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Le Zhang, Joost Willemse, Paula Yagüe, E.C. de Waal ...+2 more authors

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Branching of sporogenic aerial hyphae in *sfIA* and *sfIB* mutants of *Streptomyces coelicolor* correlates to ectopic localization of DivIVA and FtsZ in time and space

Le Zhang¹, Joost Willemse¹, Paula Yagüe², Ellen de Waal¹, Dennis Claessen¹ and Gilles P. van Wezel^{1, #}

¹Department of Molecular Biotechnology, Institute of Biology Leiden, Leiden University, PO Box 9505, Leiden, 2300 AB, The Netherlands.

²Departamento de Biología Funcional e IUOPA, Área de Microbiología, Facultad de Medicina, Universidad de Oviedo, Oviedo, 33006, Spain.

Author for Correspondence: g.wezel@biology.leidenuniv.nl; Tel: +31 71 5274310

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18 **ABSTRACT**

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

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39 INTRODUCTION

40 Streptomyces are multicellular mycelial bacteria that reproduce via sporulation ([Claessen](#)
41 [et al., 2014](#); [Flårdh and Buttner, 2009](#)). As producers of half of all known antibiotics as well
42 as many anticancer, antifungal and immunosuppressant compounds, streptomyces are of
43 great medical and biotechnological importance ([Barka et al., 2016](#); [Hopwood, 2007](#)). The
44 mycelial life style of streptomyces imposes specific requirements for the control of growth
45 and cell division ([Jakimowicz and van Wezel, 2012](#); [McCormick, 2009](#)), and they have an
46 unusually complex cytoskeleton ([Bagchi et al., 2008](#); [Celler et al., 2013](#)).

47 The *dcw* gene cluster contains various genes required for division and cell wall
48 synthesis ([Tamames et al., 2001](#); [Vicente and Errington, 1996](#)). Some genes in this cluster
49 have gained species-specific functions. An obvious example is DivIVA, which in *Bacillus*
50 *subtilis* is involved in division-site localization by preventing accumulation of the cell division
51 scaffold protein FtsZ ([Marston et al., 1998b](#)), while in Actinobacteria DivIVA is required for
52 apical growth ([Flårdh, 2003](#)). As a consequence, *divIVA* is dispensable in *B. subtilis* but
53 essential for growth in Actinobacteria ([Flårdh, 2003](#); [Letek et al., 2008](#)). Conversely, *ftsZ* is
54 essential in *B. subtilis*, but is not required for normal growth of Actinobacteria ([McCormick et](#)
55 [al., 1994](#)).

56 The control of cell division is radically different between the mycelial streptomyces
57 and the planktonic *Bacillus subtilis*, which is perhaps not surprising due to the absence of a
58 defined mid-cell position in the long hyphae of streptomyces. In rod-shaped bacteria,
59 many proteins have been identified that assist in septum-site localization, such as FtsA and
60 ZipA ([Hale and de Boer, 1997](#); [Pichoff and Lutkenhaus, 2002](#); [RayChaudhuri, 1999](#)) and ZapA
61 ([Gueiros-Filho and Losick, 2002](#)). Septum-site localization is negatively controlled, via the
62 action of Min, which prevents Z-ring assembly away from mid-cell ([Marston et al., 1998a](#);

63 [Raskin and de Boer, 1997](#)), and by nucleoid occlusion that prevents formation of the Z-ring
64 over non-segregated chromosomes ([Bernhardt and de Boer, 2005](#); [Woldringh et al., 1991](#);
65 [Wu and Errington, 2004, 2012](#)). Direct homologs of any of these control proteins are missing
66 in streptomycetes.

67 Streptomycetes have two different mechanisms of cell division. During vegetative
68 growth, divisome-independent cell division occurs, whereby occasional cross-walls separate
69 the vegetative hyphae into connected multicellular compartments. The cross-walls depend
70 on FtsZ, but not on other canonical divisome proteins such as FtsI, FtsL and FtsW ([Jakimowicz
71 and van Wezel, 2012](#); [McCormick, 2009](#); [Mistry et al., 2008](#)). Interestingly, mutants lacking
72 *ftsZ* are viable, forming long hyphae devoid of septa ([McCormick et al., 1994](#)). Intricate
73 membrane assemblies ensure that chromosome-free zones are created during septum
74 formation in vegetative hyphae, apparently protecting the DNA from damage during division
75 ([Celler et al., 2016](#); [Yagüe et al., 2016](#)). Reproductive and divisome-dependent cell division
76 occurs exclusively in sporogenic aerial hyphae. Sporulation-specific cell division in
77 *Streptomyces* may therefore be regarded as canonical cell division as it requires all
78 components of the divisome. At the onset of sporulation, up to 100 septa are formed more
79 or less simultaneously, see as spirals of FtsZ in the aerial hyphae. Cell division is positively
80 controlled, via the direct recruitment of FtsZ by the membrane-associated SsgB ([Willemse et
81 al., 2011](#)). SsgB is a member of the SsgA-like proteins, which only occur in morphologically
82 complex actinomycetes ([Jakimowicz and van Wezel, 2012](#); [Traag and van Wezel, 2008](#)). The
83 localization of SsgB depends on the orthologous SsgA protein, which activates sporulation-
84 specific cell division ([Kawamoto et al., 1997](#); [van Wezel et al., 2000](#)).

85 Four genes lie between *ftsZ* and *divIVA* in the *dcw* cluster of streptomycetes, in the
86 order *ftsZ-ylmD-ylmE-sepF-sepG-divIVA*. The small transmembrane protein SepG acts as an

87 anchor for SsgB to the membrane and also controls nucleoid organization ([Zhang et al.,](#)
88 [2016](#)). YlmDE form a likely toxin-antitoxin system, whereby YlmD acts as a toxin that has
89 detrimental effects on sporulation-specific cell division ([Zhang et al., 2018](#)). SepF is involved
90 in early division control by stimulating the polymerization of FtsZ. In *B. subtilis*, SepF forms
91 large rings of around 50 nm in diameter in vitro, and assists in bundling of FtsZ filaments
92 ([Hamoen et al., 2006](#); [Ishikawa et al., 2006](#)). SepF interacts with the membrane via its N-
93 terminal domain ([Duman et al., 2013](#)), and plays a role in both Z-ring assembly and
94 anchoring. In the actinomycete *Mycobacterium* SepF also interacts with FtsZ, and is essential
95 for division ([Gola et al., 2015](#); [Gupta et al., 2015](#)). Thus, SepF is a rare example of a cell
96 division control protein that is shared between firmicutes and by actinobacteria.

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

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109 RESULTS

110 Three *sepF*-like genes in *Streptomyces*

111 Three genes with homology to *sepF* were found on the *S. coelicolor* genome. The canonical
112 *sepF* gene (SCO2079) lies within the *dcw* cluster in close proximity to *ftsZ*, an arrangement
113 that is conserved in all Gram-positive bacteria. Two *sepF*-like (*sfl*) genes, *sflA* (SCO1749) and
114 *sflB* (SCO5967), are located elsewhere on the *S. coelicolor* chromosome. SepF is a predicted
115 213 aa protein, while SflA (146 aa) and SflB (136 aa) are significantly smaller. Thus, SflA and
116 SflB have lengths very similar to that of SepF of *Bacillus subtilis* (139 aa; accession number
117 KFK80720). Alignment of the three proteins and their comparison to SepF of *B. subtilis* and
118 *Mycobacterium smegmatis* is presented in Fig. 1; predicted α -helices and β -strands are
119 boxed with dotted and solid lines, respectively. Compared to SflA and SflB, SepF proteins of
120 *S. coelicolor* and *M. smegmatis* have an approximately 60 aa internal extension at the N-
121 terminal half. The presence of three *sepF*-like genes is common in Actinobacteria, except for
122 *Coriobacteriaceae*, which only have *sepF*. The N-terminal α -helix (aa 1-12) of *Bacillus* SepF is
123 essential for lipid binding to support cell division ([Duman et al., 2013](#)). Based on the
124 predicted secondary structure of the protein (using JPRED), this α -helix is absent in SflB ([Cole](#)
125 [et al., 2008](#)), suggesting that this protein may not bind to the membrane. Conversely, the C-
126 terminal domain of SepF, which is involved in the interaction with FtsZ ([Duman et al., 2013](#);
127 [Gola et al., 2015](#); [Gundogdu et al., 2011](#); [Gupta et al., 2015](#)), is conserved in SflA and SflB.

128

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

130 To analyze the role of SflA and SflB in growth and development of *Streptomyces*, deletion
131 mutants were created for the two genes, separately and in combination, using a strategy
132 based on the instable multi-copy plasmid pWHM3 ([Swiatek et al., 2012](#)). Briefly, the

133 +10/+426 section of *sflA* or the +10/+356 region of *sflB* (relative to the start of the respective
134 genes) was replaced by the apramycin resistance cassette, which was subsequently removed
135 using the Cre-*lox* system, leaving only the scar sequence, thereby generating an in-frame
136 deletion mutant (see Materials and Methods). The *sfl* single and double mutants sporulated
137 well on SFM agar plates, developing abundant grey-pigmented spores after 3 days of
138 growth, suggesting that these proteins are dispensable for sporulation (Fig. 2B).
139 Nonetheless, the timing of development was mildly affected in the mutants. Deletion of *sflA*
140 accelerated aerial growth and sporulation, while deletion of *sflB* delayed sporulation. In
141 *sflAB* double mutants, aerial hyphae formation was accelerated while sporulation was
142 delayed (Fig. S1).

