

1 **Divisome-dependent subcellular localization of cell-cell joining**
2 **protein SepJ in the filamentous cyanobacterium *Anabaena***

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17 **Summary**

18 **Heterocyst-forming cyanobacteria are multicellular organisms that grow as**
19 **filaments that can be hundreds of cells long. Septal junction complexes, of which**
20 **SepJ is a possible component, appear to join the cells in the filament. SepJ is a**
21 **cytoplasmic membrane protein that contains a long predicted periplasmic section**
22 **and localizes to the cell poles in the intercellular septa, but also to a position**
23 **similar to a Z ring when cell division starts suggesting a relation with the divisome.**
24 **Here we created a mutant of *Anabaena* sp. strain PCC 7120 in which the essential**
25 **divisome gene *ftsZ* is expressed from a synthetic NtcA-dependent promoter, whose**
26 **activity depends on the nitrogen source. In the presence of ammonium, low levels**
27 **of FtsZ were produced and the subcellular localization of SepJ, which was**
28 **investigated by immunofluorescence, was impaired. Possible interactions of SepJ**
29 **with itself and with divisome proteins FtsZ, FtsQ and FtsW were investigated**
30 **using the bacterial two-hybrid system. We found SepJ self-interaction and a**
31 **specific interaction with FtsQ, confirmed by co-purification and involving parts of**
32 **the SepJ and FtsQ periplasmic sections. Therefore, SepJ can form multimers and,**
33 **in *Anabaena*, the divisome has a role beyond cell division, localizing a septal**
34 **protein essential for multicellularity.**

35

36 **Introduction**

37 Although bacteria are widely considered as unicellular organisms, there are some cases
38 of true multicellularity. Multicellular bacteria have mechanisms to keep cells together
39 and distinctively exhibit the formation of cells specialized in different functions
40 (Claessen *et al.*, 2014). The heterocyst-forming cyanobacteria are true multicellular

41 bacteria, and *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena*) is becoming a model
42 to study multicellularity (Flores and Herrero, 2010). *Anabaena* grows as chains of cells
43 (known as filaments or trichomes) that can be hundreds of cells long (Rippka *et al.*,
44 1979). When *Anabaena* is grown in the absence of combined nitrogen, some
45 photosynthetic vegetative cells in the filament differentiate into N₂-fixing heterocysts
46 (Kumar *et al.*, 2010). In the developed diazotrophic filament vegetative cells and
47 heterocysts exchange nutrients including sugars and amino acids (Wolk *et al.*, 1994;
48 Haselkorn, 2007; Burnat *et al.*, 2014). Heterocyst differentiation requires the global N-
49 control transcription factor NtcA that, under nitrogen deprivation, activates transcription
50 of many genes and represses some others (Herrero *et al.*, 2013). NtcA binds to DNA at
51 sites with consensus sequence GTAN₈TAC, which are found in different contexts in
52 regulated promoters. In Class II NtcA-activated promoters, an NtcA-binding site is
53 located about 22 nucleotides upstream from a -10 promoter box in the form TAN₃T
54 (Herrero *et al.*, 2001; Picossi *et al.*, 2014). In *Anabaena*, *ntcA* expression is low when
55 ammonium is present in the growth medium, increases when nitrate is the nitrogen
56 source, and is highest in the absence of combined nitrogen (Muro-Pastor *et al.*, 2002).

57 The cyanobacteria are diderm bacteria bearing an outer membrane outside of the
58 cytoplasmic membrane and peptidoglycan layers, and in heterocyst-forming
59 cyanobacteria the outer membrane is continuous along the filament, not entering the
60 septa between adjacent cells (Wolk, 1996; Flores *et al.*, 2006; Wilk *et al.*, 2011). Hence,
61 all cells in the filament share a common periplasm (Mariscal *et al.*, 2007). Cell-cell
62 joining structures termed septal junctions (previously known as microplasmodesmata or
63 septosomes) can be observed by transmission electron microscopy and by electron
64 tomography in the intercellular septa (Lang and Fay, 1971; Giddings and Staehelin,
65 1978; Wilk *et al.*, 2011). These structures appear to be proteinaceous in nature (Wilk *et*

66 *al.*, 2011). Some genes whose mutation results in filament fragmentation have been
67 identified in *Anabaena*, including the genes in the *fraCDE* operon and *sepJ*, which
68 encode integral membrane proteins that are important for filament integrity mainly
69 under nitrogen deprivation (Bauer *et al.*, 1995; Nayar *et al.*, 2007; Flores *et al.*, 2007;
70 Merino-Puerto *et al.*, 2010). GFP fusions have shown that FraC, FraD and SepJ are
71 located at the intercellular septa, with SepJ being particularly focused in the center of
72 the septum, and that both FraC and FraD are needed for a correct localization of SepJ
73 (Flores *et al.*, 2007; Merino-Puerto *et al.*, 2010). As evidenced by experiments
74 performed with fluorescent tracers, all these proteins influence intercellular molecular
75 exchange in the cyanobacterial filament (Mullineaux *et al.*, 2008; Merino-Puerto *et al.*,
76 2011).

77 SepJ, encoded by ORF *alr2338* of the *Anabaena* genome (Kaneko *et al.*, 2001),
78 consists of 751 amino-acid residues and has three well differentiated domains: (i) an N-
79 terminal coiled-coil domain (amino acid residues 28 to 207), which could be involved in
80 protein-protein interactions and is required for proper localization of SepJ at the
81 intercellular septa, filament integrity and diazotrophic growth; (ii) a linker domain rich
82 in Pro and Ser residues (amino acid residues 208 to 410) whose deletion hardly affects
83 SepJ subcellular localization but impairs intercellular transfer of the fluorescent tracer
84 calcein; and (iii) a C-terminal permease (amino acid residues 411 to 751) similar to
85 proteins of the Drug/Metabolite Transporter (DMT) superfamily (Transporter
86 classification database number 2.A.7; <http://www.tcdb.org>) that appears to be necessary
87 for physiological intercellular molecular exchange (Flores *et al.*, 2007; Mariscal *et al.*,
88 2011). The coiled-coil and linker domains of SepJ have been predicted to be
89 periplasmic (Flores *et al.*, 2007). In addition to being detected at the cell poles in the
90 intercellular septa as mentioned above, SepJ-GFP is localized to a position similar to

91 that of a Z ring when cell division starts (Flores *et al.*, 2007; Mariscal and Flores, 2010).

92 The so-called Z ring is made up of the essential tubulin homolog FtsZ at the future site

93 of division in bacteria (Huang *et al.*, 2013).

94 The divisome is the multiprotein complex responsible for cell division in

95 bacteria (Lutkenhaus *et al.*, 2012; Egan and Vollmer, 2013; Natale *et al.*, 2013).

96 Cyanobacterial cell division genes have been studied by comparative and mutational

97 analyses, which have shown that these organisms contain some cell division genes

98 previously identified in Gram-negative bacteria, some in Gram-positive bacteria, and

99 still some others that are more specific to cyanobacteria (Miyagishima *et al.*, 2005;

100 reviewed in Cassier-Chauvat and Chauvat, 2014). In *Anabaena*, putative divisome

101 genes include *ftsZ* encoding the key Z ring protein (Doherty and Adams, 1995; Zhang *et*

102 *al.*, 1995), *zipN* (*fm2*) encoding a possible tether of FtsZ to the cytoplasmic membrane

103 (Koksharova and Wolk, 2002; Marbouty *et al.*, 2009a, 2009b), and *ftsQ* and *ftsW*

104 encoding downstream cytokinetic factors (Vicente *et al.*, 2006). Localization of

105 *Anabaena* FtsZ has been studied using GFP fusions and immunogold labeling, which

106 showed that this protein can form a ring in the middle of dividing cells (Sakr *et al.*,

107 2006; Klint *et al.*, 2007). FtsZ appears to be at low levels or absent from heterocysts

108 (Kuhn *et al.*, 2000; Klint *et al.*, 2007), but further details on its regulation are unknown.

109 Similarity between SepJ and FtsZ localization in dividing cells, together with the

110 final localization of SepJ at the cell poles, suggests that SepJ might be recruited to the

111 division ring and interact with proteins of the divisome. In this work, we addressed the

112 localization of SepJ in a conditional *ftsZ* mutant of *Anabaena*, which expresses different

113 levels of FtsZ depending on the nitrogen source. We found that SepJ localization is

114 impaired when *ftsZ* expression is down regulated resulting in low cellular levels of the

115 FtsZ protein. Moreover, using the bacterial two-hybrid system, we found evidence for

116 SepJ self-interactions and an interaction between SepJ and *Anabaena* FtsQ, a protein
117 that is known to recruit several downstream divisome elements. This interaction could
118 be confirmed by co-purification of both proteins expressed in *Escherichia coli*. Our data
119 suggest the formation of SepJ multimers and identify a role of the divisome beyond cell
120 division, contributing to the assembly of the supracellular structure of a bacterial
121 pluricellular filament.

122

123 **Results**

124 *Construction of a strain with NtcA-dependent expression of ftsZ*

125 The *ftsZ* gene is located 1,191 bp downstream of *ftsQ* in the *Anabaena* chromosome
126 (Kaneko *et al.*, 2001). There is no evidence for co-transcription of the two genes, and
127 *ftsZ* is expressed at higher levels than *ftsQ* (Flaherty *et al.*, 2011). To create a
128 conditional mutant of the essential *ftsZ* gene in *Anabaena*, we designed a construct in
129 which *ftsZ* was expressed from a synthetic NtcA-dependent promoter, which we will
130 denote P_{ND}. This promoter was designed based on known features of Class II NtcA-
131 activated promoters (Herrero *et al.*, 2001) and contains a consensus NtcA-binding site
132 located 23 bp upstream from a -10 promoter box (Fig. 1A). The P_{ND} promoter, together
133 with the C.S3 gene cassette, was inserted in the *Anabaena* chromosome 5' of nucleotide
134 52 upstream of the *ftsZ* start codon (see Fig. 1A and Experimental procedures for
135 details). An *Anabaena* clone containing only chromosomes bearing the C.S3-P_{ND}
136 construct was named strain CSFR18 (Fig. S1).

