

1 ***Sinorhizobium meliloti* FcrX coordinates cell cycle and division during free-**
2 **living growth and symbiosis**

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17 **ABSTRACT**

18

19 *Sinorhizobium meliloti* is a soil bacterium that establishes a symbiosis within root nodules of
20 legumes (*Medicago sativa*, for example) where it fixes atmospheric nitrogen into ammonia and
21 obtains in return carbon sources and other nutrients. In this symbiosis, *S. meliloti* undergoes a
22 drastic cellular change leading to a terminal differentiated form (called bacteroid) characterized
23 by genome endoreduplication, increase of cell size and high membrane permeability. The
24 bacterial cell cycle (mis)regulation is at the heart of this differentiation process. In free-living
25 cells, the master regulator CtrA ensures the progression of cell cycle by activating cell division
26 (controlled by the tubulin-like protein FtsZ) and simultaneously inhibiting supernumerary DNA
27 replication, while on the other hand the downregulation of CtrA and FtsZ is essential for
28 bacteroid differentiation during symbiosis, preventing endosymbiont division and permitting
29 genome endoreduplication. Little is known in *S. meliloti* about regulators of CtrA and FtsZ, as
30 well as the processes that control bacteroid development. Here, we combine cell biology,
31 biochemistry and bacterial genetics approaches to understand the function(s) of FcrX, a new
32 factor that controls both CtrA and FtsZ, in free-living growth and in symbiosis. Depletion of
33 the essential gene *fcrX* led to abnormally high levels of FtsZ and CtrA and minicell formation.
34 Using multiple complementary techniques, we showed that FcrX is able to interact physically
35 with FtsZ and CtrA. Moreover, its transcription is controlled by CtrA itself and displays an
36 oscillatory pattern in the cell cycle. We further showed that, despite a weak homology with FliJ-
37 like proteins, only FcrX proteins from closely-related species are able to complement *S. meliloti*
38 *fcrX* function. Finally, deregulation of FcrX showed abnormal symbiotic behaviors in plants
39 suggesting a putative role of this factor during bacteroid differentiation. In conclusion, FcrX is
40 the first known cell cycle regulator that acts directly on both, CtrA and FtsZ, thereby controlling
41 cell cycle, division and symbiotic differentiation.

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43 INTRODUCTION

44

45 *Sinorhizobium meliloti* belongs to the class of *Alphaproteobacteria* and is known for its dual
46 lifestyle: as a free-living bacterium in the soil and as a symbiotic endophyte within legumes of
47 the genera *Medicago*, *Melilotus* and *Trigonella*¹. Free-living *S. meliloti* cells thrive in the soil
48 and divide asymmetrically to produce two physiologically and morphologically different cell
49 types (Figure 1A): a smaller cell, unable to replicate its DNA and a larger cell able to replicate
50 its DNA once per cell cycle. The small cell first has to differentiate into the large cell type, upon
51 suitable nutrient conditions, before it can undergo genome replication².

52 To ensure a normal progression of the cell cycle, it has to be tightly regulated. In
53 *Alphaproteobacteria* the response regulator CtrA plays a central role in this process by binding
54 to the DNA, mostly activating or inhibiting transcription of more than a hundred target genes
55^{3,4}. The function of CtrA has been mainly studied in *Caulobacter crescentus*, an aquatic
56 bacterium that divides asymmetrically, as *S. meliloti*, giving two morphologically and
57 physiologically different daughter cells⁵; a stalked cell, able to replicate immediately after
58 division, and a non-replicative motile cell that is blocked at the G1 phase. CtrA is under a strict
59 control, as its levels oscillate during the cell cycle, reaching a maximum in two moments: i. at
60 the G1 phase (motile cell), when it inhibits DNA replication by binding to specific sites that
61 prevent origin binding of DnaA, the initiation factor of DNA replication, and ii. at the end of
62 S-phase/G2, when CtrA also activates the cell division process⁶. To permit this pattern,
63 regulation mechanisms at the transcriptional and post translational (by phosphorylation and
64 proteolysis) levels are involved^{7,8}. An homolog of CtrA is present in *S. meliloti*, where it has a
65 similar function^{9,10}. In *S. meliloti*, CtrA inhibits indirectly the DNA replication by a yet-
66 unknown process and activates the cell division by repressing the transcription of the *minCDE*
67 system¹⁰, which ultimately inhibits the polymerization of the tubulin-like FtsZ responsible of
68 cell constriction at the septum¹¹. The FtsZ protein is composed by a N terminal core region,
69 containing a GTPase domain, involved in the polymerization activity, and a C terminus
70 responsible for the interaction to other actors¹². In *S. meliloti*, FtsZ is present in two copies,
71 FtsZ1 and FtsZ2, however only FtsZ1 is essential for the Z ring formation, while the second
72 copy lacks the C-terminal domain and its deletion is not lethal¹³. As many cell cycle actors,
73 FtsZ1 is expressed at the predivisional phase of the cell cycle¹⁴.