143 Interestingly, while *S. coelicolor* M145 formed colonies with a smooth edge, those of
144 *sflA* or *sflB* mutants had a 'fluffier' phenotype, a difference that was more pronounced in
145 *sflAB* double mutants (Fig. 2B). Genetic complementation of *sflA* and *sflB* null mutants by the
146 introduction of plasmids pGWS1005 (expressing *sflA* from the *ftsZ* promoter) and pGWS1006
147 (expressing *sflB* from the *ftsZ* promoter), respectively, restored the wild-type colony
148 morphology. This indicates that the abnormal colony morphology of the mutants was indeed
149 due to the deletion of the *sfl* genes. To investigate the change in colony morphology in *sfl*
150 mutants, the tip-to-branch distance was measured in young vegetative hyphae that had
151 been grown for 20 h. This average tip-to-branch distance was $15.05 \pm 5.14 \mu\text{m}$ in the
152 parental strain M145, while it had increased significantly in *sflA*, *sflB* and *sflAB* mutants,
153 where the distance was $19.79 \pm 9.15 \mu\text{m}$, $18.84 \pm 9.06 \mu\text{m}$ and $19.89 \pm 7.12 \mu\text{m}$, respectively
154 ($p < 0.001$). The longer tip-to-branch distance in *sfl* mutants - and thus reduced compactness
155 of the mycelia - may explain the altered colony morphology of *sfl* mutants.

156 We also attempted to delete *sepF*, but failed to do so despite many attempts.
157 Therefore, CRISPRi was employed to knockdown *sepF* and obtain insights into its possible
158 functional linkage to *sflAB*. The CRISPRi system we used was modified from pCRISPR-dCas9
159 ([Tong et al., 2015](#)) by expressing Cas9 from the constitutive *gapdh* promoter, using vector
160 pSET152 that integrates at the ϕ C31 attachment site on the *S. coelicolor* chromosome (see
161 Materials and Methods section for details)([Ultee et al., 2020](#)). Introduction of control
162 constructs pGWS1050 and pGWS1353, which contain either no spacer or a spacer targeting
163 the template strand of *sepF*, respectively, did not affect growth or development of *S.*
164 *coelicolor* (Fig. 2A). Conversely, introduction of pGWS1354, which carries a spacer targeting
165 the non-template strand of *sepF*, into *S. coelicolor* M145, resulted in severe developmental
166 defects and overproduction of actinorhodin (Fig. 2A). Transmission electron microscopy
167 (TEM) showed that vegetative hyphae wherein *sepF* was knocked down using CRISPRi lacked
168 cross-walls (Fig. S2). The phenotype of *sepF* mutants was very similar to that reported for
169 *ftsZ* null mutants ([McCormick et al., 1994](#)), in line with the expected crucial role of SepF in Z-
170 ring formation in *S. coelicolor*. The severe phenotype of the *sepF* knock-down mutants
171 suggests that *sflA* and *sflB* cannot functionally compensate for the lack of *sepF*.

172

173 **Sporogenic aerial hyphae of *sflA* and *sflB* null mutants show unusual branching**

174 Surface-grown *S. coelicolor* M145 and its *sflA*, *sflB* and *sflAB* mutants were analyzed in more
175 detail by cryo-scanning electron microscopy (SEM). After three days of growth, *S. coelicolor*
176 M145 produced abundant and regular spore chains (Fig. 3A). However, strains lacking *sflA*
177 ($\Delta sflA$ and $\Delta sflAB$) produced fewer spore chains (Fig. 3B & 3D), while deletion of only *sflB* did
178 not significantly affect sporulation (Fig. 3C). Strikingly, sporogenic aerial hyphae of *sflA*, *sflB*
179 and *sflAB* null mutants branched frequently (Fig. 3E-G), a phenotype that was never seen in

180 the wild-type strain. Introduction of wild-type copies of *sfIA* or *sfIB* into the respective
181 mutants largely complemented the mutant phenotypes, and prevented branching (Fig. 3H-I).
182 Some variability in spore sizes was still observed, perhaps as the result of a difference in
183 expression level of the proteins from the chromosomal and from the plasmid-borne genes.

184 Transmission electron microscopy (TEM) was used to image thin sections at high
185 resolution. This again revealed branching spore chains in *sfIA* and *sfIB* mutants (Fig. 4,
186 arrows) and variation in spore sizes. Furthermore, while wild-type spores had a typical dark
187 (electron-dense) spore wall and well-condensed DNA, the spores of the mutants typically
188 had lighter (electron-lucent) spore walls as well as less clearly visible DNA in many of the
189 spores (Fig. 4 B-D). This suggests pleiotropic changes in spore morphogenesis and
190 maturation in *sfl* genes mutants. As was already apparent from the SEM imaging,
191 introduction of *sfIA* and *sfIB* into *sfIA* and *sfIB* null mutants, respectively, prevented
192 branching of the spore chains, although the spore walls were still relatively thin (Fig. 4 E-F).

193

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

195 To study the effect of overexpression of SepF paralogs in *S. coelicolor*, the *sfIA*, *sepF* and *sfIB*
196 genes were all cloned individually behind the *ermE* promoter region, which encompasses a
197 strong constitutive promoter and an optimized ribosome binding site (see Materials and
198 Methods for details), and the expression cassettes were then inserted in the multi-copy
199 shuttle vector pWHM3. The expression constructs were designated pGWS774, pGWS775
200 and pGWS776, respectively. pWHM3 is an unstable plasmid that is easily lost and its copy
201 number largely depends on the level of thiostrepton ([van Wezel et al., 2005](#)). The
202 thiostrepton concentration controls the copy number of pWHM3, with copy number
203 proportional to the thiostrepton concentration.

204 Plasmids pGWS774 (expressing *sflA*), pGWS775 (*sepF*), pGWS776 (*sflB*) or control
205 plasmid pWHM3 without insert were introduced into *S. coelicolor* M145. The transformants
206 were then plated onto SFM agar plates with different concentrations of thiostrepton and the
207 colony morphology investigated after 7 days of incubation (Fig. 5). On SFM media, even in
208 the absence of thiostrepton, colonies overexpressing SflA (GAL44) or SflB (GAL46) were
209 smaller than those of transformants harboring the empty plasmid (GAL70) or transformants
210 over-expressing SepF (GAL45) (Fig. 5). In the presence of thiostrepton (20 µg/mL), the size of
211 colonies over-expressing SflA or SflB were reduced further. Interestingly, spores of SflA- and
212 SflB-overexpressing strains could be easily removed from the plates with a toothpick, leaving
213 “clean” plates, suggesting they had lost the ability to attach to and invade into the agar
214 surface (Fig. 5, third row). Conversely, SepF-overexpressing colonies still grew into agar, and
215 the mycelia remained firmly attached to the plates (Fig. 5, third row). When the thiostrepton
216 concentration was increased further to 50 µg/mL, colonies of transformants with SflA or SflB
217 expression constructs were very tiny and irregularly shaped, while those with control
218 plasmid or harboring the SepF expression construct were barely affected (Fig. 5). On R5 agar
219 plates, similar tiny colonies were observed for SflA and SflB-overexpressing strains, whereby
220 the colonies more or less 'floated' on the agar surface, showing severe developmental defect
221 (Fig. S3).

222 To see if growth of the hyphae was affected, we analyzed young 9 h old vegetative
223 hyphae. Interestingly, the hyphal length of control transformants carrying empty pWHM3
224 was 8.23 ± 3.57 , while SflA- and SflB-overexpressing strains had a distance from germination
225 site to hyphae tip of only 2.70 ± 1.59 µm and 2.70 ± 1.60 µm, respectively. The hyphal length
226 of SepF-overexpressing colonies was less reduced, reaching on average 5.30 ± 1.70 µm.

227 Taken together, we conclude that *sfIA* or *sfIB*, and to a lesser extent *sepF*, play a role in the
228 control of tip growth.

229

230 **Altered localization of DivIVA and FtsZ in *sfIA* and *sfIB* mutants**

231 Streptomyces grow via extension of the hyphal tip, although the molecular mechanism of
232 polar growth is still largely unknown ([Jakimowicz and van Wezel, 2012](#)). DivIVA is required
233 for tip growth, whereby it localizes at apical sites and at new branches ([Flärdh, 2003](#); [Hempel](#)
234 [et al., 2008](#)). Therefore, DivIVA is a very good indicator for active tip growth, and we used
235 this to study the onset of branching in the hyphae of wild-type and mutant strains. Construct
236 pGWS800, harboring *Streptomyces venezuelae divIVA-egfp* under the control of its native
237 promoter, was introduced into *sfIA* and *sfIB* null mutants. In wild-type cells, DivIVA-eGFP
238 accumulated at tips of aerial hyphae, with 93% of the foci observed at apical sites. In aerial
239 hyphae of *sfIA* and *sfIB* null mutants, DivIVA-eGFP foci were more widely distributed, not
240 only at apical sites, but also along hyphae at the places without apparent branching,
241 suggesting the emergence of new branching points (Fig. 6). In *sfIA* and *sfIB* mutants, 21% and
242 64% of the DivIVA-eGFP signals were observed along the lateral wall, respectively. Strikingly,
243 DivIVA-eGFP localized abundantly in maturing spore chains of *sfIA* and *sfIB* mutants, while in
244 wild-type spore chains no DivIVA-eGFP was observed (Fig. 6). The ectopic localization of
245 DivIVA-eGFP in the absence of *sfIA* or *sfIB* suggests that their gene products play a role in the
246 control of DivIVA localization and hence in determining apical growth of the hyphae in
247 *Streptomyces*. This is consistent with the functional correlation of SflAB with tip growth and
248 hyphal length.