137 Because NtcA-dependent promoters are most active when the cells are incubated
138 in the absence of a source of combined nitrogen and least active in the presence of
139 ammonium, strain CSFR18 was expected to grow well diazotrophically and, as a
140 consequence of insufficient *ftsZ* expression, poorly in the presence of ammonium. Tests

141 of growth on solid medium showed poorer growth in the presence of ammonium than
142 fixing N₂ or in the presence of nitrate (Fig. 1B). Strain CSFR18 was therefore routinely
143 maintained on solid BG11 (nitrate-containing) medium. When CSFR18 cells grown on
144 BG11 medium were inoculated in liquid medium, growth was observed for about 5 days
145 independently of the nitrogen source. Although the growth rates were somewhat slower
146 than those of the wild type, exponential growth was not much affected (Fig. S2).
147 Microscopic inspection of the cultures showed, however, an altered morphology, mainly
148 in ammonium-containing media, in which the mutant cells were significantly larger than
149 the wild-type cells (Fig. 1C). In contrast to many bacteria in which lack of FtsZ results
150 in cell elongation (Margolin, 2009), the cylindrical *Anabaena* cells got enlarged, being
151 longer and wider than the control cells, in response to decreased expression of *ftsZ*. In
152 the presence of nitrate the cells of the mutant were also larger than the wild-type cells,
153 but in the diazotrophic cultures mutant and wild-type cells were similar in size (cellular
154 areas are summarized in the legend to Fig. 1). The final appearance of the cultures was
155 very different as observed after 7 days of incubation under the different nitrogen
156 regimes (Fig. 1D). The culture of the mutant containing nitrate as the nitrogen source
157 was yellowish, which is indicative of an altered physiology, the culture with ammonium
158 was largely lysed (hence the lack of turbidity and the blue color reflecting the release of
159 phycobiliproteins from the cells), and only the diazotrophic culture was similar to the
160 corresponding wild-type culture.

161 The observations described above are consistent with NtcA-dependent
162 expression of *ftsZ* in strain CSFR18, with a limited expression mainly in ammonium-
163 containing cultures. Transcript levels of *ftsZ* were determined after two days of
164 incubation in liquid medium with the different nitrogen sources. Levels of *ftsZ*
165 transcript were about 23%, 60% and 89% in the mutant as compared to the wild type in

166 media containing ammonium, nitrate or no combined nitrogen, respectively (Fig. 2A).
167 The low level of *ftsZ* expression in cells of CSFR18 incubated in the presence of
168 ammonium corroborates that the P_{ND} promoter substitutes for the natural *ftsZ* promoter
169 in this strain. Our results also show that in the wild type, *ftsZ* expression is about 2-fold
170 higher in the diazotrophic cultures than in cultures containing combined nitrogen.
171 Western blot analysis performed with an antibody raised against the FtsZ protein of
172 *Anabaena* expressed in *E. coli* confirmed that the FtsZ levels in strain CSFR18 were
173 higher in diazotrophic than in nitrate-containing cultures, and lowest in ammonium-
174 containing cultures, with the levels in the absence of combined nitrogen being similar in
175 the mutant and the wild type (Fig. 2B).

176 Subcellular localization of FtsZ in the wild type and strain CSFR18 was
177 addressed by immunofluorescence with the *Anabaena* FtsZ antibodies. In the wild type,
178 localization of FtsZ in a ring at the middle of the cells could be readily observed in
179 vegetative cells, but not in heterocysts (Fig. 3). (We repeatedly found poor labeling in
180 ammonium-grown wild-type cells, but the reason for this is unknown.) In strain
181 CSFR18, FtsZ ring labeling was readily observed in diazotrophic filaments, in which a
182 number of vegetative cells, but not heterocysts, were labeled (Fig. 3). In this strain, an
183 FtsZ ring was observed with difficulty in some cells of the filaments incubated with
184 nitrate, but it was not observed in the big cells produced after incubation in the presence
185 of ammonium. These results are consistent with the different levels of FtsZ observed by
186 western blot analysis in the cells of CSFR18 incubated with different nitrogen sources.

187

188 *SepJ* localization in strain CSFR18

189 Once a strain with regulated expression of *ftsZ* was available and conditions leading to
190 production of low FtsZ cellular levels were established, we addressed the localization of

191 SepJ under those conditions. Localization of SepJ has previously been investigated
192 using a SepJ-GFP fusion (Flores *et al.*, 2007; Mariscal *et al.*, 2011). For this work,
193 however, we set up a protocol to study the subcellular localization of the native SepJ
194 protein by immunofluorescence, using antibodies raised against the coiled-coil domain
195 of SepJ (anti SepJ-CC antibodies; Mariscal *et al.*, 2011). These antibodies localized
196 SepJ at the cell poles in filaments grown with nitrate as the nitrogen source (Fig. 4).
197 Additionally, SepJ was observed, less focused, in the middle of enlarged cells that were
198 apparently dividing (see N₂-grown cells in Fig. 4).

199 In strain CSFR18, specific localization of SepJ at the cell poles was only
200 observed in filaments that had been incubated without combined nitrogen (Fig. 4). In
201 filaments incubated for 2 days in ammonium-containing medium, the SepJ signal, seen
202 as spots, was delocalized. In filaments incubated with nitrate, SepJ could be observed
203 localized in the cell poles, but also some SepJ signal was observed disperse (Fig. 4 and
204 not shown). Because of the low levels of FtsZ protein present in the cells incubated with
205 ammonium, these observations suggest that the correct localization of SepJ at the cell
206 poles needs the presence of FtsZ in the cells at normal, or close to normal, levels.

207

208 *Treatment with berberine*

209 Berberine is a plant alkaloid that has been shown to interfere with the assembly of the
210 FtsZ ring (Domadia *et al.*, 2008; Boberek *et al.*, 2010). To assess in a different way the
211 possible role of FtsZ in the localization of SepJ, we treated *Anabaena* cells with
212 berberine and performed immunofluorescence tests with the anti FtsZ and anti SepJ-CC
213 antibodies. Incubation of cells grown using nitrate as the nitrogen source with 0.1 mM
214 berberine for 24 h hampered the formation of the FtsZ ring (Fig. 5). Longer incubations
215 (≥ 48 h) or incubation with higher berberine concentrations (≥ 0.2 mM) resulted in cell

216 lysis. The filaments with cells lacking an FtsZ ring showed SepJ labeling more spaced
217 than the non-treated filaments (Fig. 5). Mean distance between SepJ spots was 3.0 ± 0.7
218 μm (number of intervals counted, $n = 76$) in untreated filaments and $5.1 \pm 2.5 \mu\text{m}$ ($n =$
219 74) in berberine-treated filaments (the significance of the difference between untreated
220 and treated filaments was assessed by the Student's t test; $P < 10^{-10}$). Whereas spots
221 observed with the anti SepJ-CC antibodies may correspond to SepJ proteins placed at
222 the intercellular septa before the treatment with berberine, implying a remarkable
223 stability of SepJ, elongated cells in which no SepJ signal is evident may result from lack
224 of SepJ localization related to lack of FtsZ assembly. Although indirect effects of
225 berberine cannot be ruled out, these results are consistent with a dependence of SepJ
226 localization on the FtsZ ring as deduced above with the CSFR18 mutant.

227

228 *Protein-protein interactions tested with the bacterial two-hybrid system*

229 The dependence of SepJ subcellular localization on FtsZ could result from a direct
230 interaction between these two proteins or from an interaction of SepJ with other
231 protein(s) of the divisome that require FtsZ for proper localization. To identify possible
232 direct interactions of SepJ with FtsZ or some other divisome proteins, we used the
233 bacterial two-hybrid system (BACTH), which permits a visual screening for interactions
234 on X-gal-containing plates and an estimation of the strength of those interactions by
235 quantitative determination of β -galactosidase activity (Karimova *et al.*, 1998). Fusions
236 of SepJ and divisome proteins FtsZ, FtsQ and FtsW, all of them from *Anabaena*, to the
237 two complementary fragments (T18 and T25) of the catalytic domain of adenylate
238 cyclase were prepared and cloned together in different combinations. The predicted
239 topology of the protein fusions used is schematized in Fig. 6, and β -galactosidase
240 activities are presented in Table 1.

