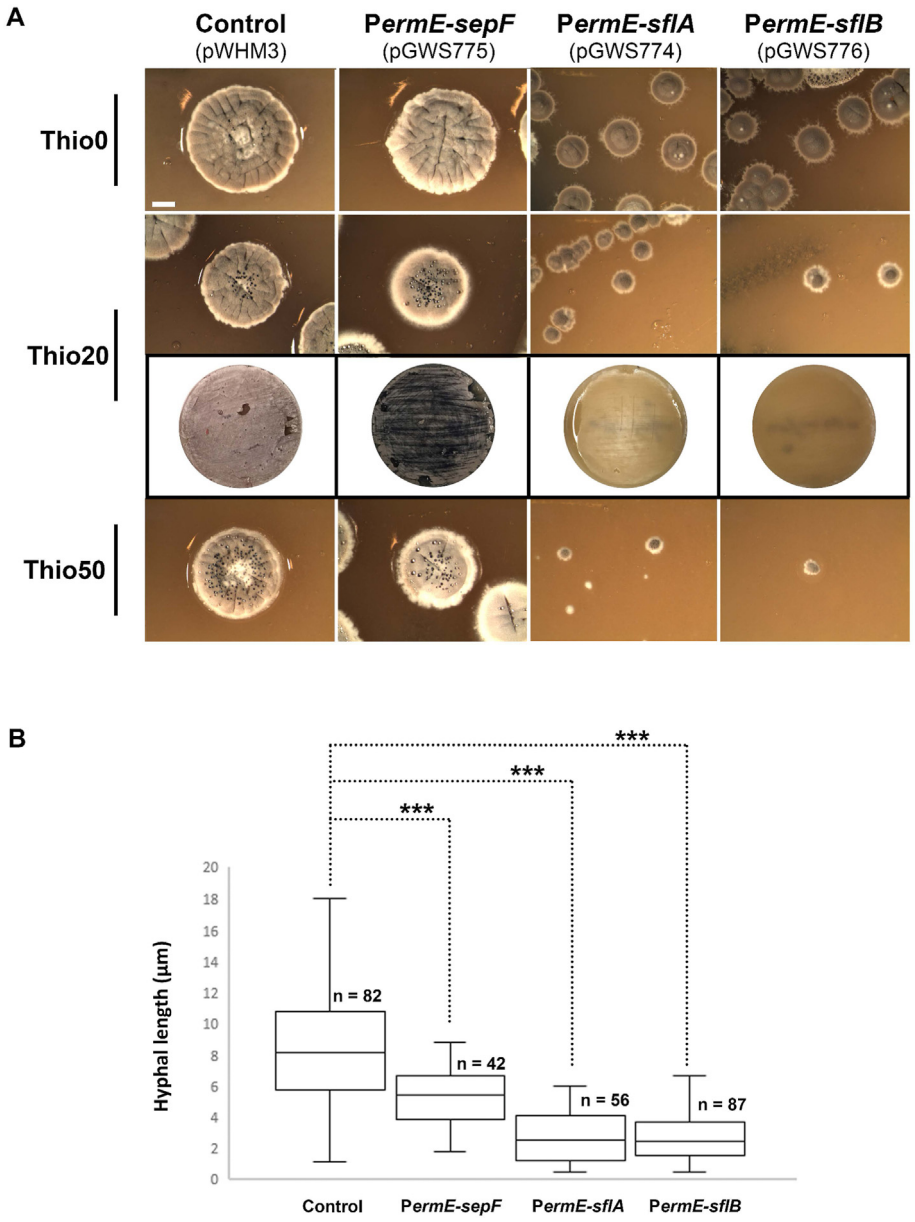

Fig. 4. Effect of enhanced expression of *sepF* and *sfl* genes on colony morphology. (A) Stereomicrographs of GAL70 (*S. coelicolor* M145 + empty plasmid pWHM3), GAL44 (M145 + pGWS774, expressing *sflA*), GAL45 (M145 + pGWS775, expressing *sepF*) and GAL46 (M145 + pGWS776, expressing *sflB*) were grown on SFM agar containing different concentrations of thiostrepton (0–50 mg/ml). Plates were incubated for 7 days at 30 °C. Over-expression of *sflA* or *sflB* resulted in tiny colonies, whereby colonies could be entirely lifted from the agar surface, suggestive of loss of attachment; overexpression of *sepF* did not affect colony size or adherence. Bar, 2 mm. **(B)** Measurement of hyphal length. Data are presented as median and interquartile range in boxplots, with whiskers spread to the maximal and minimal values. The numbers (n) of hyphae measured for each strain are indicated. The shown two-tailed P values between each mutant and the parental strain were calculated using student's *t*-test. The two-tailed P values were far below 0.001(***), which shows that the length of the mutant hyphae deviated significantly from those of wild-type hyphae.

phenotypes, and prevented branching (Fig. 2H–I). Some variability in spore sizes was still observed, perhaps as the result of a difference in expression level of the proteins from the chromosomal and from the plasmid-borne genes.