249 To establish how FtsZ localizes in *sfl* mutants, construct pKF41 expressing FtsZ-eGFP
250 ([Grantcharova et al., 2005](#)) was introduced into *S. coelicolor* and its *sfIA*, *sfIB* and *sfIAB*

251 mutants. In sporogenic aerial hyphae, FtsZ formed typical ladder-like patterns in all strains.
252 Canonical Z-ladders were formed in *sfl* null mutants, although occasional misplaced septa
253 were seen in *sflA* null mutants (Fig. 7, left). However, while FtsZ foci and rings disassembled
254 and were absent in mature spore chains of wild-type *S. coelicolor*, they persisted in late
255 sporogenic aerial hyphae of *sflA* and *sflB* mutants (Fig. 7, right). Prolonged Z-rings were
256 observed in 46%, 28% and 72% of the premature spores of *sflA*, *sflB* and *sflAB* mutants,
257 respectively, while they were not seen in wild-type spores. This corresponds very well to the
258 ratios of incomplete septa in non-separated spores, which were 68% and 13% for *sflA* and
259 *sflB* mutants, respectively, 79% for *sflAB* mutant and only 1% for the parent *S. coelicolor*
260 M145. Taken together, the ectopic and continued localization of DivIVA and FtsZ in *sfl* null
261 mutants throughout sporulation strongly suggests that SflA and SflB play an important role
262 in controlling the dynamics of apical growth and cell division during *Streptomyces*
263 development, and in particular ensure timely disassembly of DivIVA and FtsZ foci.

264

265 **Localization of SflA and SflB in *S. coelicolor***

266 To analyze the localization of the SepF paralogs, constructs were created in the integrative
267 vector pSET152 containing either paralogue fused in frame behind *egfp* expressed from the
268 *ftsZ* promoter region (see Materials and Methods). Constructs expressing eGFP-SflA, eGFP-
269 SepF or eGFP-SflB from and were called pGWS784, pGWS785 and pGWS786 respectively. To
270 analyze the colocalization of SepF and Sfl proteins, the gene for E2-Crimson was fused in
271 frame with *sflA* and that for dTomato fused in frame with *sepF* (See Materials and Methods).
272 The constructs expressing E2-Crimson-SflA or dTomato-SepF were named pGWS1380 and
273 pGWS1383, respectively.

274 In young aerial hyphae, no specific localization of eGFP-SepF was observed prior to
275 the onset of septum synthesis (Fig. 8A top row). Eventually, SepF-eGFP localized in a ladder-
276 like pattern, similar to Z-ladders, which co-stained with the septa as seen by membrane
277 staining using FM5-95 (Fig. 8A middle row). During spore maturation, no SepF-eGFP signal
278 was detected (Fig. 8A bottom row). This indicates that SepF localizes in canonical fashion to
279 sporulation septa, consistent with its role in Z-ring formation. Interestingly, eGFP-SflA and
280 eGFP-SflB formed ring-like structures before septation had initiated (top row in Fig. 8B and
281 8C, respectively). When cell division had started, as visualized by membrane staining, eGFP-
282 SflA and eGFP-SflB signals had largely disappeared (middle rows of Fig. 8B and Fig 8C,
283 respectively). During spore maturation, when invagination between spores was clearly
284 visible, the two proteins re-appeared at the junction between the adjacent spores (Fig. 8BC,
285 bottom row). Thus, SflA and SflB localized specifically prior to and after the completion of
286 septum synthesis, while SepF localized in rings primarily at the time when SflAB foci were
287 no visible.

288 The distinct localization patterns of SepF and Sfl proteins led us to investigate their
289 colocalization. Indeed, SflA and SflB colocalized with each other, but most of the time they
290 did not colocalize with SepF. However, on rare occasions, we did see colocalization between
291 SepF and SflA or SflB, whereby they formed ring-like structures in sporogenic aerial hyphae
292 (Fig. S5). This is consistent with experiments in *S. venezuelae*, which showed that both SepF
293 and SflB (named SepF2 in *S. venezuelae*) colocalized with FtsZ, which indirectly confirmed
294 the colocalization between SepF as SflB ([Schlimpert et al., 2017](#)). Live imaging of the
295 sporulation process in solid-grown aerial hyphae is very difficult, due to the mobile nature of
296 the airborne hyphae. We have been able to image the recruitment of FtsZ by SsgB, but this
297 was during a short time frame and these are highly abundant proteins. Capturing the specific

298 time when SflAB and SepF colocalize using live imaging was not feasible. Still, our results do
299 show that while SepF and SflAB localized in differentially in terms of timing, there is a short
300 time window when colocalization occurs. Dispersal of SflAB then marks the start of cell
301 division.

302

303

304 **DISCUSSION**

305 A major question in the developmental biology of *Streptomyces* that we seek to address is,
306 how do *Streptomyces* ensure that septa are controlled in time and space in the long and
307 multinucleoid hyphae? We have shown previously that in streptomycetes the correct
308 localization of FtsZ is governed by a system of positive control, whereby the actinomycete-
309 specific SsgA and SsgB proteins recruit FtsZ to the septum sites to initiate sporulation-
310 specific cell division ([Willemse et al., 2011](#)). As a consequence, deletion of either *ssgA* or
311 *ssgB* blocks sporulation ([Keijser et al., 2003](#); [van Wezel et al., 2000](#)). Additionally, SepG
312 (YlmG in *B. subtilis*) is an auxiliary protein that allows SsgB to dock to the membrane ([Zhang
313 et al., 2016](#)). In this work we present a new piece of this jigsaw, which points at the possible
314 existence of a layer of negative control during *Streptomyces* sporulation, revolving around
315 the SepF-like proteins SflA and SflB.

316 The most eye-catching change in morphogenesis due to the deletion of either *sflA* or
317 *sflB* was the extensive branching of the aerial hyphae and in particular of spore chains, which
318 we have never seen in any of our wild-type streptomycetes. The tip-to-branch distance of
319 vegetative hyphae was also extended in *sflA* and *sflB* null mutants, which likely contributes
320 to the 'fluffy' morphology of the mutant colonies. Conversely, constitutive expression of SflA
321 and SflB from the *ermE* promoter inhibited growth and reduced adhesion of the colonies to

322 the agar surface, with as possible explanation that tip extension and hence also branching is
323 impaired in the vegetative mycelium (Fig. 5 and Fig. S3). These data strongly suggest an
324 inverse correlation between the expression level of SflA and SflB and polarisome activity.
325 Indeed, we found mislocalization of DivIVA in *sflA* and *sflB* null mutants, with many foci
326 along the lateral wall of aerial hyphae, instead of only apical localization. While in wild-type
327 hyphae virtually all DivIVA-eGFP foci were located at the apex, in total 21% and 64% of the
328 foci were observed along the lateral wall in *sflA* and *sflB* mutants, respectively. Since DivIVA
329 drives tip growth and thus also branching, this likely explains the observed branching spore
330 chains frequently observed in *sfl* null mutants (Fig. 3 and Fig. 4). The inhibition of growth
331 following the constitutive expression of SflA or SflB in vegetative hyphae suggests that
332 expression of these proteins throughout the life cycle directly or indirectly inhibits DivIVA
333 during vegetative growth, which will then result in growth inhibition, as DivIVA is essential
334 for tip growth.

335 Typical ladders of Z-rings were produced in young sporogenic aerial hyphae of both
336 wild-type *S. coelicolor* and in *sflA* or *sflB* null mutants, though the distance between adjacent
337 Z-rings in mutants varied more in the mutants. Importantly, besides for DivIVA, we also
338 noticed strongly prologued and ectopic localization of FtsZ in mutants lacking *sflA* and/or
339 *sflB*. While Z-ladders disappeared in mature spore chains of the parental strain *S. coelicolor*
340 M145, ladders and foci persisted in the different *sfl* mutants during spore maturation,
341 strongly suggesting that either the septa had not yet been completed or that disassembly of
342 the FtsZ polymers was compromised (Fig. 7). SflA and SflB reappeared at the interface
343 between adjacent spores, perhaps to allow the disassembly of SepF, and hence
344 destabilization of the Z-rings. The C-terminal part of SepF interacts with FtsZ in *B. subtilis* and
345 *M. smegmatis* ([Duman et al., 2013](#); [Gola et al., 2015](#); [Gupta et al., 2015](#); [Hamoen et al.,](#)

346 [2006](#)). Though the Sfl proteins share significant homology with SepF in their C-terminal parts
347 (Fig. 1), only SepF interacts with FtsZ ([Schlimpert et al., 2017](#)). Interestingly, in
348 *Mycobacterium smegmatis* and *B. subtilis*, overexpression of SepF is lethal and largely blocks
349 cell division, and it was suggested that this was due to interference of free SepF with the
350 assembly of lateral cell division proteins ([Gao et al., 2017](#); [Gola et al., 2015](#)). In *S. coelicolor*
351 however, overexpression of SepF barely showed any effect, while overexpression of its
352 paralogs SflA or SflB led to growth inhibition.

353 Taking into account the distinct localization patterns of SepF and SflAB, and the *in vitro*
354 interaction between these three proteins (Figure S4; ([Schlimpert et al., 2017](#))), the activity of
355 SflAB in terms of the disassembly of FtsZ filaments may be mediated via the disassembly of
356 SepF rings. SflB lacks the N-terminal α -helix that is required for membrane lipid binding
357 ([Duman et al., 2013](#)), suggesting that SflB will require SflA for membrane-specific
358 localization. Extensive analysis using fluorescence microscopy showed that SflA and SflB foci
359 are primarily formed before and after the cell division process, which is when SepF and FtsZ
360 rings are formed. However, we occasionally observed colocalization of SflA or SflB with SepF
361 (Fig. S5). Indeed, as discussed above, two-hybrid analysis also revealed interaction of SepF
362 with the Sfl proteins. In the absence of SflAB, foci of FtsZ and SepF persist after spores have
363 been formed, strongly suggesting that SflAB play a role in the termination of the cell division
364 process.