241 We first checked whether SepJ interacts with itself by cloning SepJ fused to the
242 N-termini of T25 (SepJ-T25) and T18 (SepJ-T18). Whereas appropriate control
243 combinations with empty T18 or T25 plasmids were negative, a strong interaction was
244 detected for the SepJ-T25/SepJ-T18 pair (Table 1). This result shows that SepJ can be
245 involved in protein-protein interactions when fused to either T18 or T25. As described
246 in the Introduction, *Anabaena* SepJ bears three well-defined domains: a coiled-coil
247 domain and a linker domain that likely reside in the periplasm and an integral
248 membrane (permease) domain (schematically depicted in Fig. 6). To test a possible role
249 of specific protein domains in the interaction, we prepared truncated versions of SepJ
250 lacking (i) a substantial part (amino acid residues 463 to 748, leaving only one putative
251 transmembrane segment) of the permease domain, denoted SepJ(Δ TM), (ii) most of the
252 predicted periplasmic section, including both the coiled-coil and linker domains (amino
253 acid residues 40 to 410), denoted SepJ(Δ pp), (iii) the linker domain (amino acid
254 residues 223 to 410), denoted SepJ(Δ linker), and (iv) most of the coiled-coil domain
255 (amino acid residues 40 to 201), denoted SepJ(Δ CC). These proteins were fused to the
256 N-termini of T25 and T18, and appropriate controls of interaction with empty T18 and
257 T25, respectively, were negative (Table 1). SepJ(Δ TM) did not show self-interaction or
258 interaction with the whole SepJ, and SepJ(Δ pp) showed a very low self-interaction and
259 no interaction with the whole SepJ (Table 1). In contrast, SepJ(Δ linker) and SepJ(Δ CC)
260 showed weak and strong self-interactions, respectively, and appreciable interactions
261 with the whole SepJ in both cases. Because SepJ is a cytoplasmic membrane protein, it
262 is possible that the truncated SepJ(Δ TM) protein is not properly incorporated into the
263 membrane making any interaction not possible. In contrast, interactions observed with
264 SepJ(Δ linker) and SepJ(Δ CC) indicate that these proteins were properly produced to

265 work appreciably. These results show an important role of the linker domain in SepJ
266 self-interactions.

267 The SepJ-T18 plasmid (or the T18 plasmid as a control) was then tested with
268 FtsZ-T25 (FtsZ fused to the N-terminus of T25), T25-FtsW (FtsW fused to the C-
269 terminus of T25), and T25-FtsQ (FtsQ fused to the C-terminus of T25) (see schemes in
270 Fig. 6). Whereas all controls with empty T18 were negative, in the SepJ-divisome
271 protein pairs tested no interaction was detected with FtsZ, a weak interaction of
272 uncertain statistical significance was detected with FtsW, and a strong interaction was
273 detected with FtsQ (Table 1). Whereas the negative result with FtsZ does not provide
274 evidence for interaction and the result with FtsW leaves the possibility of an interaction
275 open, the positive result with FtsQ suggests interaction of this protein with SepJ.

276 FtsQ from *E. coli* has one transmembrane segment and a periplasmic section
277 consisting of two domains, α and β , that mediate interactions with other proteins (Chen
278 *et al.*, 1999; van den Ent *et al.*, 2008; Villanelo *et al.*, 2011), and *Anabaena* FtsQ is
279 predicted to have similar domains (Fig. S3). To investigate possible domain-specific
280 interactions of SepJ with FtsQ, the SepJ truncated proteins were tested. Whereas
281 SepJ(Δ TM) and SepJ(Δ linker) did not interact, and SepJ(Δ pp) showed a very weak
282 interaction with FtsQ, SepJ(Δ CC) showed a strong interaction (Table 1). Whereas, as
283 noted above, lack of proper integration of SepJ(Δ TM) into the cytoplasmic membrane
284 cannot be ruled out, these results suggest a role of the SepJ linker domain in interaction
285 with FtsQ.

286 To test whether one or the two of the FtsQ periplasmic domains have a role in
287 interaction with SepJ, we prepared truncated versions of FtsQ, FtsQ($\Delta\alpha$) and FtsQ($\Delta\beta$)
288 (Fig. S3), fused to the C-terminus of T25. Whereas control tests with T18 were
289 negative, tests with SepJ-T18 showed a very weak interaction with FtsQ($\Delta\alpha$) and a very

290 strong interaction with FtsQ($\Delta\beta$), suggesting that the α domain, but not the β domain is
291 needed for the FtsQ-SepJ interaction.

292

293 *Co-purification of SepJ and FtsQ*

294 To corroborate the interaction of SepJ and FtsQ, an *E. coli* strain carrying compatible
295 plasmids expressing SepJ-GFP and His₆-FtsQ, respectively, was prepared. Because a
296 part of the predicted periplasmic section of SepJ appears necessary for the interaction, a
297 plasmid expressing a SepJ-GFP fusion protein without most of this section (Δ pp-SepJ-
298 GFP) was also used. As controls, *E. coli* strains with a plasmid expressing one of the
299 proteins (SepJ-GFP, Δ pp-SepJ-GFP or His₆-FtsQ) and the second plasmid without an
300 insert were constructed. Cell-free extracts were prepared by breaking down the cells in a
301 French pressure cell (see Experimental procedures), incubated with anti GFP antibodies
302 (anti-GFP MicroBeads) and passed through a magnetic-activated cell sorting (MACS)
303 column, and the material retained was eluted and subjected to SDS-PAGE. It should be
304 noted that the material retained in the column should consist of inside-out membrane
305 micro-vesicles (normally produced by French pressure cell breakage; see e.g., Altendorf
306 and Staehelin, 1974), in which the cytoplasmic-exposed GFP is available for interaction
307 with the antibodies. As shown in Fig. 7A, His₆-FtsQ, detected with anti His-tag
308 antibodies, was retained in the case of extracts containing also SepJ-GFP, but much less
309 in those containing Δ pp-SepJ-GFP or not in the case of control extracts lacking SepJ.
310 The presence of SepJ-GFP or Δ pp-SepJ-GFP in the corresponding preparations was
311 corroborated with anti-GFP antibodies (Fig. 7B). These results indicate that FtsQ was
312 recovered at substantial levels only in micro-vesicles containing the whole SepJ protein,
313 thus corroborating an interaction of SepJ with FtsQ that requires the predicted
314 periplasmic section of SepJ to take place.

315

316 **Discussion**

317 SepJ is a key protein in *Anabaena* multicellularity, since mutants lacking SepJ show a
318 strong filament fragmentation phenotype (Nayar *et al.*, 2007; Flores *et al.*, 2007) and are
319 impaired in the intercellular transfer of a fluorescent tracer (Mullineaux *et al.*, 2008;
320 Mariscal *et al.*, 2011). SepJ-GFP fusions have been shown to localize to the cell poles at
321 the intercellular septa in the filaments of *Anabaena* (Flores *et al.*, 2007; Mariscal *et al.*,
322 2011), and immunofluorescence analysis performed in this work with an antibody
323 raised against the coiled-coil domain of *Anabaena* SepJ has confirmed the same
324 localization for native SepJ (Fig. 4). This same approach has recently permitted the
325 localization of SepJ in the complex intercellular septa of the true-branching, heterocyst-
326 forming filamentous cyanobacterium *Mastigocladus laminosus* (Nürenberg *et al.*,
327 2014), indicating that localization of SepJ at the intercellular septa may be a universal
328 feature in heterocyst-forming cyanobacteria.

329 In contrast to SepJ-GFP, which is observed as a single fluorescence spot in the
330 septa between adjacent vegetative cells (Flores *et al.*, 2007), two spots, one in each of
331 the adjacent cells, are frequently observed in the immunofluorescence analysis (Fig. 4)
332 indicating that SepJ localizes to both poles in each cell. Two spots have also been
333 observed in immunofluorescence analysis with anti-GFP antibodies in a strain
334 producing SepJ-GFP (Mariscal and Flores, 2010). (The two foci at the intercellular
335 septa likely result from shrinking of the cells during preparation for
336 immunofluorescence that involves a dehydration step.) Therefore, to produce a single
337 fluorescence spot from the SepJ-GFP fusion, in which the GFP is predicted to reside
338 next to the cytoplasmic face of the cytoplasmic membrane (Flores *et al.*, 2007), SepJ
339 from adjacent cells must be very close to each other. On the other hand, our BACTH

340 analysis has unraveled a strong self-interaction of SepJ, for which the linker domain
341 appears to be very important, indicating that SepJ can form multimers in the cells
342 producing it. All these observations are consistent with the idea that SepJ is part of a
343 septal junction complex in which SepJ multimers from adjacent cells interact,
344 presumably through the SepJ coiled-coil domains that, as described previously
345 (Mariscal *et al.*, 2011), are required to keep SepJ at the cell poles.

346 SepJ-GFP is also seen to localize in a ring, similar to a Z ring, when cell division
347 starts (Flores *et al.*, 2007; Mariscal and Flores, 2010), and a related location has also
348 been confirmed here for native SepJ by immunofluorescence (Fig. 4). Localization in a
349 Z ring and progressive focusing to the new cell poles as the septum is synthesized
350 during cell division suggested a relation with the divisome. Because *ftsZ* is an essential
351 gene in most bacteria including *Anabaena* (Zhang *et al.*, 1995), we constructed strain
352 CSFR18 in which, based on expression from a synthetic NtcA-dependent promoter, the
353 FtsZ levels depend on the provided nitrogen source. This strain produces very low
354 levels of FtsZ after incubation for a few days in the presence of ammonium, resulting in
355 malformed cells that eventually lyse. However CSFR18 can be maintained in the
356 presence of nitrate, although the highest levels of FtsZ, similar to the wild-type levels
357 and readily seen to form a Z ring, are observed in the vegetative cells of diazotrophic
358 filaments. Thus, we could study the localization of SepJ, tested by immunofluorescence,
359 as a function of FtsZ abundance in filaments of strain CSFR18 grown with nitrate and
360 incubated for a few days in medium with nitrate, ammonium or lacking a source of
361 combined nitrogen. Our results show that the correct localization of SepJ requires the
362 presence of close to normal FtsZ levels, which are best attained in the diazotrophic
363 filaments of strain CSFR18 (Fig. 4). In a complementary approach, we observed that
364 treatment of *Anabaena* cells with berberine impedes FtsZ ring formation, as previously

365 shown for *E. coli* (Domadia *et al.*, 2008; Boberek *et al.*, 2010), and affects the correct
366 localization of SepJ. All these results together suggest that FtsZ has a role in the
367 subcellular localization of SepJ.