Transmission electron microscopy (TEM) was used to image thin sections at high resolution. This again revealed branching spore chains in *sflA* and *sflB* mutants (Fig. 3, arrows) and variation in spore sizes. Furthermore, while wild-type spores had a typical dark (electron-dense) spore wall and well-condensed DNA, the spores of the mutants typically had lighter (electron-lucent) spore walls as well as less clearly visible DNA in many of the spores (Fig. 3B–D). This suggests pleiotropic changes in spore morphogenesis and maturation in *sfl* genes mutants. As was already apparent from the SEM imaging, introduction of *sflA* and *sflB* into *sflA* and *sflB* null mutants, respectively, prevented branching of the spore chains, although the spore walls were still relatively thin (Fig. 3E–F).

3.4. 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, which encompasses a strong constitutive promoter and 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. pWHM3 is an unstable plasmid that is easily lost and its copy number largely depends on the level of thiostrepton [39]. The thiostrepton concentration controls the copy number of pWHM3, with copy number proportional to the thiostrepton concentration.

Plasmids pGWS774 (expressing *sflA*), pGWS775 (*sepF*), pGWS776 (*sflB*) or control plasmid pWHM3 without insert were introduced into *S. coelicolor* M145. The transformants were then plated onto SFM agar plates with different concentrations of thiostrepton and the colony morphology investigated after 7 days of incubation (Fig. 4A). On SFM media, even in the absence of thiostrepton, colonies overexpressing SflA (GAL44) or SflB (GAL46) were smaller than those of transformants harboring the empty plasmid (GAL70) or transformants over-expressing SepF (GAL45) (Fig. 4A). In the presence of thiostrepton (20 μg/mL), the size of colonies over-expressing SflA or SflB were reduced further. Interestingly, spores of SflA- and SflB-overexpressing strains could be easily removed from the plates with a toothpick, leaving "clean" plates, suggesting they had lost the ability to attach to and invade into the agar surface (Fig. 4A, third row). Conversely, SepF-overexpressing colonies still grew into agar, and the mycelia remained firmly attached to the plates (Fig. 4A, third row). When the thiostrepton concentration was increased further to 50 μg/mL, colonies of transformants with SflA or SflB expression constructs were very tiny and irregularly shaped, while those with control plasmid or harboring the SepF expression construct were barely affected (Fig. 4A). On R5 agar plates, similar tiny colonies were observed for SflA and SflB-overexpressing strains, whereby the colonies more or less 'floated' on the agar surface, showing severe developmental defect (Fig. S4).

To see if growth of the hyphae was affected, we analyzed young vegetative hyphae (grown for 9 h). Interestingly, the hyphal length of control transformants carrying empty pWHM3 was 8.23 ± 3.57, while SflA- and SflB-overexpressing strains had a distance from germination site to hyphae tip of only 2.70 ± 1.59 μm and 2.70 ± 1.60 μm, respectively (Fig. 4B). The hyphal length of SepF-overexpressing colonies was less reduced, reaching on average 5.30 ± 1.70 μm (Fig. 4B). Taken together, we conclude that *sflA* or *sflB*, and to a lesser extent *sepF*, play a role in the control of tip growth.