365 We propose a model wherein SflA and SflB negatively affect the polymerization of
366 SepF, thereby preventing the polymerization of SepF prior to the onset of cell division, and
367 stimulating the depolymerization of SepF polymers after completion of cell division. During
368 the onset of sporulation-specific cell division, SepF-rings assembly is initiated, initially
369 whereby colocalizing with SflAB, which keep SepF inactive. Dispersal of SflAB then allows the

370 formation of SepF rings, while SsgB localizes to recruit FtsZ, thus marking the start of cell
371 division. Once completed, SflAB take up their positions again and assist in dispersing SepF,
372 which leads to the destabilization of FtsZ filaments and their disassembly. The continued
373 presence of SepF polymers in *sflAB* null mutants after the completion of sporulation-specific
374 cell division would stabilize FtsZ filaments and continue to anchor them to the membrane,
375 explaining why FtsZ polymers did not disassemble during spore maturation in these mutants.

376 Surprisingly, the localization of DivIVA was also disturbed in the aerial hyphae. While
377 DivIVA is known to interact with a range of different protein partners, no interaction
378 between DivIVA and either SepF or FtsZ has so far been reported ([Halbedel and Lewis, 2019](#)).
379 Interestingly, DivIVA homologue GpsB was recently shown to interact with SepF in *Listeria*
380 *monocytogenes* ([Cleverley et al., 2019](#)), while in *Staphylococcus aureus* GpsB was shown to
381 interact with FtsZ to stimulate the formation of FtsZ bundles ([Eswara et al., 2018](#)).
382 Biochemical experiments are required to establish how SflA and SflB affect the localization
383 and/or polymerization of SepF, FtsZ and DivIVA.

384 Taken together, our work shows that SflAB control growth and cell division of the
385 aerial hyphae of *Streptomyces*. Over-expression of the proteins strongly inhibits growth of
386 the colonies, while in the absence of *sflA* and/or *sflB* DivIVA localizes ectopically, resulting in
387 unusual branching of aerial hyphae. Besides controlling the localization and activity of
388 DivIVA, SflAB also interact with - and control the localization of - SepF and hence of FtsZ. In
389 the absence of SflAB, Z-rings and foci persist in mature spore chains. Thus SflAB ensure the
390 correct localization of key cell division proteins in time and space during sporulation-specific
391 cell division of *Streptomyces*.

392

393

394 MATERIALS AND METHODS

395 Bacterial strains and media

396 The bacterial strains used in this work are listed in Table S1. *E. coli* strains JM109 ([Sambrook](#)
397 [et al., 1989](#)) and ET12567 ([MacNeil et al., 1992](#)) were used for routine cloning and for
398 isolation of non-methylated DNA, respectively. *E. coli* transformants were selected on LB
399 agar media containing the relevant antibiotics and grown O/N at 37°C. *Streptomyces*
400 *coelicolor* A3(2) M145 was used as parental strain to construct mutants. All media and
401 routine *Streptomyces* techniques are described in the *Streptomyces* manual ([Kieser et al.,](#)
402 [2000](#)). Yeast extract-malt extract (YEME) and tryptic soy broth with 10% sucrose (TSBS) were
403 the liquid media for standard cultivation. Regeneration agar with yeast extract (R2YE) was
404 used for regeneration of protoplasts and with appropriate antibiotics for selection of
405 recombinants ([Kieser et al., 2000](#)). Soy flour mannitol (SFM) agar plates were used to grow
406 *Streptomyces* strains for preparing spore suspensions and for morphological characterization
407 and microscopy.

408

409 Plasmids and constructs and oligonucleotides

410 All plasmids and constructs described in this work are summarized in Table S2. The
411 oligonucleotides are listed in Table S3.

412

413 *Constructs for CRISPRi*

414 As described previously, the 20 nt target sequence(spacer) was introduced into sgRNA
415 scaffold by PCR using forward primers SepF_TF or SepF_NTF together with the reverse
416 primer SgTermi_R_B ([Ultee et al., 2020](#)) . The generated PCR products were cloned into
417 pGWS1049 via restriction sites NcoI and BamHI to generate constructs pGWS1351 and

418 pGWS1352. Subsequently, DNA fragments containing sgRNA scaffold and *Pgapdh-dcas9* of
419 constructs pGWS1049, pGWS1351 and pGWS1352 were digested with EcoRI and XbaI and
420 cloned into pSET152 using the same restriction enzymes. The generated constructs
421 pGWS1050 (no target sequence), pGWS1353(targeting template strand of *sepF*) and
422 pGWS1354 (targeting non-template strand of *sepF*) were used in CRISPRi system.

423

424 *Constructs for creating deletion mutants*

425 Construction for in-frame deletion were based on the instable vector pWHM3 ([Vara et al.,](#)
426 [1989](#)), essentially as described previously ([Swiatek et al., 2012](#)). For the deletion of *sfIA*, its
427 upstream region -1336/+9 (using primers *sfIA*_LF-1339 and *sfIA* _LR+9) and downstream
428 region +427/+1702 (using primers *sfIA* _RF_427 and *sfIA* _RR+1702) were amplified by PCR
429 from *S. coelicolor* M145 genomic DNA and cloned into pWHM3 as EcoRI-BamHI fragments,
430 and the apramycin resistance cassette *aac(3)IV* flanked by *loxP* sites inserted in between.
431 This resulted in plasmid pGWS750 that was used for deletion of *sfIA* (SCO1749). The
432 presence of *loxP* sites allows efficient removal of apramycin resistance cassette by Cre-
433 recombinase ([Fedoryshyn et al., 2008](#)). The same strategy was used to create construct
434 pGWS751 for the deletion of *sfIB* (SCO5967). This plasmid contained the -1258/+9 and
435 +357/+1917 regions relative to *sfIB*, and the apramycin resistance cassette inserted in-
436 between. The *sfIA* and *sfIB* double mutant (GAL16) was constructed in the background of a
437 *sfIA* in-frame deletion mutant (GAL14) by deleting *sfIB*. For complementation of the *sfIA* null
438 mutant, pGWS1005 was used, an integrative vector based on pSET152 and harboring the
439 entire coding region (+1/+468, amplified using primers *sfIA*_F+1 and *sfIA*_R+468) of *sfIA*
440 under control of the *ftsZ* promoter. Similarly, pGWS1006 was used for genetic
441 complementation of *sfIB* mutants, with pSET152 harboring the entire coding region

442 (+1/+438, amplified using primers sflB-F+1 and sflB_R+438) of *sflB* under control of the *ftsZ*
443 promoter.

444

445 *Constructs for the expression of eGFP or E2-Crimson or dTomato fusion proteins*

446 The eGFP gene was amplified by PCR from pKF41 using primers eGFP_F+1 and
447 eGFP_R_717_Linkers, adding a 12 bp linker in primer eGFP_R_717_Linkers. The PCR fragments
448 were digested with *StuI* and *BamHI*, and fused behind *EcoRI* and *StuI* digested fragment
449 containing *ftsZ* promoter region excised from pGWS755 ([Zhang et al., 2016](#)). The fused
450 *EcoRI-StuI-BamHI* fragment was then cloned into pSET152 via *EcoRI* and *BamHI*. Coding
451 genes of *sflA* (amplified from *S. coelicolor* genomic DNA using primers *sflA_F+1* and
452 *sflA_R+441*), *sepF* (primers *sepF_F+1* and *sepF_R+639*) and *sflB* (primers *sflB_F+1* and
453 *sflB_R+411*) were cloned in to the above construct via *BamHI* and *XbaI* to generate
454 constructs pGWS784, pGWS785 and pGWS786, respectively. In pGWS786, the *BamHI* site
455 between *egfp* and *sflB* was lost by fusion to the *BglIII* site in PCR-amplified *sflB* DNA. The *E2-*
456 *Crimson* gene was amplified by PCR from pTEC19 ([Takaki et al., 2013](#)) using primers
457 *E2Crimson_F_EEV* and *E2Crimson_linker_R_BH*. The PCR fragment was digested with *EcoRV*
458 and *BamHI*, and cloned into pGWS784 via *StuI* and *BamHI* to replace the gene for eGFP.
459 Subsequently, the *EcoRI-XbaI* fragment containing *P_{ftsZ}-E2-Crimson-sflA* was cloned into
460 pHJL401 to generate pGWS1380. Similarly, *dTomato* gene was amplified by PCR from pLenti-
461 V6.3 Ultra-Chili (Addgene plasmid # 106173) using primers *dTomato_F_EEV* and
462 *dTomato_linker_R_BH*. The *EcoRV* and *BamHI* digested PCR was clone into *StuI* and *BamHI*
463 digested pGWS785. Subsequently, the *EcoRI-XbaI* fragment containing *P_{ftsZ}-E2-dTomato-sepF*
464 was cloned into pHJL401 to generate pGWS1383.

465 The coding region of *divIVA* (excluding the stop codon) together with its 393 bp
466 upstream region were amplified by PCR from *S. venezuelae* genomic DNA using primers BglII-
467 *divIVA*-SV-FW and NdeI-*divIVA*-SV-REV. The PCR product was cloned as BglII-NdeI fragment
468 into pIJ8630 to generate construct pGWS800, which expresses DivIVA-eGFP under the
469 control of the *divIVA* promoter.