368 Dependence of SepJ localization on FtsZ can be indirect, since FtsZ has a
369 scaffolding role for the divisome. We therefore addressed, using BACTH, the possible
370 direct interaction of SepJ with FtsZ and two downstream divisome proteins, FtsQ and
371 FtsW, all of them from *Anabaena*. A strong interaction was observed only between
372 SepJ and FtsQ, consistent with FtsQ recruiting SepJ to the divisome, which is
373 reminiscent of the FtsQ role in *E. coli* at recruitment of downstream cell division
374 proteins (Chen *et al.*, 2002). The interaction between SepJ and FtsQ could be confirmed
375 by co-purification of the two proteins expressed in *E. coli* (Fig. 7), which also showed a
376 role of the predicted periplasmic section of SepJ in this interaction. This is consistent
377 with the results of BACTH analysis, which suggest a role of the linker domain of SepJ
378 in a specific interaction with the periplasmic α domain of FtsQ. This domain exhibits
379 high similarity to polypeptide transport-associated (POTRA) domains (van den Ent *et*
380 *al.*, 2008). Although we cannot rule out that interactions between the transmembrane
381 segments of these proteins occur, our results support a specific interaction between parts
382 of the long extra-membrane section of SepJ and the periplasmic section of FtsQ. A
383 corollary of this observation is that the section of SepJ containing the coiled-coil and
384 linker domains is periplasmic, as predicted previously (Flores *et al.*, 2007). We
385 therefore suggest that SepJ localization at the cell poles in the intercellular septa
386 depends on the divisome, involving an interaction with FtsQ. Nonetheless, interactions
387 of SepJ with other divisome proteins may also take place, some of which may be
388 functionally redundant as is not uncommon in interactions between divisome proteins
389 (Lutkenhaus *et al.*, 2012). A more ample analysis of interactions between SepJ and

390 divisome proteins will need however an increased knowledge of the *Anabaena*
391 divisome. Localization of SepJ at the cell poles may additionally be stabilized by the
392 above-discussed interactions between the coiled-coil domains of SepJ proteins from
393 adjacent cells.

394 In filamentous cyanobacteria, when cell division is completed, the peptidoglycan
395 layers of the two adjacent cells remain fused in a substantial number of the filament's
396 septa allowing the isolation of murein sacculi corresponding to several cell units
397 (Lehner *et al.*, 2011), and the outer membrane does not enter into the septum between
398 adjacent cells (Wolk, 1996; Flores *et al.*, 2006; Wilk *et al.*, 2011). Thus, the divisome of
399 this type of cyanobacteria must differ in composition and/or regulation of its activity
400 from the divisome of unicellular bacteria, including unicellular cyanobacteria, which
401 performs splitting of septal peptidoglycan and invagination of the outer membrane to
402 complete cell division. Because SepJ or a SepJ-like protein is found in most filamentous
403 cyanobacteria (Mariscal *et al.*, 2011; Nürenberg *et al.*, 2014), an interaction of SepJ
404 with the divisome might contribute to the characteristic cell division of these organisms.
405 Besides SepJ, the *fraCDE* operon is often conserved in filamentous cyanobacteria
406 (Merino-Puerto *et al.*, 2013), and products of this operon have also been observed in the
407 place of the Z ring (FraC, observed with a FraC-GFP fusion; Merino-Puerto *et al.*,
408 2010) or in the growing intercellular septa (FraD, observed by means of immunogold
409 labeling; Merino-Puerto *et al.*, 2011), making it possible that these proteins interact with
410 the divisome as well. Specific late events during cell division may be at the basis of the
411 multicellular character of these bacteria, in which the divisome appears to have a role
412 localizing proteins essential for multicellularity.

413

414

415

416 **Experimental procedures**

417 *Strains and growth conditions*

418 *Anabaena* sp. strain PCC 7120 (also known as *Nostoc* sp. strain PCC 7120) and strain
419 CSFR18 were grown in BG11 (containing NaNO₃), BG11₀ (free of combined nitrogen)
420 or BG11₀ + ammonium (BG11₀ containing 4 mM NH₄Cl and 8 mM TES-NaOH buffer,
421 pH 7.5) media at 30°C in the light (25 μE m⁻² s⁻¹ from fluorescent lamps), in shaken (80-
422 90 rpm) liquid cultures or in medium solidified with 1% Difco agar. The BG11-based
423 medium contained ferric citrate instead of the ferric ammonium citrate used in the
424 original recipe (Rippka *et al.*, 1979). Media for strain CSFR18 was supplemented with 5
425 μg ml⁻¹ streptomycin sulfate (Sm) and 5 μg ml⁻¹ spectinomycin dihydrochloride
426 pentahydrate (Sp).

427 *Escherichia coli* DH5α and XL1-Blue (Stratagene) were used for plasmid
428 constructions. Strains HB101 and ED8654 were used for conjugation with *Anabaena*.
429 Strain BTH101 (*cya*-99) was used for BACTH analysis. Strain BL21-lacIq was used for
430 production of *Anabaena* FtsZ and co-purification assays. All *E. coli* strains were grown
431 in LB medium, supplemented when appropriate with antibiotics at standard
432 concentrations (Ausubel *et al.*, 2014; Karimova *et al.*, 2005).

433

434 *Plasmid construction and genetic procedures*

435 DNA was isolated from *Anabaena* sp. by the method of Cai and Wolk (1990). Plasmid
436 pCSFR15, carrying *ftsZ* (ORF *alr3858*) under the control of the synthetic NtcA-
437 regulatable promoter, P_{ND}, was prepared by PCR and standard cloning procedures.
438 pCSFR15 is a pMBL-based plasmid that contains a fragment upstream of *alr3858*
439 (*Anabaena* chromosome coordinates 4,655,349 to 4,655,844), amplified by PCR using
440 primers *alr3858*-3 and *alr3858*-4 (all oligodeoxynucleotide primers are described in

441 Table S1) and cloned between *Apa*I and *Sal*I sites; the C.S3 cassette (Elhai and Wolk,
442 1988; C.S3 is derived from the Ω cassette described by Prentki and Krisch, 1984)
443 cloned into *Bam*HI; a synthetic *NtcA*-regulated promoter generated by PCR using Pro-
444 s*NtcA*-1 and Pro-s*NtcA*-2 overlapping primers and cloned into *Spe*I and *Eco*RV sites;
445 and the 5' region of *alr3858* (coordinates 4,655,850 to 4,656,703), amplified by PCR
446 using primers *alr3858*-1 and *alr3858*-2 and cloned between *Sac*I and *Xho*I sites. The
447 insert of pCSFR15 was corroborated by sequencing and digested with *Pvu*II, and the
448 fragment containing the C.S3-P_{ND} construct was transferred to pRL278 previously
449 digested with *Xho*I and treated with the Klenow fragment producing pCSFR18. This
450 plasmid was transferred by conjugation, performed as described (Elhai *et al.*, 1997), to
451 *Anabaena* sp. strain PCC 7120 with selection for resistance to Sm and Sp. Cultures of
452 exconjugants obtained were used to select for clones resistant to 5% sucrose (Cai and
453 Wolk, 1990), and individual Suc^R colonies were checked by PCR. Clones in which the
454 C.S3-P_{ND} construct was inserted into *ftsZ* upstream region were isolated, and a clone
455 homozygous for the chromosomes bearing this construct was selected for further
456 analysis and named strain CSFR18.

457 For bacterial two-hybrid (BACTH) analysis, all genes were amplified using
458 *Anabaena* DNA as template. The following primers were used: *all0154*-9 and *all0154*-
459 10 to amplify *ftsW* (ORF *all0154*); *alr3857*-7 and *alr3857*-8 to amplify *ftsQ* (ORF
460 *alr3857*); and *alr3858*-13 and *alr3858*-14 to amplify *ftsZ*. The PCR products
461 corresponding to *alr0154* and *alr3857* were cloned in pKT25 using *Pst*I and *Bam*HI,
462 and that corresponding to *alr3858* was cloned in pKNT25 using the same enzymes. For
463 the *sepJ* gene (ORF *alr2338*), a PCR product amplified using *alr2338*-13 and *alr2338*-
464 35 primers was cloned in pCSVM97 (bearing the complete *sepJ* gene with the stop
465 codon substituted by a *Xho*I restriction site; unpublished) using *Pst*I and *Xba*I,

466 generating plasmid pCSE216. The pCSE216 insert was then transferred to pUT18 and
467 pKNT25 using PstI and SmaI. In addition, primers alr2338-35 and alr2338-36 were
468 used to amplify *sepJ*-truncated versions using genomic DNA from *Anabaena* strains
469 CSVM25, CSVM26, CSVM85 (Mariscal *et al.*, 2011) and CSVM90 (bearing a *sepJ*
470 gene encoding a SepJ protein lacking amino acid residues 463 to 748; unpublished).
471 The resulting PCR products were cloned in pUT18 and pKNT25 using PstI and SmaI
472 and sequenced. As a result, the following plasmids were generated: pCSFR30
473 (producing T25-FtsQ), pCSFR31 (producing T25-FtsW), pCSFR32 (producing FtsZ-
474 T25), pCSE221 (producing SepJ-T18), pCSE222 (producing SepJ-T25), pCSE226
475 (producing SepJ_CSVM25-T18), pCSE227 (producing SepJ_CSVM26-T18), pCSE228
476 (producing SepJ_CSVM90-T18), pCSE231 (producing SepJ_CSVM25-T25), pCSE236
477 (producing SepJ_CSVM90-T25), pCSE237 (producing SepJ_CSVM26-T25), pCSE239
478 (producing SepJ_CSVM85-T18) and pCSE240 (producing SepJ_CSVM85-T25). For
479 simplicity, SepJ_CSVM25 is denoted SepJ(Δ CC), SepJ_CSVM26 is denoted
480 SepJ(Δ pp), SepJ_CSVM85 is denoted SepJ(Δ linker), and SepJ_CSVM90 is denoted
481 SepJ(Δ TM).