3.5. Altered localization of DivIVA and FtsZ in sflA and sflB mutants

Streptomycetes grow via extension of the hyphal tip, although the molecular mechanism of polar growth is still largely unknown [5]. DivIVA is required for tip growth, whereby it localizes at apical sites and at new branches [12,40]. Therefore, DivIVA is an excellent marker for active tip growth, which was used to study the onset of branching in wild-type and mutant hyphae. Construct pGWS800, harboring *S. venezuelae divIVA-egfp* under the control of its native promoter, was introduced into *sflA* and *sflB* null mutants. In wild-type cells, DivIVA-eGFP accumulated at tips of aerial hyphae, with 93% of the foci observed at apical sites (n = 45). In aerial hyphae of *sflA* and *sflB* null mutants, DivIVA-eGFP foci were more widely distributed, not only at apical sites, but also along hyphae at the places without apparent branching, suggesting the emergence of new branching points (Fig. 5A). In *sflA* and *sflB* mutants, 21% (n = 56) and 64% (n = 99) of the DivIVA-eGFP signals were observed along the lateral wall, respectively. Strikingly, DivIVA-eGFP localized abundantly in maturing spore chains of *sflA* and *sflB* mutants, while in wild-type spore chains no DivIVA-eGFP was observed (Fig. 5A). The ectopic localization of DivIVA-eGFP in the absence of *sflA* or *sflB* suggests that their gene products play a role in the control of DivIVA localization and hence in determining apical growth of the hyphae in *Streptomyces*. This is consistent with the functional correlation of SflAB with tip growth and hyphal length (see above).

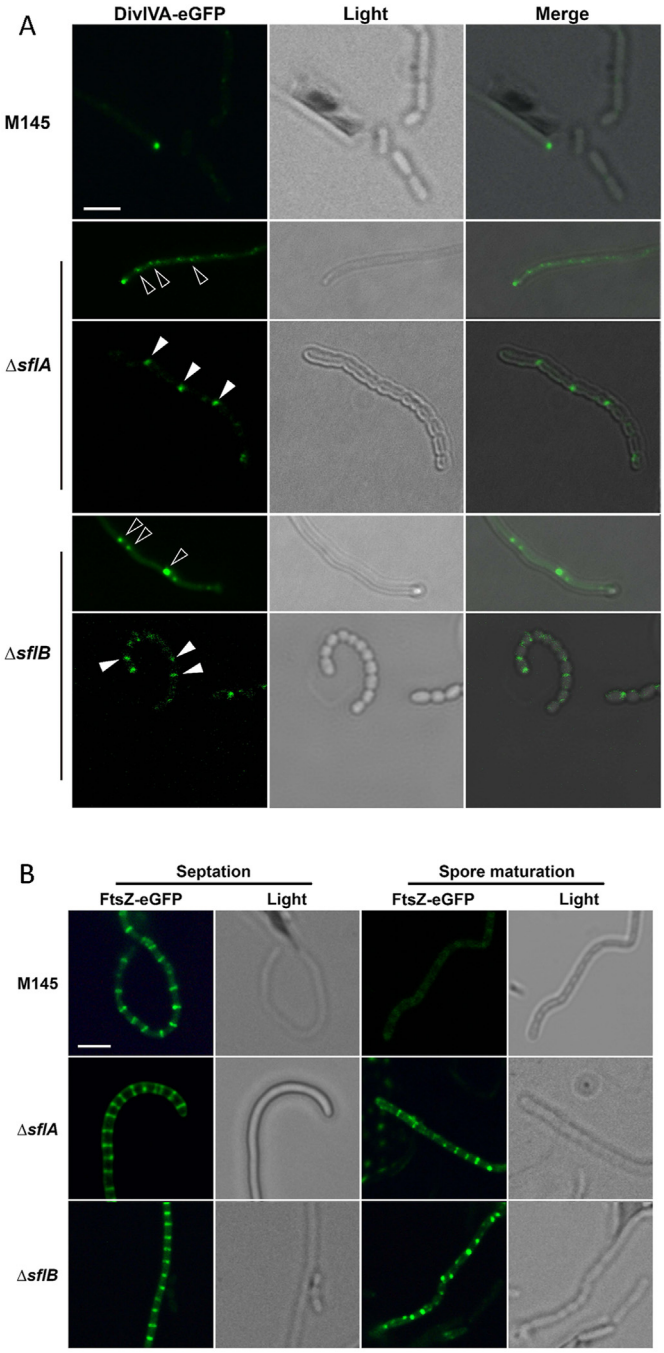

Fig. 5. Localization of DivIVA and FtsZ in *S. coelicolor* and its *sfl* null mutants. A. Localization of DivIVA. In wild-type aerial hyphae, DivIVA localized mainly at the tips, while the protein was more dispersed in *sfl* mutants (indicated with empty arrow heads). DivIVA-eGFP could not be detected in mature spore chains of wild-type cells, but DivIVA-eGFP foci were frequently seen in mature spore chains *sfl* mutants (indicated with filled arrow heads). This likely explains the branching of the aerial hyphae seen in Figs. 3 and 4. Bar, 2 μm. **B. Localization of FtsZ.** In the parental strain, FtsZ forms ladder-like structures in sporogenic aerial hyphae and disappears once septation is completed. Conversely, in *sfl* mutants FtsZ ladders persist, even in mature spore chains. Bar, 2 μm.