470

471 *Constructs for enhanced gene expression*

472 To obtain enhanced expression of *sepF*, *sfIA* and *sfIB*, the genes were inserted behind the
473 constitutive *ermE* promoter and an optimized ribosome binding site using plasmid pHM10a
474 ([Motamedi et al., 1995](#)). For this, DNA fragments harboring the entire *sfIA*, *sepF* or *sfIB*
475 coding region were amplified by PCR from *S. coelicolor* M145 genomic DNA using primer
476 pairs *sfIA*_F+4 and *sfIA*_R+447, *sepF*_F+4 and *sepF*_R+648 and *sfIB*_F+4 and *sfIB*_R+417,
477 respectively, and cloned into pHM10a digested with NdeI-HindII or NdeI-BamHI. The inserts
478 of the pHM10a-based constructs were subsequently transferred as BglII-HindII or BglII-
479 BamHI fragments to BamHI-HindII or BamHI digested pWHM3 to generate pGWS774 (for
480 expression of *sfIA*), pGWS775 (for *sepF*) and pGWS776 (for *sfIB*).

481

482 *Constructs for BACTH screening*

483 The coding region of *sfIA* was amplified from *S. coelicolor* M145 genomic DNA using primer
484 pair *sfIA*-fw and *sfIA*-rv, and cloned as an XbaI-KpnI fragment into pUT18C and pKT25 to
485 generate pBTH166 and pBTH167, respectively. *sepF* was amplified using primers *sepF*-fw and
486 *sepF*-rv and cloned into pUT18C and pKT25 as an XbaI-XmaI fragment to generate pBTH110
487 and pBTH111, respectively. *sfIB* was amplified from *S. coelicolor* M145 genomic DNA using
488 primer pair *sfIB*-fw and *sfIB*-rv, cloned as an XbaI-KpnI fragment into pUT18C and pKT25, so

489 as to generate pBTH170 and pBTH171, respectively. *sigR* was amplified from *S. coelicolor*
490 M145 genomic DNA using primer pair SCO5216-fw and SCO5216-rv and cloned into pUC18
491 as an XbaI-XmaI fragment to generate pBTH17. Similarly, *rsrA* was amplified using primers
492 SCO5217-fw and SCO5217-rv and cloned into pKT25 as XbaI-XmaI fragment to generate
493 pBTH23.

494

495 **Microscopy**

496 *Light microscopy*

497 Sterile cover slips were inserted at an angle of 45 degrees into SFM agar plates, and spores
498 of *Streptomyces* strains were carefully inoculated at the intersection angle. After incubation
499 at 30°C for 3 to 5 days, cover slips were positioned on a microscope slide prewetted 5 µl of
500 1xPBS. Fluorescence and corresponding light micrographs were obtained with a Zeiss
501 Axioscope A1 upright fluorescence microscope (with an AxioCam Mrc5 camera at a
502 resolution of 37.5 nm/pixel). The green fluorescent images were created using 470/40 nm
503 band pass (bp) excitation and 525/50 bp detection, for the red channel 550/25 nm bp
504 excitation and 625/70 nm bp detection was used ([Willemse and van Wezel, 2009](#)). DAPI was
505 detected using 370/40 nm excitation with 445/50 nm emission band filter. For staining of
506 the cell wall (peptidoglycan) we used FITC-WGA, for membrane staining FM5-95 and for DNA
507 staining DAPI (all obtained from Molecular Probes). For stereomicroscopy we used a Zeiss
508 Lumar V12 stereomicroscope. All images were background corrected setting the signal
509 outside the hyphae to zero to obtain a sufficiently dark background. These corrections were
510 made using Adobe Photoshop CS4.

511

512 *Electron microscopy*

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

518

519 **BATCH complementation assay**

520 For BACTH complementation assays, vectors pKT25 and pUT18C harboring genes of interest
521 were used in various combinations to co-transform *E. coli* BTH101 cells carrying plasmid
522 pRARE (Novagen). The transformants were plated onto LB medium containing ampicillin
523 (100 µg/mL), kanamycin (50 µg/mL) chloramphenicol (50 µg/mL) and were incubated for 24–
524 36 h at 30°C. Then 3 independent representative co-transformants were grown on M63
525 minimal medium agar plates containing proper antibiotics ampicillin 50 µg/ mL, kanamycin
526 25 µg/ mL and chloramphenicol 25 µg/ mL. This medium allows growth of co-transformants
527 only if the co-expressed proteins interact with each other. Co-transformation of pBTH17
528 (*sigR*) and pBTH23 (*rsrA*) was used as positive control, while co-transformation of empty
529 plasmids pUT18 and pKT25 was used as negative control.

530

531 **Computer analysis**

532 For DNA and protein searches used StrepDB (<http://strepdb.streptomyces.org.uk/>) and
533 STRING (<http://string.embl.de>). Alignment was built using Clustal Omega
534 (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) and Boxshade program. Secondary structures of
535 proteins were predicted using JPRED
536 (http://www.compbio.dundee.ac.uk/jpred4/index_up.html).

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702

703

704 **FIGURE LEGENDS**

705 **Figure 1. Alignment of SepF proteins.** Amino acid sequences of SepF proteins from *B. subtilis*
706 (SepFbs), *M. smegmatis* (SepFms) and *S. coelicolor* (SepFsc), and two SepF paralogs of *S.*
707 *coelicolor* (SflA and SflB) were aligned using Boxshade program. Identical residues are
708 shaded in black; conservative changes are shaded in grey. α -helices and β -strands in the
709 predicted secondary structures (via JPRED) of are boxed by red dotted line and solid line,
710 respectively. Essential amino acids for FtsZ interaction were highlighted with star.

711

712 **Figure 2. Phenotypic analysis of *sepF* and *sfl* mutants.** *sepF* knockdown mutant shows
713 severe developmental defect when the spacer in CRISPRi system targets non-template
714 strand **(A)**. Stereomicrographs show representative colonies of *S. coelicolor* M145, its *sflA*
715 and *sflB* null mutants and complemented strains. Strains were grown on SFM agar plates for
716 three days at 30°C. Note that colonies of *sfl* mutants were ‘fluffier’ than those of the
717 parental strain M145, and expression of wild-type SflA or SflB restore smooth colony edge to
718 the corresponding mutants Bar, 1 mm **(B)**.

719

720 **Figure 3. Cryo-scanning electron micrographs of spore chains of *S. coelicolor* M145, its *sfl***
721 **mutants and complemented *sfl* mutants.** Wild-type *S. coelicolor* M145 **(A)** sporulated
722 abundantly after three days of incubation, while mutants lacking either *sflA* **(B & E)** or *sflAB*

723 **(D & G)** showed reduced sporulation; the *sfIB* null mutant **(C & F)** produced comparable
724 amount of spores as the parental strain. Most notable change in all mutants was that the
725 spore chains frequently branched, while spore chains in genetically complemented *sfIA* **(H)**
726 and complemented *sfIB* **(I)** did not show any branching. Cultures were grown on SFM agar
727 plates for 5 days at 30°C. Bar, 1 µm.

728

729 **Figure 4. Transmission electron micrographs of spore chains of *S. coelicolor* M145, its *sfI***
730 **mutants and complemented *sfI* mutants.** While spore chains of wild type M145 **(A)** do not
731 branch and contain regularly sized spores, mutant lacking either *sfIA* **(B)**, *sfIB* **(C)** or *sfIAB* **(D)**
732 produce irregular spores and spore chains frequently branch, in line with the SEM images
733 (Figure 3). Complemented *sfIA* **(E)** and complemented *sfIB* **(F)** produced unbranched spore
734 chain as wild type. Cultures were grown on SFM agar plates for 5 days at 30°C. Arrows
735 indicate branching points of spore chains. Bar, 1 µm.

736

737 **Figure 5. Effect of enhanced expression of *sepF* and *sfI* genes on colony morphology.**
738 Stereomicrographs showing the phenotype of GAL70 (*S. coelicolor* M145 + empty plasmid
739 pWHM3 control), GAL44 (M145 + pGWS774, expressing *sfIA*), GAL45 (M145 + pGWS775,
740 expressing *sepF*) and GAL46 (M145 + pGWS776, expressing *sfIB*) were grown on SFM plates
741 containing different concentrations of thiostrepton (0-50 mg/ml). Plates were incubated for
742 7 days at 30°C. Over expression of *sfIA* or *sfIB* resulted in tiny colonies and no mycelium left
743 on the plates after spore collection suggested the loss of attachment to agar, while
744 overexpression of *sepF* didn't affect colonial size and adherence. It should be noted that the
745 tiny colonies produced by *sfIA* or *sfIB* overexpressing strains still show gray color, suggested
746 that the sporulation were not inhibited. Bar, 2 mm.

747

748 **Figure 6. Localization of DivIVA-eGFP in *S. coelicolor* and its *sfl* null mutants.** In aerial
749 hyphae, DivIVA localized in wild-type cells mainly at tips while it was more dispersed in *sfl*
750 mutants (indicated as empty arrow heads). DivIVA-eGFP was not detected in maturing spore
751 chains of wild type cells, but it was often seen in that of *sfl* mutants (indicated as filled arrow
752 heads). Bar, 2 μ m.

753

754 **Figure 7. Localization of FtsZ-eGFP in *S. coelicolor* and its *sfl* null mutants.** FtsZ forms
755 ladder-like structure in sporogenic aerial hyphae of wild type and disappears in later
756 developmental stage. While in *sfl* mutants FtsZ ladder remains longer even in spore maturing
757 stage. Bar, 2 μ m.