482 Also for BACTH analysis, to produce a version of *Anabaena* FtsQ with the α
483 domain deleted, two DNA fragments, one encoding amino acid residues 1 to 59 and the
484 other one residues 128 to 281, were amplified by PCR using primer pairs alr3857-
485 7/alr3857-10 and alr3857-11/alr3857-8 respectively. Both DNA fragments were used as
486 template in an overlapping PCR using primers alr3857-7 and alr3857-8. The fragment
487 obtained was digested with PstI and BamHI and inserted into pKT25 with the same
488 enzymes producing pCSFR45, which encodes FtsQ($\Delta\alpha$) fused to the C terminus of the
489 T25 subunit. To produce a version of *Anabaena* FtsQ lacking the β domain (lacking
490 amino acid residues 128 to 281) and fused to the C-terminus of the T25 subunit, a DNA

491 fragment obtained by PCR using primers alr3857-7 and alr3857-9 (which includes a
492 termination codon) was cloned in pKT25 using PstI and BamHI. This plasmid was
493 called pCSFR46.

494 To produce *Anabaena* FtsZ protein and obtain an antibody against it, the *ftsZ*
495 gene was amplified using *Anabaena* DNA as template and primers alr3858-7 and
496 alr3858-8, and the PCR product was cloned in vector pCOLADuet-1 (Novagen) using
497 BamHI and XhoI, producing plasmid pCSFR22.

498 For co-purification assays, plasmids bearing genes encoding GFP-tagged SepJ
499 (or GFP-tagged SepJ without most of its predicted periplasmic section, denoted Δ pp-
500 SepJ-GFP) and His-tagged FtsQ were constructed. The *Anabaena ftsQ* gene was
501 amplified using primers alr3857-13 and alr3857-14, and the PCR product was digested
502 with BamHI and XhoI and cloned in pACYCDuet (Novagen) using the same enzymes,
503 producing plasmid pCSFR50 (six histidine residues added to the N terminus of FtsQ).
504 To produce SepJ-GFP and Δ pp-SepJ-GFP a SacI-EcoRI fragment from pCSAL33
505 (bearing the *gfp-mut2* gene; A. López-Lozano and A. Herrero) was cloned in pCSE221
506 or in pCSE227, producing pCSFR51 and pCSFR52 respectively.

507

508 *Expression and purification of Anabaena FtsZ*

509 Plasmid pCSFR22, which contains the *Anabaena ftsZ* gene fused to a sequence
510 encoding a His₆ tag under an IPTG-inducible promoter, was transferred to *E. coli* BL21-
511 lacIq. A pre-inoculum of this strain grown overnight in LB medium supplemented with
512 50 μ g of kanamycin sulfate (Km) ml⁻¹ and 2% glucose was washed with LB medium
513 and used to inoculate 1 L of LB medium + Km. The culture was incubated at 37°C up to
514 an OD₆₀₀ of 0.6. Protein expression was induced by addition of 1 mM isopropyl- β -D-1-
515 thiogalactopyranoside (IPTG). After 3 h at 37°C, cells were collected and resuspended

516 in a buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl and 10% glycerol (5
517 ml/g of cells). DNaseI and protease inhibitor cocktail *complete Mini EDTA-free* (Roche)
518 were added just before breakage of the cells by passage twice through a French pressure
519 cell at 20,000 psi. After centrifugation at 15,000 g (10 min, 4°C), the His₆-FtsZ protein
520 was purified from the supernatant by chromatography through a 5-ml His-Select column
521 from Sigma, using imidazole to elute the retained proteins. Samples obtained after
522 purification were subjected to SDS-PAGE, excised from the gel, electro-eluted and
523 concentrated (Stirred Ultrafiltration Cell, Millipore). An amount of 1.4 mg of purified
524 protein was used in subcutaneous injection of a rabbit to produce antibodies in the
525 ‘Centro de Producción y Experimentación Animal’, Universidad de Sevilla (Seville,
526 Spain). Antiserum was recovered 90 days after the first injection and stored at -80°C
527 until used.

528

529 *Protein sample preparation and western blots*

530 Samples containing 5 µg of chlorophyll *a* were taken from cultures of *Anabaena* strains
531 incubated in the presence of different nitrogen sources for 48 h. Total proteins were
532 precipitated by incubating samples in 10% trichloroacetic acid at 4°C for at least 30
533 min, subsequent centrifugation at 13,200 g (4°C, 30 min) and finally washed with cold
534 acetone. The protein pellet was dried for 15 min and then resuspended in a buffer
535 containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl and 10% glycerol. After that,
536 samples were mixed with 1 volume of 2x sample buffer, incubated at 95°C for 15 min,
537 run in a 10% Laemmli SDS-PAGE system, and transferred to PVDF membrane filters
538 as previously reported (Mariscal *et al.*, 2011). For detection of *Anabaena* FtsZ, the
539 filters were incubated overnight in blocking buffer containing 10 mM Tris-HCl (pH
540 7.5), 150 mM NaCl, 5% non-fat milk powder and 0.05% Tween-20. Afterwards,

541 primary anti-FtsZ serum (diluted 1:1000 in blocking buffer) was added, incubated at
542 30°C for 1 h and washed three times with TBS. Secondary antibody (anti-rabbit IgG
543 conjugated to peroxidase from Sigma) was then added at a dilution 1:10,000 in blocking
544 buffer, incubated 1 h at 30°C and washed three times with TBS. Detection was
545 performed with a chemiluminiscence kit (WesternBright™ ECL, Advansta) and
546 exposure to hyperfilm (GE Healthcare).

547 For co-purification assays, *E. coli* strains expressing *Anabaena* FtsQ fused to a
548 His₆ tag and SepJ or Δpp-SepJ fused to GFP, or control plasmid vectors, were induced
549 with IPTG as described above. After 4 h at 37°C, cells were collected and resuspended
550 in 5 mL of PBS containing 140 mM NaCl, 1.5 mM KH₂PO₄, 2.7 mM KCl (pH 7.4) and
551 one tablet of protease inhibitor cocktail *complete Mini EDTA-free* (Roche). Cells were
552 disrupted by passage twice through a French pressure cell at 20,000 psi. After
553 centrifugation at 15,000 *g* (10 min, 4°C), cell extracts were incubated with μMACS
554 Anti-GFP MicroBeads (Miltenyi Biotec) for 1 h. Afterwards, the mixture was loaded
555 into a MACS column (Miltenyi Biotec) and the column was washed with 3 mL of PBS
556 buffer. Elution of the GFP-tagged protein (SepJ or Δpp-SepJ) and its interacting
557 protein(s) was accomplished with buffer containing 50 mM Tris-HCl (pH 6.8), 50 mM
558 DTT, 1% SDS, 1 mM EDTA, 0.005% bromphenol blue and 10% glycerol. The eluate
559 was subjected to electrophoresis in a 10% Laemmli SDS-PAGE system. SepJ-GFP and
560 Δpp-SepJ-GFP were detected by western blot as described above using an anti-GFP
561 antibody (A6455 from Invitrogen) diluted 1:2,000. His₆-tagged FtsQ was detected using
562 anti-His HRP-conjugated antibody (Qiagen) following the instructions from the
563 supplier.

564

565 *Growth rates*

566 The growth rate, μ , which corresponds to $\ln 2/t_d$, where t_d is the doubling time, was
567 calculated from the increase of protein concentration determined by a modified Lowry
568 procedure (Markwell *et al.*, 1978) in 0.2-ml samples from shaken cultures. The growth
569 rate was followed for a period of 5 days, between cellular densities corresponding to 5
570 to about 100 μg of protein (0.2-4 μg of chlorophyll *a*) per ml. Chlorophyll *a* content of
571 cultures was determined by the method of Mackinney (1941).

572

573 *Analysis of ftsZ expression by RT-qPCR*

574 RNA was isolated as described previously (Mohamed and Jansson, 1989) from cultures
575 of *Anabaena* strains incubated in the presence of different nitrogen sources. RNA (100
576 ng) was used for retrotranscription using Quantitect Reverse Transcription Kit (Qiagen).

577 cDNA obtained was used to carry out real time PCR using *iCycler iQ Real Time PCR*

578 *Detection System* equipped with the *iCycler iQ v 3.0* software from BioRad. PCR

579 amplification was carried out using SensiFASTTM SYBR & Fluorescein Kit (BioLine)

580 following the instructions from the supplier. The amplification protocol was as follows:

581 1 cycle at 95°C for 2 min, 30 cycles of: 95°C for 15 s, 67°C for 20 s and 72°C for 30 s.

582 After this protocol was ended, a melting point calculation protocol was done in order to

583 check that only the correct product was amplified in each tube. The expression of

584 *alr0599* and *all5167* (Flaherty *et al.*, 2011) was used as internal standards to normalize

585 the values obtained for *alr3858* (*ftsZ*). To study expression of these genes, the following

586 primer pairs were used: *alr0599-1/alr0599-2*, *all5167-1/all5167-2*, and *alr3858-*

587 *9/alr3858-10*, respectively.