To establish how FtsZ localizes in *sfl* mutants, construct pKF41 expressing FtsZ-eGFP [19] was introduced into *S. coelicolor* and its *sflA*, *sflB* and *sflAB* mutants. In sporogenic aerial hyphae, FtsZ formed

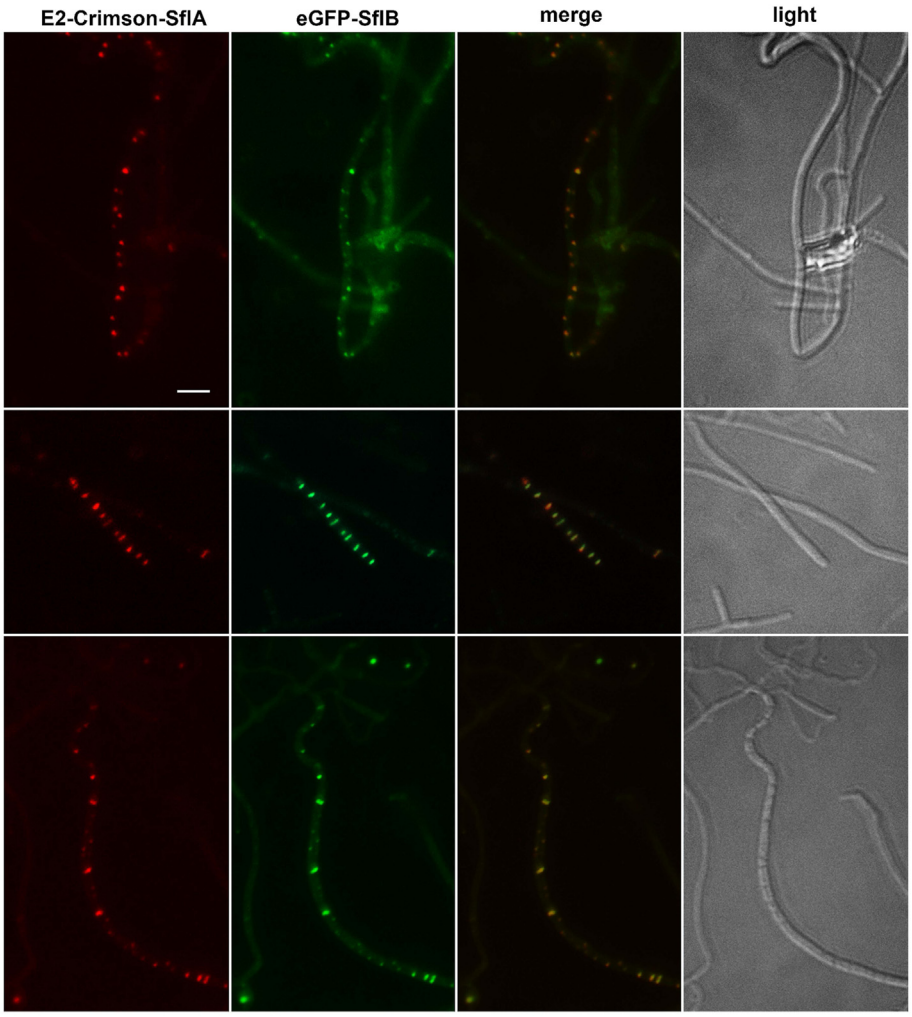

Fig. 6. Colocalization of SflA and SflB in *S. coelicolor*. Sporogenic aerial hyphae of *S. coelicolor* M145 were imaged by fluorescence microscopy visualizing the E2-Crimson, dTomato or eGFP fusion proteins and corresponding light micrograph. E2-Crimson-SflA and eGFP-SflB localized in a dynamic pattern and colocalized with each other along development. They formed random bright foci in early aerial hyphae and developed into regular-spaced ladder-like structure in late aerial hyphae. In pre-mature spore chains, SflA and SflB were often observed in-between spores. Bar, 2 μm.