758

759

760

761 **Figure 8. Localization of SepF, SflA and SflB in *S. coelicolor*.**

762 Fluorescence micrographs present three consecutive stages, namely prior to the onset of cell
763 division (top panel), septum formation (middle panel) and spore maturation (bottom panel).

764 Sporogenic aerial hyphae of *S. coelicolor* M145 were imaged by fluorescence microscopy
765 visualizing the respective eGFP fusion proteins (green), membrane (stained with FM5-95;

766 red) and corresponding light micrographs. As expected, eGFP-SepF (**A**) localizes in a ladder-
767 like pattern that overlaps the sporulation septa. Foci of eGFP-SflA (**B**) forms foci along aerial

768 hyphae prior to septum synthesis, re-appearing during spore maturation at invagination

769 sites. Foci of eGFP-SflB (**C**) localize in a ladder-like pattern prior to septum synthesis, vanish

770 as septal membranes formed and re-emerge during spore maturation. Bar, 2 μ m.

771


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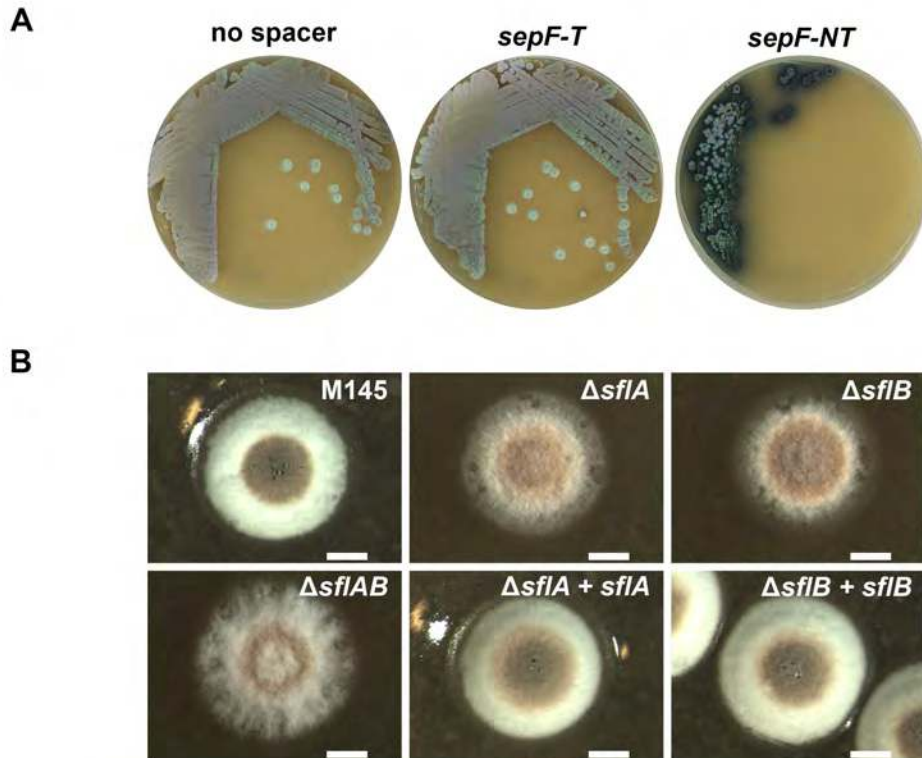
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Sf1A 1  -MG...V...K...A...S...A...L...G...V...I...D...N...D...I...R...Y...D...D...Y...S...E...G...P...E...S...D...A...--...W...V...T...D...P...R...-----V...K...V...A...S...D...V...A
Sf1B 1  -----V...K...S...G...E...P...V...N...S...H...V...T...E...Q...L...G...L...A...Q...V...V...L...R...E...R...D...A...M...P...S...A...V...G...-----H...R...A...M...P...E...A...E
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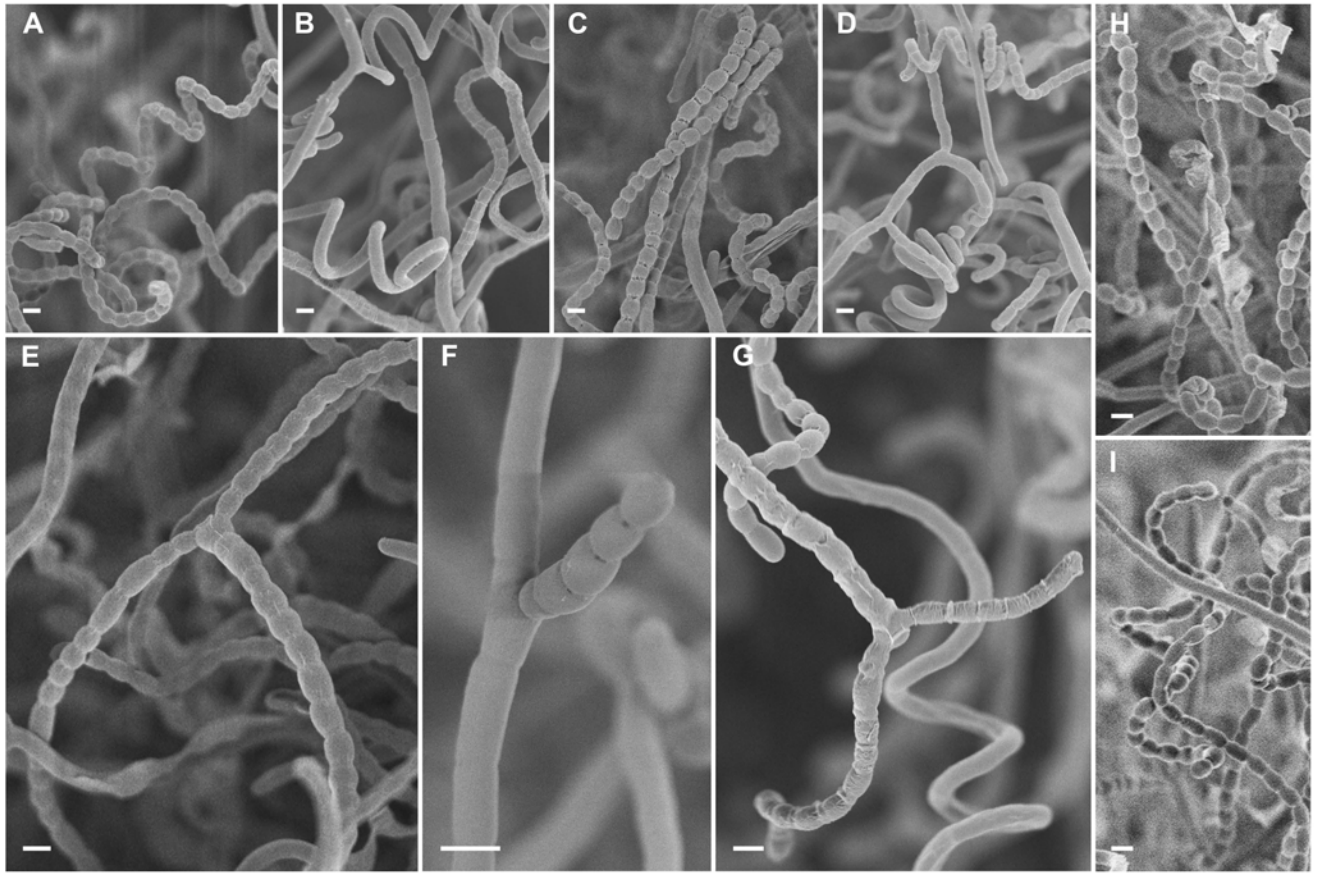
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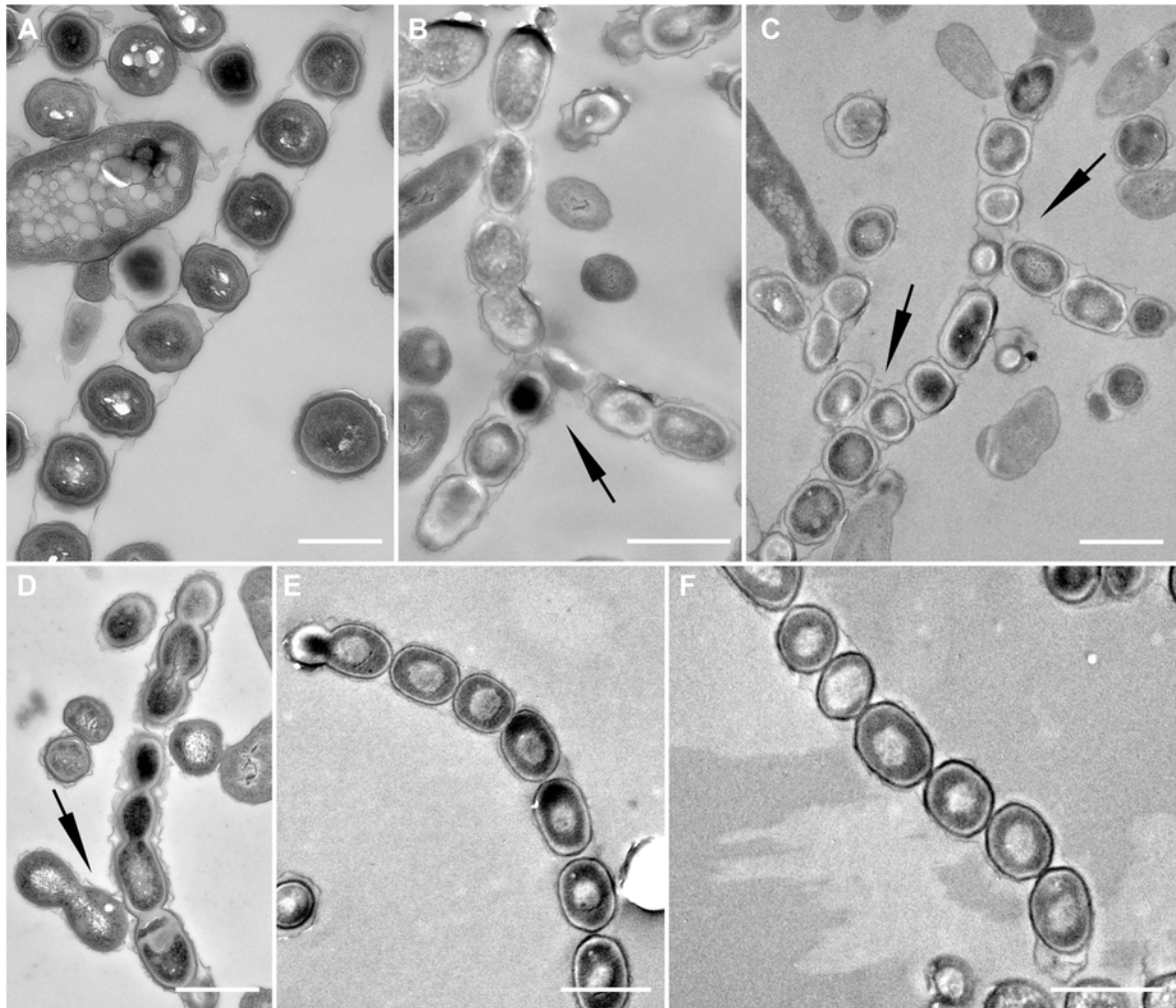
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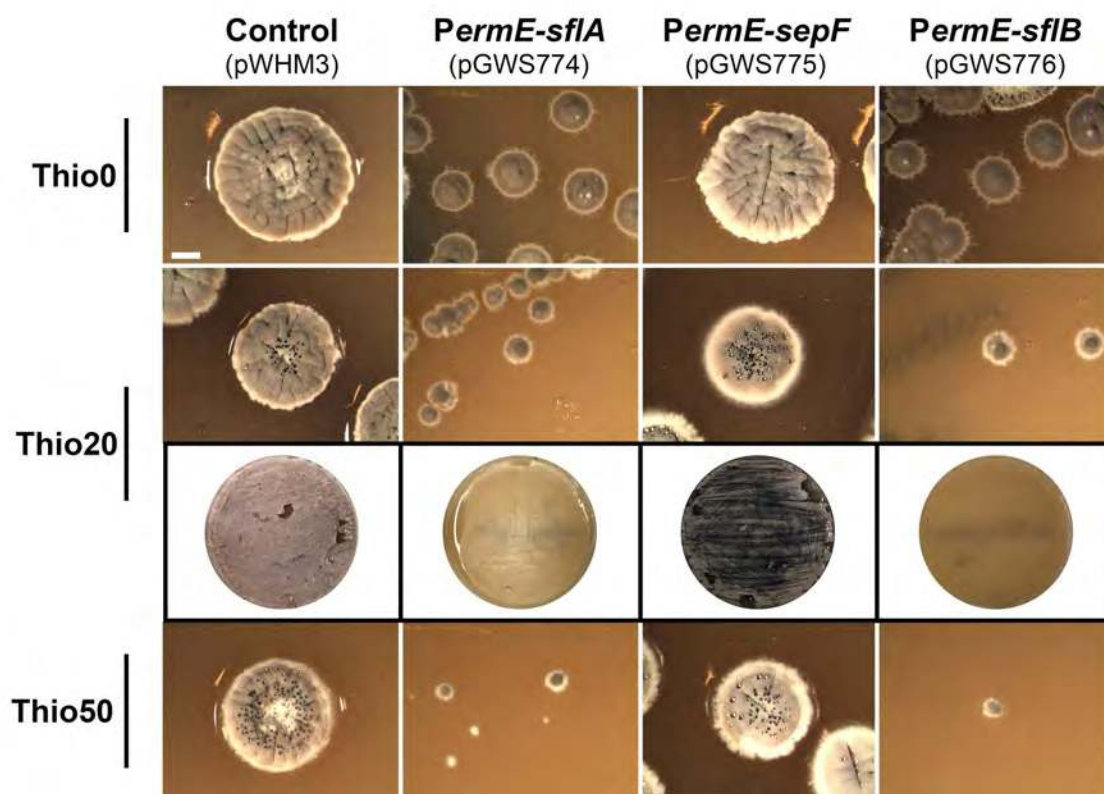
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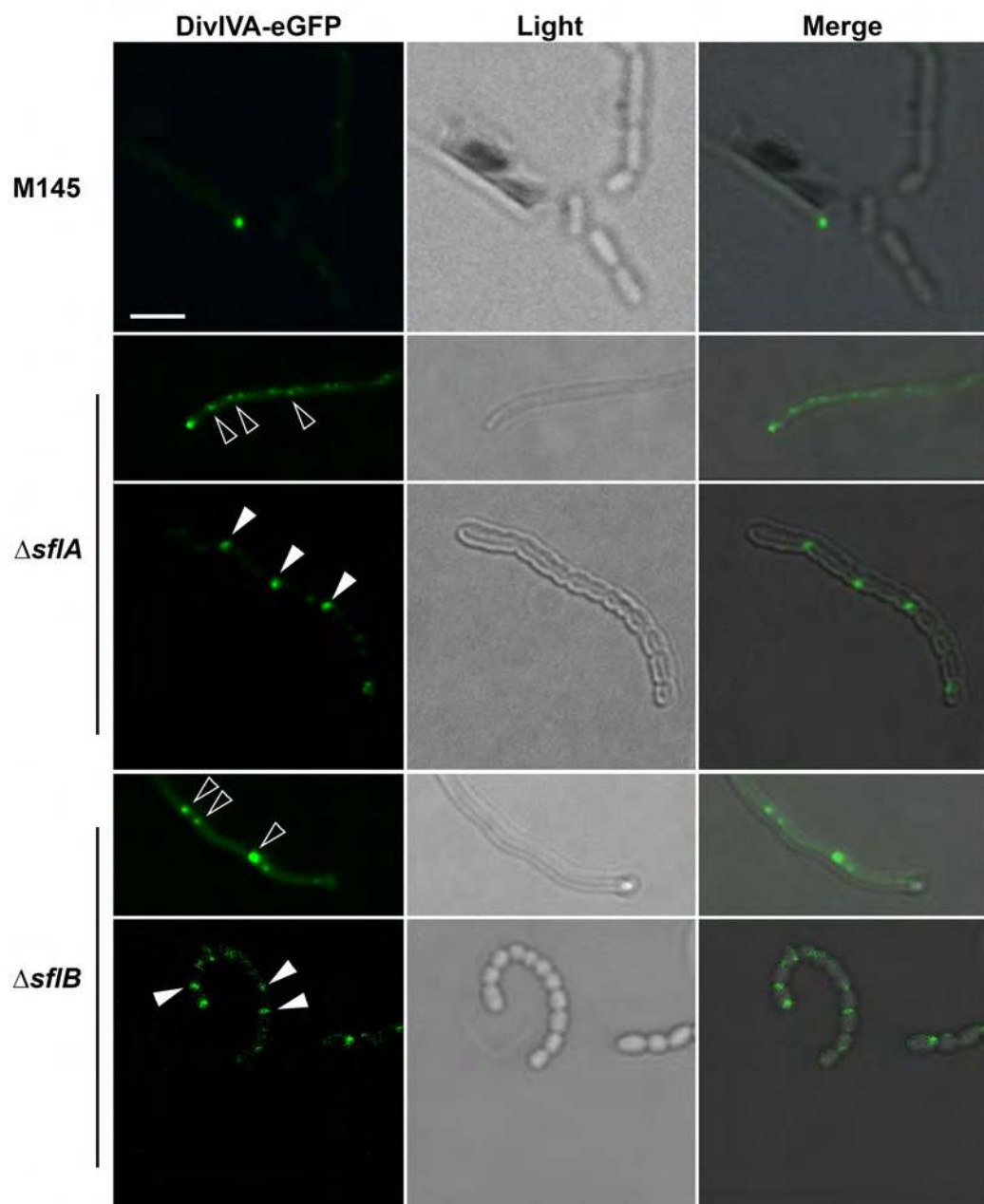
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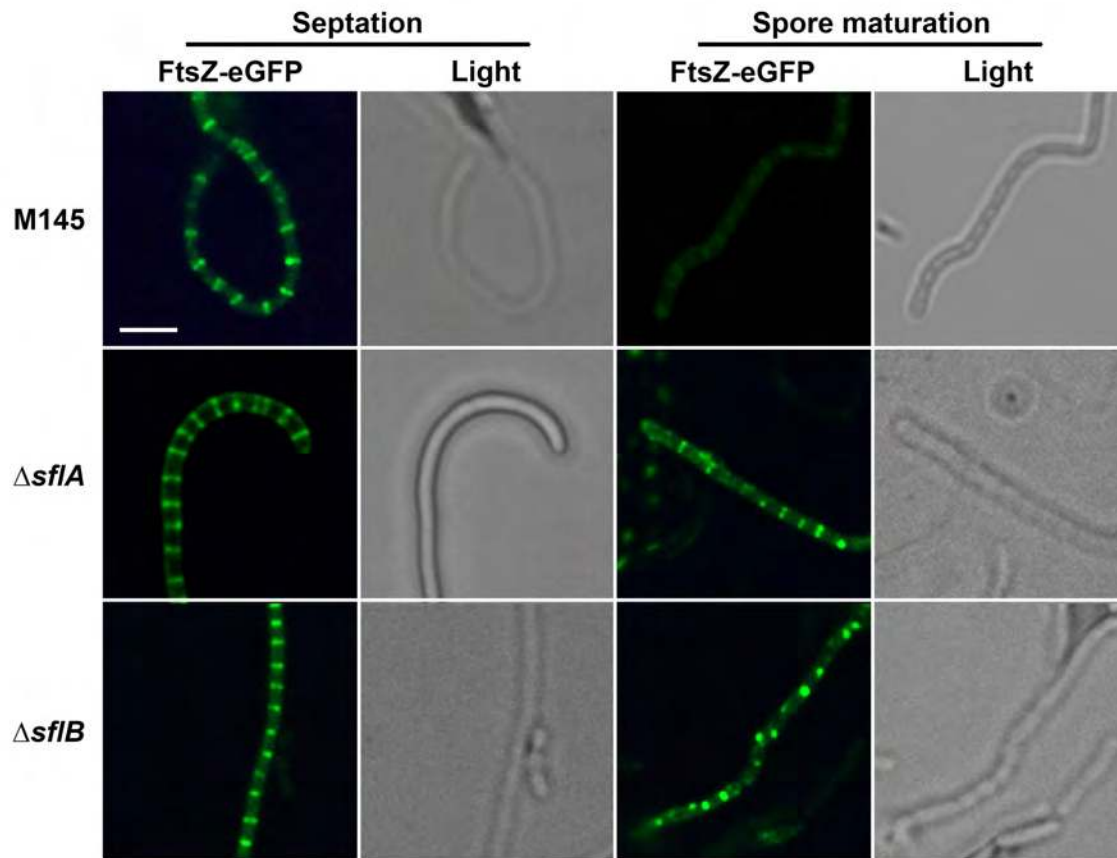