588 The mathematical treatment of data to calculate relative gene expression was

589 performed according to Pfaffl (2001) using the formula: Relative gene expression =

590 $2^{-\Delta\Delta C_t}$. Where $\Delta\Delta C_t$ corresponds to the increase in the threshold cycle of the problem

591 gene with respect to the increase in the threshold cycle of the housekeeping genes
592 (*alr0599* and *all5167*). The final quantification value for each condition indicates the
593 relative change of gene expression in strain CSFR18 and the wild type with respect to
594 the wild-type strain grown with nitrate as nitrogen source.

595

596 *Immunolocalization and fluorescence microscopy*

597 For immunolocalization of SepJ or FtsZ, cells from 1.5 ml of liquid cultures were
598 collected by centrifugation, placed atop a poly-L-lysine pre-coated microscope slide and
599 covered with a 45- μ m pore-size Millipore filter. Afterwards, the filter was removed and
600 the slide was let to dry at room temperature and, then, immersed in 70% ethanol
601 at -20°C for 30 min and dried 15 min at room temperature. The cells were washed twice
602 (2 min each time, room temperature) by covering the slide with PBS-T (PBS
603 supplemented with 0.05% Tween-20). Subsequently, the slides were treated with a
604 blocking buffer (5% milk powder in PBS-T) for 15 min. Cells on the slides were then
605 incubated with a primary antibody (anti-SepJ-CC [Mariscal *et al.*, 2011], diluted in
606 blocking buffer 1:250, or anti-FtsZ serum, diluted 1:100) for 90 min, washed three
607 times with PBS-T, incubated 45 min in the dark with secondary anti-rabbit antibody
608 conjugated to fluorescein isothiocyanate (FITC) (Sigma, 1:500 dilution in PBS-T) and
609 washed three times with PBS-T. After dried, several drops of FluorSave (Calbiochem)
610 were added atop, covered with a coverslip and sealed with nail lack. Fluorescence was
611 imaged using a Leica DM6000B fluorescence microscope and an ORCA-ER camera
612 (Hamamatsu). Fluorescence was monitored using a FITC L5 filter (excitation, band-
613 pass (BP) 480/40 filter; emission, BP 527/30 filter). Images were analyzed using the
614 ImageJ software (<http://imagej.nih.gov/ij>).

615

616 *Treatment with berberine*

617 Cultures of wild-type *Anabaena* grown in BG11 medium and containing about 1 μg
618 chlorophyll *a* ml^{-1} were incubated in the presence of 0.1 to 1 mM berberine
619 hemisulphate (Sigma) at 30°C for 24 to 72 h. After incubation, cells were harvested by
620 centrifugation and the localization of FtsZ and SepJ was studied by
621 immunofluorescence as described above.

622

623 *BACTH complementation assays*

624 Plasmids used for BACTH assays (Karimova *et al.*, 2005) were co-transformed into
625 BTH101 (*cya-99*). The transformants were plated onto LB medium containing selective
626 antibiotics, 40 $\mu\text{g ml}^{-1}$ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and
627 0.5 mM IPTG and, then, incubated at 30°C for 24 to 36 h. Efficiencies of interactions
628 between different hybrid proteins were quantified by measuring β -galactosidase activity
629 in liquid cultures. Bacteria were grown in LB medium in the presence of 0.5 mM IPTG
630 and appropriate antibiotics at 30°C for 16 h. Before the assays, the cultures were diluted
631 1:5 into buffer Z (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl and 1 mM MgSO_4).
632 To permeabilize cells, 30 μl of toluene and 35 μl of a 0.1% SDS solution were added to
633 2.5 ml of bacterial suspension. The tubes were vortexed for 10 s and incubated with
634 agitation at 37°C for 45 min for evaporation of toluene. For the enzymatic reaction, 875
635 μl of permeabilized cells were added to buffer Z supplemented with β -mercaptoethanol
636 (25 mM final concentration), to a final volume of 3.375 ml. The tubes were incubated at
637 30°C in a water bath for at least 5 min. The reaction was started by adding 875 μl of 0.4
638 mg ml^{-1} *o*-nitrophenol- β -galactoside (ONPG) in buffer Z without β -mercaptoethanol. 1-
639 ml samples, taken at times up to 10 min, were added to 0.5 ml of 1 M Na_2CO_3 to stop
640 the reaction. $A_{420 \text{ nm}}$ was recorded, and the amount of *o*-nitrophenol produced was

641 calculated using an extinction coefficient $\epsilon_{420\text{ nm}} = 4.5\text{ mM}^{-1}\text{ cm}^{-1}$ and referred to the
642 amount of total protein, determined by a modified Lowry procedure (Markwell *et al.*,
643 1978). The *o*-nitrophenol produced per mg of protein versus time was represented, and
644 β -galactosidase activity was deduced from the slope of the linear function.

645

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650

651 **Conflict of interest**

652 The authors declare that they have no conflict of interest.

653

654 **References**

655

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838

839 **Figure legends**

840

841 **Fig. 1.** Genomic structure and phenotype of *Anabaena* sp. strain CSFR18 (C.S3-P_{ND}-
842 *ftsZ*). (A) Schematic (not to scale) of the *ftsQ-ftsZ* genomic region in strain CSFR18,
843 including, shown in blue color, the sequence of the DNA fragment bearing the
844 introduced synthetic NtcA-dependent promoter: blue bracket, NtcA-binding site; red
845 bracket, -10 promoter hexamer; black arrow, predicted transcription start site. Sequence
846 in red corresponds to the end of C.S3 and sequence in green to the *ftsZ* 5' and upstream
847 region (the three first codons of the gene are underlined). C.S3 includes the Ω cassette
848 that encodes resistance to Sm and Sp (gene orientation indicated by the white arrow)
849 and bears transcriptional terminators in both ends, indicated by white exes (Elhai and
850 Wolk, 1988). (B) Test of growth on solid media. Samples from BG11-grown filaments
851 of strains PCC 7120 (wild type) and CSFR18 were spotted at different dilutions in solid
852 media with the indicated nitrogen source, incubated under growth conditions and
853 photographed after 7 days. (C, D) Cultures of BG11-grown filaments of the indicated
854 strains were incubated under growth conditions in liquid media with the indicated
855 nitrogen sources and visualized by light microscopy after 5 days (C) or photographed
856 after 7 days (D). Size bars in C, 3 μm . The area of the cells was determined in the
857 different liquid cultures with the following results: nitrate-containing cultures, $12.51 \pm$
858 $0.53 \mu\text{m}^2$ for the wild type and $22.9 \pm 1.14 \mu\text{m}^2$ for the mutant; ammonium-containing
859 cultures, $13.36 \pm 0.68 \mu\text{m}^2$ for the wild type and $45.37 \pm 3.61 \mu\text{m}^2$ for the mutant;
860 cultures without combined nitrogen, $12.18 \pm 0.33 \mu\text{m}^2$ for the wild type and 10.9 ± 0.50
861 μm^2 for the mutant (37 cells for each strain and growth condition were measured).
862 Student's *t* test indicated that the differences between the mutant and the wild type were
863 significant in the cultures containing nitrate ($P < 10^{-11}$) or ammonium ($P < 10^{-12}$).

864

865 **Fig. 2.** Expression of *ftsZ* in *Anabaena* strains PCC 7120 (wild type) and CSFR18
866 (C.S3-P_{ND-ftsZ}). (A) Levels of *ftsZ* transcript in strains CSFR18 and PCC 7120, relative
867 to those in nitrate-grown PCC 7120 (wild type) cells. RNA was isolated from BG11-
868 grown filaments incubated for 48 hours under culture conditions with the indicated
869 nitrogen source, and RT-qPCR was performed as described in Experimental procedures.
870 (B) Western blot analysis of FtsZ. BG11-grown filaments of the indicated strain were
871 incubated for 48 hours under culture conditions with the indicated nitrogen source, and
872 extracts were prepared, loaded into SDS-PAGE gels (60 µg protein per lane),
873 electrophoresed and probed with antibodies raised against the *Anabaena* FtsZ protein as
874 described in Experimental procedures.

875

876 **Fig. 3.** Immunofluorescence localization of FtsZ in *Anabaena* strains PCC 7120 (wild
877 type) and CSFR18 (C.S3-P_{ND-ftsZ}). BG11-grown filaments of the indicated strain were
878 incubated for 48 hours under culture conditions with the indicated nitrogen source,
879 prepared for immunofluorescence analysis with anti *Anabaena* FtsZ protein antibodies,
880 and visualized by fluorescence microscopy as described in Experimental procedures.
881 Arrows point to heterocysts. Size bar, 3 µm; magnification was the same for all
882 micrographs. Merged bright-field and fluorescence images are shown.

883

884 **Fig. 4.** Immunofluorescence localization of SepJ in *Anabaena* strains PCC 7120 (wild
885 type) and CSFR18 (C.S3-P_{ND-ftsZ}). BG11-grown filaments of the indicated strain were
886 incubated for 48 h under culture conditions with the indicated nitrogen source, prepared
887 for immunofluorescence analysis with antibodies raised against the coiled-coil domain
888 of the *Anabaena* SepJ protein, and visualized by fluorescence microscopy as described

889 in Experimental procedures. Size bars, 3 μm . Arrowheads point to places, in dividing
890 cells, where the immunofluorescence signal is in a position similar to that of a Z ring.
891 Bright-field, fluorescence (SepJ) and merged images are shown.