74 In symbiosis with *Medicago* plants, *S. meliloti* colonizes special root organs, called nodules.
75 There, it fixes atmospheric nitrogen into ammonium that is assimilated by the plant, while it

76 receives in return dicarboxylic acids and other nutrients ¹⁵. In nodules, after a stage of
77 multiplication, *S. meliloti* undergoes a drastic cellular change into a terminally differentiated
78 form called bacteroid. This process takes place intracellularly, inside the nodule plant cells,
79 where differentiated bacteroids are characterized by genome endoreduplication, cell
80 enlargement and high membrane permeability ¹⁶. Previous studies have shown an implication
81 of the bacterial cell cycle regulation in this differentiation process (Pini et al., 2013-2015;
82 Kobayashi et al., 2009). Indeed, CtrA and FtsZ are absent in bacteroids ^{17,18} and mutants that
83 overexpress CtrA are characterized by a symbiotic defect ¹⁷. Interestingly, the depletion of *ctrA*
84 in *S. meliloti*, similarly to *C. crescentus*, leads to a cell elongation/enlargement and
85 endoreduplication phenotype that is strikingly similar to bacteroids formed in nodules. The
86 mechanism leading to the downregulation of CtrA and FtsZ in bacteroids is not known yet but
87 studies showed the involvement of plant-produced Nodule-specific Cysteine-Rich (NCR)
88 peptides ^{19,20} (Figure 1A). Legumes such as *Medicago truncatula* produce a wide spectrum of
89 NCR peptides (about 600) that are implicated in the disruption of several cellular processes
90 including the cell cycle, thus resulting in the terminal differentiation of *S. meliloti* (Mergaert *et*
91 *al.*, 2006; Van de Velde *et al.*, 2010; Alunni & Gourion, 2016). Indeed, the treatment of a wild
92 type strain of *S. meliloti* with the NCR247 showed a down regulation of CtrA and its regulon.
93 Farkas and colleagues also highlighted a physical interaction between this NCR and FtsZ
94 (Farkas *et al.*, 2014). Overall these data strongly suggest that the regulation of bacterial cell
95 cycle and cell division are playing a major role in the symbiosis process ²¹.
96 Here we characterized the role of a new cell cycle regulator, named FcrX, elucidating its role
97 with respect to CtrA and FtsZ1 and FtsZ2, its regulation by transcription, its conservation across
98 the class *Alphaproteobacteria* and finally, its role during symbiosis.
99

100 RESULTS

101

102 **The *fcrX* gene is essential and controls cell cycle in *Sinorhizobium meliloti***