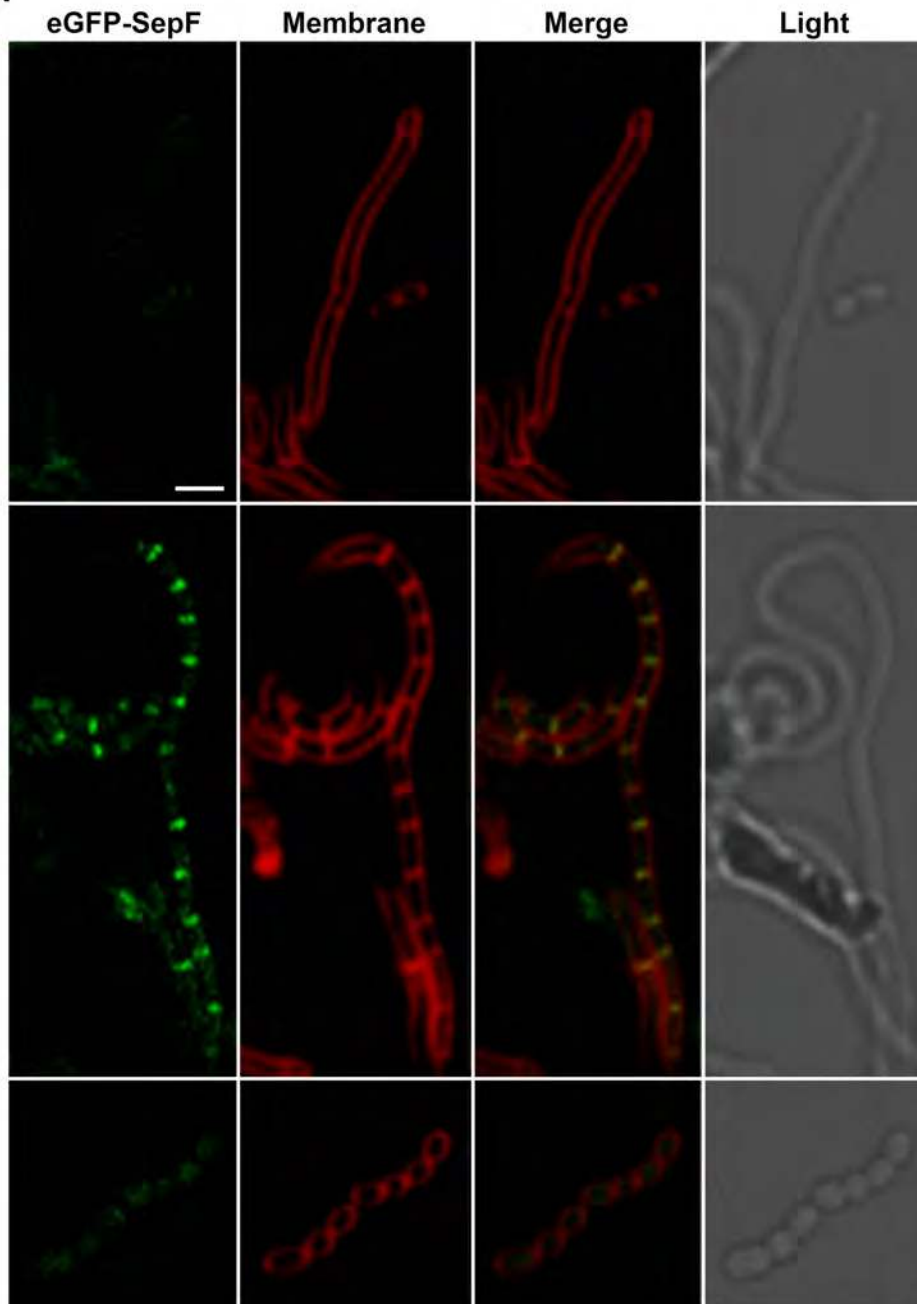




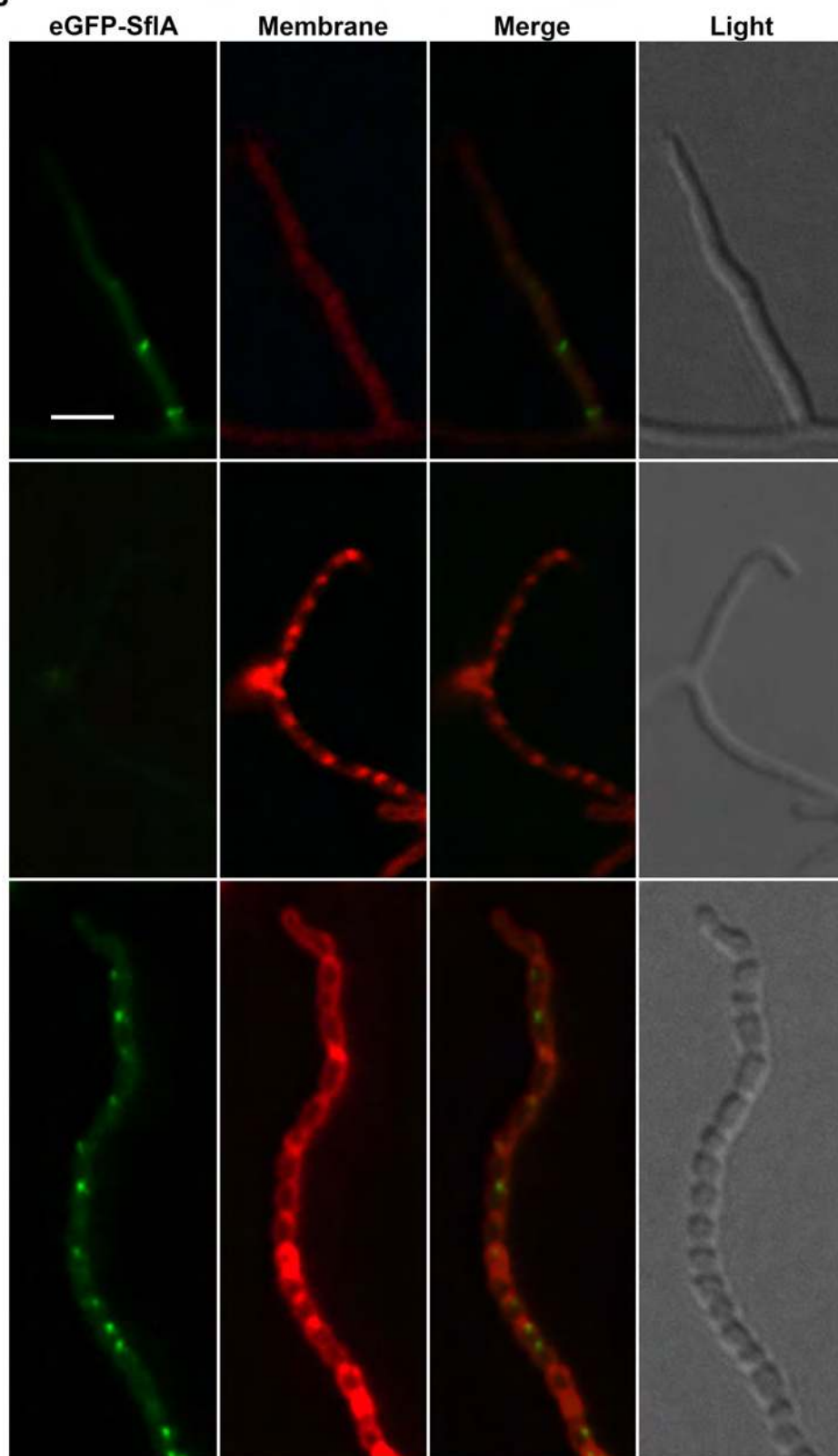




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