892

893 **Fig. 5.** FtsZ and SepJ localization in berberine-treated *Anabaena* filaments. Filaments
894 grown in BG11 medium were treated (+) or not (-) with 0.1 mM berberine for 24 h and
895 subjected to immunofluorescence analysis with anti FtsZ and anti SepJ-CC antibodies
896 as described in Experimental procedures. Size bar, 3 μm ; magnification was the same
897 for the four micrographs. Merged bright-field and fluorescence images are shown

898

899 **Fig. 6.** Schematic of the protein fusions used in BACTH analysis. The T25 and T18
900 fragments of the catalytic subunit of adenylate cyclase are represented as block arrows
901 indicating the orientation (N-terminal to C-terminal) of the polypeptide. The SepJ
902 protein (751 amino acid residues; blue) consists of three domains: N-terminal coiled-
903 coil domain (CC), linker and C-terminal permease (likely containing 9 or 11
904 transmembrane segments). *Anabaena* FtsQ (281 amino acid residues; green) is
905 predicted to contain the same domains as *E. coli* FtsQ: an N-terminal transmembrane
906 segment and periplasmic α (POTRA) and β domains (van den Ent *et al.*, 2008).
907 *Anabaena* FtsZ (428 amino acid residues; yellowish) is a predicted soluble protein.
908 *Anabaena* FtsW (396 amino acid residues; red) is predicted to have 8 transmembrane
909 segments with its N- and C-termini in the cytoplasmic side of the cytoplasmic
910 membrane. N denotes the N-terminus in each fusion protein.

911

912

913 **Fig. 7.** Joint extraction from *E. coli* of SepJ-GFP and His₆-tagged *Anabaena* FtsQ. Total
914 extracts from cells of *E. coli* expressing SepJ-GFP or Δpp-SepJ-GFP and/or His₆-tagged
915 *Anabaena* FtsQ were allowed to interact with anti-GFP MicroBeads and loaded into a
916 MACS column, and the retained material was then eluted and subject to SDS-PAGE.
917 Tagged *Anabaena* FtsQ (about 33 kDa) was identified using an anti-pentahistidine
918 antibody (A), and tagged SepJ was identified using an anti-GFP antibody (B). For each
919 lane the proteins expressed in the corresponding *E. coli* strain are shown: SepJ refers to
920 SepJ-GFP; Δpp, SepJ-GFP without most of the predicted SepJ periplasmic section;
921 FtsQ, His₆-tagged *Anabaena* FtsQ. φ, plasmid vector without insert. White triangles
922 point to signals corresponding to the SepJ-GFP fusion protein (about 108 kDa) and
923 black triangles point to Δpp-SepJ-GFP (about 68 kDa). The SepJ protein generates
924 forms moving to different extents in SDS-PAGE gels (Mariscal *et al.*, 2011). Some
925 degradation of the SepJ-GFP fusion proteins releasing at least two forms of GFP (about
926 27 kDa) and, in the case of the complete protein, possibly also a protein lacking the
927 predicted periplasmic section appears to have taken place.

928

929

T18 fusion	T25 fusion	β -Galactosidase activity (nmol ONP [mg protein] ⁻¹ min ⁻¹)	Student's <i>t</i> test
		Mean \pm SD (n)	<i>P</i>
<i>Negative control</i>			
T18	T25	9.70 \pm 2.06 (8)	
<i>SepJ self-interactions</i>			
T18	SepJ-T25	9.35 \pm 1.38 (8)	0.6969
SepJ-T18	T25	10.04 \pm 2.45 (8)	0.7705
SepJ-T18	SepJ-T25	199.47 \pm 57.95 (7)	4 E-07 (*)
T18	SepJ(Δ TM)-T25	10.76 \pm 0.63 (4)	0.3489
T18	SepJ(Δ app)-T25	12.05 \pm 1.81 (4)	0.0829
T18	SepJ(Δ linker)-T25	9.76 \pm 1.10 (4)	0.9589
T18	SepJ(Δ CC)-T25	12.44 \pm 1.65 (4)	0.0443
SepJ(Δ TM)-T18	T25	10.18 \pm 4.17 (6)	0.7791
SepJ(Δ app)-T18	T25	9.32 \pm 2.09 (6)	0.7395
SepJ(Δ linker)-T18	T25	10.51 \pm 4.25 (6)	0.6431
SepJ(Δ CC)-T18	T25	11.47 \pm 3.74 (6)	0.2791
SepJ(Δ TM)-T18	SepJ(Δ TM)-T25	14.33 \pm 4.34 (4)	0.0278
SepJ(Δ TM)-T18	SepJ-T25	11.85 \pm 1.89 (4)	0.1110
SepJ-T18	SepJ(Δ TM)-T25	15.16 \pm 5.36 (4)	0.0256
SepJ(Δ app)-T18	SepJ(Δ app)-T25	16.69 \pm 1.81 (4)	0.0002 (*)
SepJ(Δ app)-T18	SepJ-T25	11.48 \pm 1.41 (4)	0.1548
SepJ-T18	SepJ(Δ app)-T25	11.27 \pm 2.02 (4)	0.2399
SepJ(Δ linker)-T18	SepJ(Δ linker)-T25	25.50 \pm 3.39 (4)	1 E-06 (*)
SepJ(Δ linker)-T18	SepJ-T25	45.16 \pm 9.31 (4)	8 E-07 (*)
SepJ-T18	SepJ(Δ linker)-T25	73.83 \pm 14.08 (3)	2 E-07 (*)
SepJ(Δ CC)-T18	SepJ(Δ CC)-T25	154.65 \pm 30.36 (3)	1 E-07 (*)
SepJ(Δ CC)-T18	SepJ-T25	69.36 \pm 16.90 (3)	3 E-06 (*)
SepJ-T18	SepJ(Δ CC)-T25	74.01 \pm 17.07 (4)	6 E-07 (*)
<i>SepJ-divisome protein interactions</i>			
T18	FtsZ-T25	8.51 \pm 1.70 (4)	0.3451
T18	T25-FtsW	9.30 \pm 1.19 (4)	0.7298
T18	T25-FtsQ	8.69 \pm 2.32 (6)	0.4053
SepJ-T18	FtsZ-T25	7.50 \pm 1.53 (4)	0.0903
SepJ-T18	T25-FtsW	28.68 \pm 23.14 (4)	0.0359
SepJ-T18	T25-FtsQ	207.50 \pm 118.67 (5)	0.0005 (*)
SepJ(Δ TM)-T18	T25-FtsQ	10.34 \pm 1.86 (4)	0.6161
SepJ(Δ app)-T18	T25-FtsQ	15.64 \pm 5.42 (4)	0.0181
SepJ(Δ linker)-T18	T25-FtsQ	10.18 \pm 4.00 (3)	0.7930
SepJ(Δ CC)-T18	T25-FtsQ	161.87 \pm 50.86 (4)	5 E-06 (*)
T18	T25-FtsQ($\Delta\alpha$)	5.72 \pm 4.01 (2)	0.0680
T18	T25-FtsQ($\Delta\beta$)	7.14 \pm 4.49 (2)	0.2298
SepJ-T18	T25-FtsQ($\Delta\alpha$)	15.10 \pm 8.48 (4)	0.1057
SepJ-T18	T25-FtsQ($\Delta\beta$)	302.06 \pm 121.31 (3)	4 E-05 (*)

Table 1. Quantification of SepJ self-interactions and interactions between SepJ and some divisome proteins assessed by BACTH.

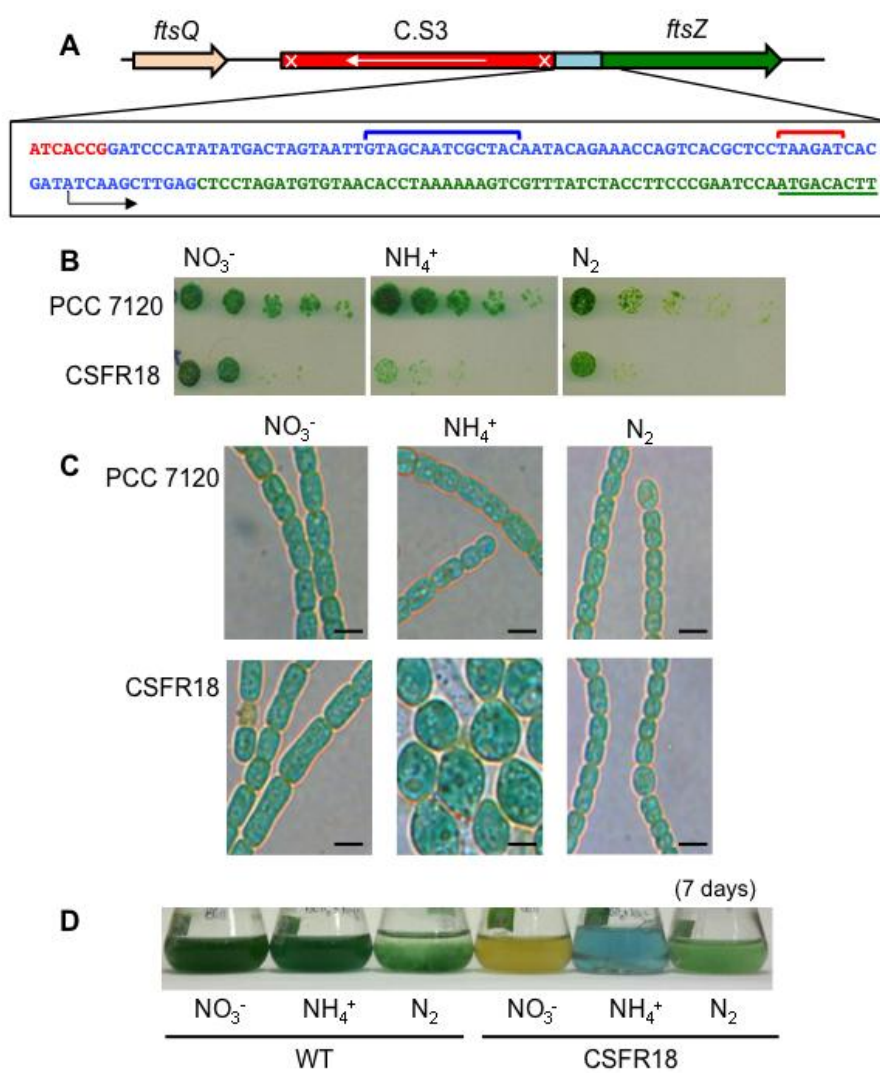
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The interactions of the proteins fused to the T18 and T25 vectors cloned in *E. coli* were measured as β -galactosidase activity in liquid cultures as described in Experimental procedures. The protein fused to the N- or the C-terminus of T18 or T25 is indicated in each case (N-terminus, protein-T18 or protein-T25; C-terminus, T18-protein or T25-protein). Non-fused T18/T25 plasmid pair was used as negative control. A T18-zip/T25-zip positive control produced an activity of about 600 nmol ONP (mg protein)⁻¹ min⁻¹. The mean and standard deviation of the results from the number of experiments indicated (n) is presented. The difference between each plasmid combination and the T18/T25 plasmid pair was assessed by the Student's *t* test (*P* indicated in each case); an asterisk (*) highlights differences significant at *P* ≤ 0.0005.