typical ladder-like patterns in all strains. Canonical Z-ladders were formed in *sfl* null mutants, although occasional misplaced septa were seen in *sflA* null mutants (Fig. 5B, left). However, while FtsZ foci and rings disassembled and were absent in mature spore chains of wild-type *S. coelicolor*, they persisted in late sporogenic aerial hyphae of *sflA* and *sflB* mutants (Fig. 5B, right). Prolonged Z-rings were observed in 46% (n = 108), 28% (n = 46) and 72% (n = 39) of the premature spores of *sflA*, *sflB* and *sflAB* mutants, respectively, while they were not seen in wild-type spores. This corresponds very well to the ratios of incomplete septa in non-separated spores, which were 68% and 13% for *sflA* and *sflB* mutants, respectively, 79% for *sflAB* mutant and only 1% for the parent *S. coelicolor* M145 (more than 150 spores were examined for each strain). Taken together, the ectopic and continued localization of DivIVA and FtsZ in *sfl* null mutants throughout sporulation strongly suggests that SflA and SflB play an important role in controlling the dynamics of apical growth and cell division during *Streptomyces* development, and in particular ensure timely disassembly of DivIVA and FtsZ foci.

3.6. Localization of SflA and SflB in S. coelicolor

To analyze the localization of the SflA and SflB, constructs were created in the integrative vector pSET152 or multi-copy vector pHJL401 containing either paralogue fused in frame behind *egfp or E2-Crimson* expressed from the *ftsZ* promoter region (see Materials and Methods).

In young aerial hyphae, both SflA and SflB localized as foci and the two proteins colocalized (Fig. 6, top row). Eventually, SflA and SflB colocalized in a ladder-like pattern during later aerial development, shown as widening of the hyphae (Fig. 6, middle row). During spore maturation, SflA and SflB again co-localized at invagination sites along the hyphae (Fig. 6, bottom row). Since SflA and SflB colocalized during aerial growth and sporulation, we investigated their interaction *in vitro*. Indeed, Sfl proteins showed clear interaction between each other in bacterial two-hybrid experiment (Fig. S5). Additionally, Sfl proteins also showed interaction with SepF (Fig. S5), which led us to study their colocalization. In most of the cases, SflA and SflB colocalized with each while didn't colocalize with SepF. However, colocalization between SepF and Sfl

proteins were also observed on occasions when they formed ring-like structures in aerial hyphae (Fig. S6). Consistently, in *S. venezuelae*, both SepF and SflB (named SepF2 in *S. venezuelae*) colocalized with FtsZ, which indirectly confirmed the colocalization between SepF and SflB [41].

4. Discussion

A major question in the developmental biology of *Streptomyces* that we seek to address is, how do *Streptomyces* ensure that septa are controlled in time and space in the long and multinucleoid hyphae? In this work we present a new piece of this jigsaw, which points at the possible existence of a layer of negative control during *Streptomyces* sporulation, revolving around the SepF-like proteins SflA and SflB.

The most obvious change in morphogenesis due to the deletion of either *sflA* or *sflB* was the extensive branching of the aerial hyphae and in particular of spore chains, which we have never seen in any of our wild-type streptomycetes. Conversely, constitutive expression of SflA and SflB from the *ermE* promoter inhibited growth and reduced adhesion of the colonies to the agar surface, with as possible explanation that tip extension and hence also branching is impaired in the vegetative mycelium. These data suggest an inverse correlation between the expression level of SflA and SflB and polarisome activity. Indeed, we found mislocalization of DivIVA in *sflA* and *sflB* null mutants, with many foci along the lateral wall of aerial hyphae, instead of only apical localization. While in wild-type hyphae virtually all DivIVA-eGFP foci were located at the apex, in total 21% and 64% of the foci were observed along the lateral wall in *sflA* and *sflB* mutants, respectively. Since DivIVA drives tip growth and thus also branching, this likely explains the observed branching spore chains frequently observed in *sfl* null mutants. The inhibition of growth following the constitutive expression of SflA or SflB in vegetative hyphae suggests that expression of these proteins throughout the life cycle directly or indirectly inhibits DivIVA during vegetative growth, which will then result in growth inhibition, as DivIVA is essential for tip growth.