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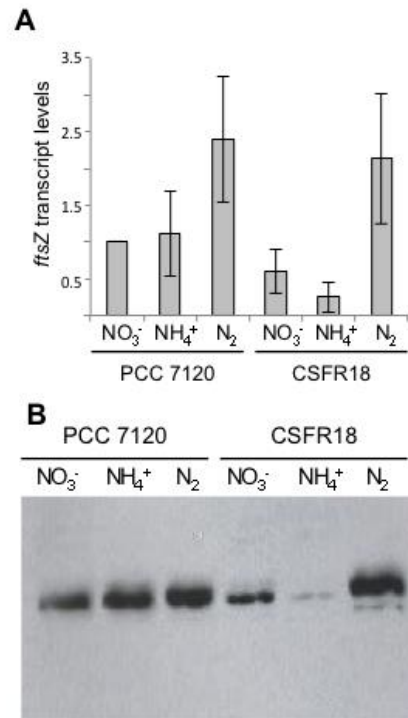
Fig. 1



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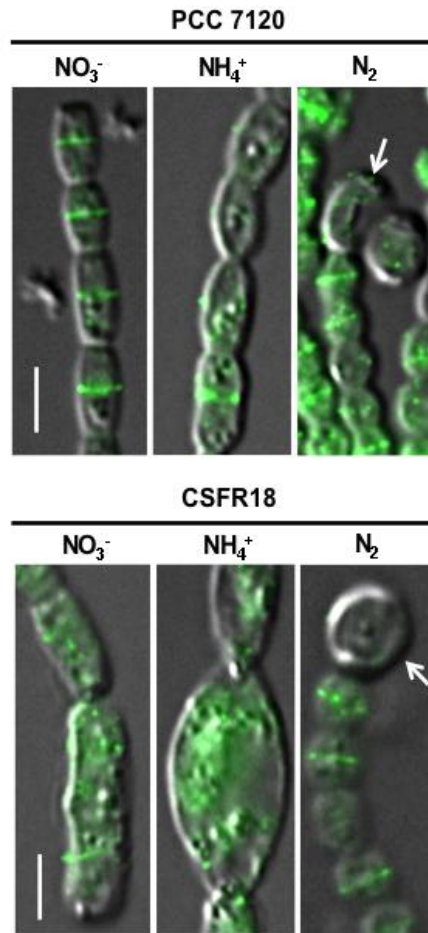
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Fig. 2

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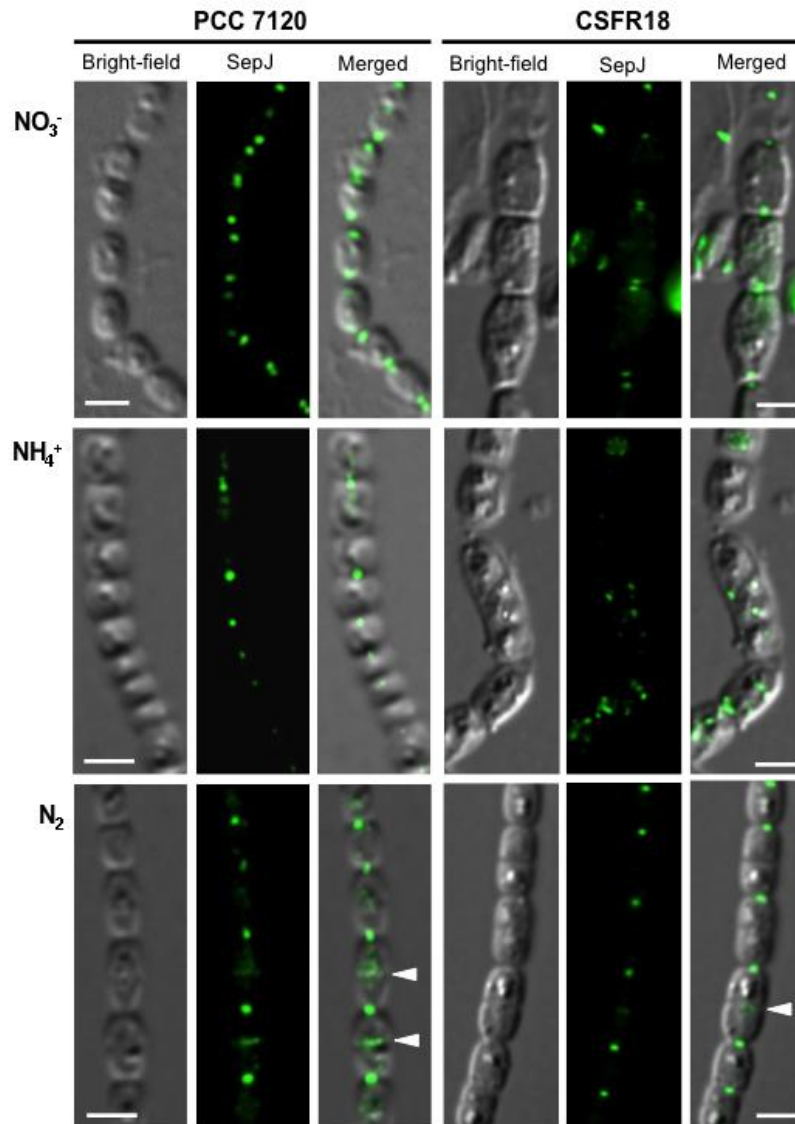
Fig. 3



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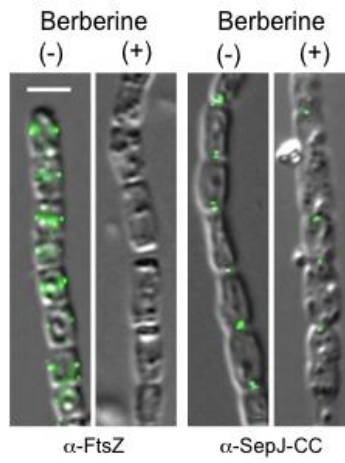
Fig. 4



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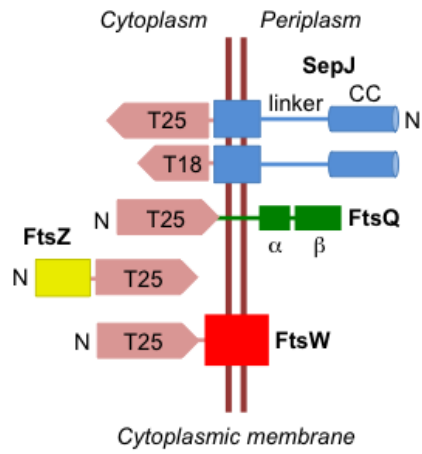
Fig. 5



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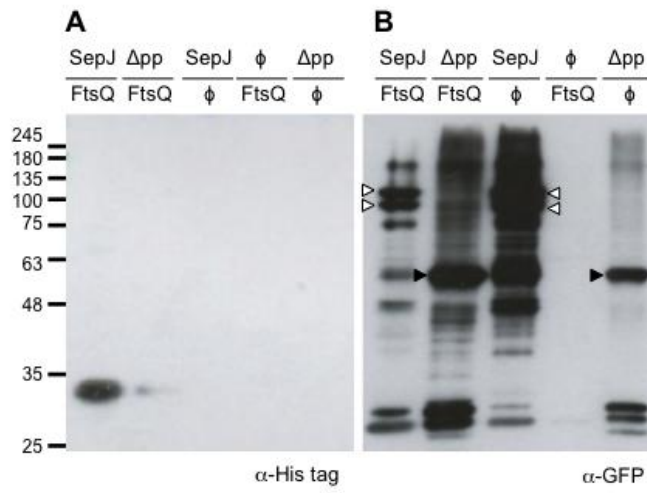
Fig. 6



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Fig. 7



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M

30-Jan-2015

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Dear Enrique,

I am happy to tell you that your manuscript entitled Divisome-dependent subcellular localization of cell-cell joining protein SepJ in the filamentous cyanobacterium *Anabaena* has been accepted for publication in *Molecular Microbiology* without further review. I have also requested that your colour fees be waived as agreed previously.

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