Typical ladders of Z-rings were produced in young sporogenic aerial hyphae of both wild-type *S. coelicolor* and in *sflA* or *sflB* null mutants, though the distance between adjacent Z-rings in mutants varied more in the mutants. Importantly, besides for DivIVA, we also noticed strongly prologued and ectopic localization of FtsZ in mutants lacking *sflA* and/or *sflB*. While Z-ladders disappeared in mature spore chains of the parental strain *S. coelicolor* M145, ladders and foci persisted in the different *sfl* mutants during spore maturation, strongly suggesting that either the septa had not yet been completed or that disassembly of the FtsZ polymers was compromised. SflA and SflB reappeared at the interface between adjacent spores, perhaps to allow the disassembly of SepF, and hence destabilization of the Z-rings.

The phenotypes of the *sflA* and *sflB* mutants suggest that the proteins play a role in Z-ring disassembly. Their colocalization during aerial development thereby indicates that SflAB do so as a complex. SflB lacks the N-terminal α-helix that is required for membrane lipid binding [27], suggesting that SflB requires SflA for membrane-specific localization. One model to explain the observed phenotypes is that SflAB compete with SepF itself, or stimulate the depolymerization of SepF polymers after completion of cell division. The localization of DivIVA was also disturbed in the aerial hyphae. While DivIVA is known to interact with a range of different protein partners, no interaction between DivIVA and either SepF or FtsZ has so far been reported [42]. Interestingly, DivIVA homologue GpsB interacts with SepF in *Listeria monocytogenes* [43], while in *Staphylococcus aureus* GpsB was shown to interact with FtsZ to stimulate the formation of FtsZ bundles [44]. Biochemical experiments are required to establish how SflA and SflB affect the localization and/or polymerization of SepF, FtsZ and DivIVA.

Taken together, our work shows that SflAB control growth and cell division of the aerial hyphae of *Streptomyces*. Over-expression of the proteins inhibits growth of the colonies, while in the absence of *sflA* and/or *sflB* DivIVA localizes ectopically, resulting in unusual branching of aerial hyphae. In the absence of SflAB, Z-rings and foci persist in mature spore chains. Thus SflAB play a role in ensuring the correct localization of key cell division proteins in time and space during sporulation-specific cell division of Streptomyces.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gilles van Wezel reports financial support was provided by Netherlands Organisation for Applied Scientific Research. Gilles van Wezel reports a relationship with Netherlands Organisation for Applied Scientific Research that includes: funding grants.

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Appendix A. Supplementary data

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References

[1] D. Claessen, D.E. Rozen, O.P. Kuipers, L. Sogaard-Andersen, G.P. van Wezel, Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies, Nat. Rev. Microbiol. 12 (2014) 115–124.
[2] K. Flärdh, M.J. Buttner, *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium, Nat. Rev. Microbiol. 7 (2009) 36–49.
[3] E.A. Barka, P. Vatsa, L. Sanchez, N. Gavaut-Vaillant, C. Jacquard, H.P. Klenk, C. Clément, Y. Oudouch, G.P. van Wezel, Taxonomy, physiology, and natural products of the *Actinobacteria*, Microbiol. Mol. Biol. Rev. 80 (2016) 1–43.
[4] D.A. Hopwood, *Streptomyces* in Nature and Medicine: the Antibiotic Makers, Oxford University Press, New York, 2007.
[5] D. Jakimowicz, G.P. van Wezel, Cell division and DNA segregation in *Streptomyces*: how to build a septum in the middle of nowhere? Mol. Microbiol. 85 (2012) 393–404.
[6] J.R. McCormick, Cell division is dispensable but not irrelevant in *Streptomyces*, Curr. Opin. Biotechnol. 12 (2009) 689–698.
[7] S. Bagchi, H. Tomenius, L.M. Belova, N. Ausmees, Intermediate filament-like proteins in bacteria and a cytoskeletal function in *Streptomyces*, Mol. Microbiol. 70 (2008) 1037–1050.
[8] K. Celler, R.I. Koning, A.J. Koster, G.P. van Wezel, Multidimensional view of the bacterial cytoskeleton, J. Bacteriol. 195 (2013) 1627–1636.
[9] M. Vicente, J. Errington, Structure, function and controls in microbial division, Mol. Microbiol. 20 (1996) 1–7.
[10] J. Tamames, M. González-Moreno, J. Mingorance, A. Valencia, M. Vicente, Bringing gene order into bacterial shape, Trends Genet. 17 (2001) 124–126.
[11] A.L. Marston, H.B. Thomaides, D.H. Edwards, M.E. Sharpe, J. Errington, Polar localization of the MinD protein of *Bacillus subtilis* and its role in selection of the mid-cell division site, Genes Dev. 12 (1998) 3419–3430.
[12] K. Flärdh, Essential role of DivIVA in polar growth and morphogenesis in *Streptomyces coelicolor* A3(2), Mol. Microbiol. 49 (2003) 1523–1536.
[13] M. Letek, E. Ordonez, J. Vaquera, W. Margolin, K. Flardh, L.M. Mateos, J.A. Gil, DivIVA is required for polar growth in the MreB-lacking rod-shaped actinomycete *Corynebacterium glutamicum*, J. Bacteriol. 190 (2008) 3283–3292.
[14] J.R. McCormick, E.P. Su, A. Driks, R. Losick, Growth and viability of *Streptomyces coelicolor* mutant for the cell division gene *ftsZ*, Mol. Microbiol. 14 (1994) 243–254.
[15] B.V. Mistry, R. Del Sol, C. Wright, K. Findlay, P. Dyson, FtsW is a dispensable cell division protein required for Z-ring stabilization during sporulation septation in *Streptomyces coelicolor*, J. Bacteriol. 190 (2008) 5555–5566.
[16] P. Yagüe, J. Willemse, R.I. Koning, B. Rioseras, M.T. Lopez-Garcia, N. Gonzalez-Quinonez, C. Lopez-Iglesias, P.V. Shliaha, A. Rogowska-Wrzesinska, A.J. Koster, O.N. Jensen, G.P. van Wezel, A. Manteca, Subcompartmentalization by cross-membranes during early growth of *Streptomyces* hyphae, Nat. Commun. 7

(2016), 12467.
[17] K. Celler, R.I. Koning, J. Willemse, A.J. Koster, G.P. van Wezel, Cross-membranes orchestrate compartmentalization and morphogenesis in *Streptomyces*, Nat. Commun. (2016) in press.
[18] M.J. Bush, K.A. Gallagher, G. Chandra, K.C. Findlay, S. Schlimpert, Hyphal compartmentalization and sporulation in *Streptomyces* require the conserved cell division protein SepX, Nat. Commun. 13 (2022) 71.
[19] N. Grantcharova, U. Lustig, K. Flärdh, Dynamics of FtsZ assembly during sporulation in *Streptomyces coelicolor* A3(2), J. Bacteriol. 187 (2005) 3227–3237.
[20] J. Willemse, J.W. Borst, E. de Waal, T. Bisseling, G.P. van Wezel, Positive control of cell division: FtsZ is recruited by SsgB during sporulation of *Streptomyces*, Genes Dev. 25 (2011) 89–99.
[21] B.A. Traag, G.P. van Wezel, The SsgA-like proteins in actinomycetes: small proteins up to a big task, Antonie Leeuwenhoek 94 (2008) 85–97.
[22] G.P. van Wezel, J. van der Meulen, S. Kawamoto, R.G. Luiten, H.K. Koerten, B. Kraal, *ssgA* is essential for sporulation of *Streptomyces coelicolor* A3(2) and affects hyphal development by stimulating septum formation, J. Bacteriol. 182 (2000) 5653–5662.
[23] S. Kawamoto, H. Watanabe, A. Hesketh, J.C. Ensign, K. Ochi, Expression analysis of the *ssgA* gene product, associated with sporulation and cell division in *Streptomyces griseus*, Microbiology 143 (1997) 1077–1086.
[24] L. Zhang, J. Willemse, D. Claessen, G.P. van Wezel, SepG coordinates sporulation-specific cell division and nucleoid organization in *Streptomyces coelicolor*, open biology 6 (2016), 150164.
[25] S. Ishikawa, Y. Kawai, K. Hiramatsu, M. Kuwano, N. Ogasawara, A new FtsZ-interacting protein, YlmF, complements the activity of FtsA during progression of cell division in *Bacillus subtilis*, Mol. Microbiol. 60 (2006) 1364–1380.
[26] L.W. Hamoen, J.-C. Meile, W. de Jong, P. Noirot, J. Errington, SepF, a novel FtsZ-interacting protein required for a late step in cell division, Mol. Microbiol. 59 (2006) 989–999.
[27] R. Duman, S. Ishikawa, I. Celik, H. Strahl, N. Ogasawara, P. Troc, J. Löwe, L.W. Hamoen, Structural and genetic analyses reveal the protein SepF as a new membrane anchor for the Z ring, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) E4601–E4610.
[28] S. Gola, T. Munder, S. Casonato, R. Manganelli, M. Vicente, The essential role of SepF in mycobacterial division, Mol. Microbiol. 97 (2015) 560–576.
[29] S. Gupta, S.K. Banerjee, A. Chatterjee, A.K. Sharma, M. Kundu, J. Basu, Essential protein SepF of mycobacteria interacts with FtsZ and MurG to regulate cell growth and division, Microbiology 161 (2015) 1627–1638.
[30] J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual, 1989.
[31] D.J. MacNeil, K.M. Gewain, C.L. Ruby, G. Dezeny, P.H. Gibbons, T. Maeneil, Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector, Gene 111 (1992) 61–68.
[32] T. Kieser, M.J. Bibb, M.J. Buttner, K.F. Chater, D.A. Hopwood, Practical *Streptomyces* Genetics, 2000.
[33] J. Willemse, G.P. van Wezel, Imaging of *Streptomyces coelicolor* A3(2) with reduced autofluorescence reveals a novel stage of FtsZ localization, PLoS One 4 (2009), e4242.
[34] S. Colson, G.P. van Wezel, M. Craig, E.E.E. Noens, H. Nothaft, A.M. Mommaas, F. Titgemeyer, B. Joris, S. Rigali, The chitobiose-binding protein, DasA, acts as a link between chitin utilization and morphogenesis in Streptomyces coelicolor, Microbiology 154 (2008) 373–382.
[35] A. Piette, A. Derouaux, P. Gerkens, E.E. Noens, G. Mazzucchelli, S. Vion, H.K. Koerten, F. Titgemeyer, E. De Pauw, P. Leprince, G.P. van Wezel, M. Galleni, S. Rigali, From dormant to germinating spores of *Streptomyces coelicolor* A3(2): new perspectives from the crp null mutant, J. Proteome Res. 4 (2005) 1699–1708.
[36] C. Cole, J.D. Barber, G.J. Barton, The Jpred 3 secondary structure prediction server, Nucleic Acids Res. 36 (2008) W197–W201.
[37] M.E. Gundogdu, Y. Kawai, N. Pavlendova, N. Ogasawara, J. Errington, D.J. Scheffers, L.W. Hamoen, Large ring polymers align FtsZ polymers for normal septum formation, EMBO J. 30 (2011) 617–626.
[38] M.A. Swiatek, E. Tenconi, S. Rigali, G.P. van Wezel, Functional analysis of the N-acetylglucosamine metabolic genes of *Streptomyces coelicolor* and role in the control of development and antibiotic production, J. Bacteriol. 194 (2012) 1136–1144.
[39] G.P. van Wezel, K. Mahr, M. Konig, B.A. Traag, E.F. Pimentel-Schmitt, A. Willimek, F. Titgemeyer, GlcP constitutes the major glucose uptake system of *Streptomyces coelicolor* A3(2), Mol. Microbiol. 55 (2005) 624–636.
[40] A.M. Hempel, S.B. Wang, M. Letek, J.A. Gil, K. Flardh, Assemblies of DivIVA mark sites for hyphal branching and can establish new zones of cell wall growth in *Streptomyces coelicolor*, J. Bacteriol. 190 (2008) 7579–7583.
[41] S. Schlimpert, S. Wasserstrom, G. Chandra, M.J. Bibb, K.C. Findlay, K. Flardh, M.J. Buttner, Two dynamin-like proteins stabilize FtsZ rings during Streptomyces sporulation, P Natl Acad Sci USA 114 (2017) E6176–E6183.
[42] S. Halbedel, R.J. Lewis, Structural basis for interaction of DivIVA/GpsB proteins with their ligands, Mol. Microbiol. 111 (2019) 1404–1415.
[43] R.M. Cleverley, Z.J. Rutter, J. Rismondo, F. Corona, H.T. Tsui, F.A. Alatawi, R.A. Daniel, S. Halbedel, O. Massidda, M.E. Winkler, R.J. Lewis, The cell cycle regulator GpsB functions as cytosolic adaptor for multiple cell wall enzymes, Nat. Commun. 10 (2019) 261.
[44] P.J. Eswara, R.S. Brzozowski, M.G. Viola, G. Graham, C. Spanoudis, C. Trebino, J. Jha, J.I. Aubee, K.M. Thompson, J.L. Camberg, K.S. Ramamurthi, An essential *Staphylococcus aureus* cell division protein directly regulates FtsZ dynamics, Elife 7 (2018).