103 The *fcrX* gene (SMc00655, 351 bp ORF coding for a 116 aa predicted protein) is adjacent to
104 *ctrA* in the *S. meliloti* chromosome in a head-to-head orientation, with a *ca.* 500 bp long
105 intergenic sequence that controls the transcription of both genes. The *fcrX* gene appears
106 essential in free-living conditions, based on Tn-seq results (Figure S1A). In order to study the
107 function of *fcrX*, we constructed a depletion strain by deleting the chromosomal copy of *fcrX*
108 and expressing an extra copy of *fcrX* on a plasmid under the control of an IPTG-inducible *lac*
109 promoter^{17,22}. This conditional depletion strain of FcrX grew best in a medium supplemented
110 with 100 μ M IPTG (Figure S1B) and showed upon removal of IPTG dramatic growth defects
111 (Figure 1B and Figure S1B), as well as a reduced capacity to form colonies (Figure 1C),
112 indicating that *fcrX* is essential for the viability of *S. meliloti*. To gain more insights in FcrX
113 function(s), we investigated the FcrX-depleted cells by microscopy and flow cytometry.
114 Interestingly, the absence of FcrX induced the production of DNA-free small cells, referred to
115 as minicells, as revealed by DNA staining by Syto9 and microscopy observation (Figure 1D).
116 The accumulation of minicells in an FcrX-depleted suspension was confirmed by
117 DNA/membrane double staining with DAPI/Potomac Gold dyes and flow cytometry analysis
118 (Figure S2-S3B, 14.99% of total events with IPTG against 36.91% of total events without
119 IPTG). Thus upon depletion of *fcrX*, an accumulation of small cells with no DNA was observed.
120 We monitored the depletion of FcrX by time-lapse microscopy in order to understand the
121 development of minicells at the cellular level. Minicells tend to form only in daughter cells
122 (small cell) while the mother (large) cell remains able to divide efficiently producing daughter
123 cells, until two additional cycles and then completely stops dividing (Figure 1E). This minicell
124 phenotype can be interpreted as the consequence of an imbalanced cell cycle, with an excess of
125 division and a block of DNA replication, suggesting that FcrX may coordinate these two
126 processes in *S. meliloti*. Consistently, the absence of FcrX led to increased levels of CtrA, FtsZ1
127 and FtsZ2 proteins, as shown by western blot analysis (Figure 2A), indicating that FcrX indeed
128 negatively controls the accumulation of CtrA and FtsZ1/2. This result strengthens our
129 hypothesis of the implication of FcrX in the regulation of cell cycle and cell division.

130

131 **FcrX interacts with CtrA and FtsZ1/2**

132 The 3D structure prediction of FcrX performed with the AlphaFold 2 algorithm showed that the
133 FcrX protein may be composed of two alpha helices in a coiled coil configuration (Figure S4).

134 The structure did not reveal the presence of a DNA binding domain, suggesting that its putative
135 mode of action on CtrA and FtsZ1/2 may be by a direct interaction at the protein level. To verify
136 this hypothesis, an affinity column experiment was realized using His6-FcrX loaded nickel
137 columns in order to identify potential FcrX-interacting proteins. The presence of FtsZ1/2 and
138 FcrX itself, together with other proteins listed in Table S1 was detected in the affinity column
139 eluate by mass spectrometry (Figure S5). The presence of CtrA on the other hand was only
140 detected by western blot (Figure 2B), as its low abundance makes it hardly detectable by mass
141 spectrometry. In order to confirm this result, we carried out a bacterial two-hybrid (BACTH)
142 experiment using the *Escherichia coli* carrier (Figure 2C) (Karimova *et al.*, 1998). FcrX, CtrA
143 and FtsZ1/2 proteins were fused to domains 18 and 25 of the adenylate cyclase from *Bordetella*
144 *pertussis* in C- and N-terminal orientations and all possible combinations of prey and bait were
145 introduced in the *E. coli* strain HB101. In this system, FcrX interacts with CtrA and with both
146 copies of FtsZ (1 and 2). FtsZ2 lacks the C-terminal domain that is usually implicated in protein-
147 protein interactions and recruitment of FtsZ partners²³. Thus, the observed interaction between
148 FcrX and FtsZ2 may be explained by either an unusual interaction between the N-terminal
149 domain or by the presence of a functional full-length FtsZ homolog in *E. coli* (FtsZ_{Ec}), which
150 could promote the formation of a FcrX-FtsZ_{Ec}-FtsZ2 ternary complex. We also tested the
151 interaction of FcrX with itself to verify the possible dimerization of the protein, as observed
152 with the affinity column. As shown in the (Figure 2C) a strong FcrX-FcrX interaction was
153 observed, which is consistent with a putative oligomeric FcrX structure.

154

155 **Subcellular localization of FcrX and its interactors**

156 To gain more insights about the function of FcrX we decided to investigate its subcellular
157 localization and whether it colocalizes with its interactors, FtsZ being shown to have a mid-cell
158 localization and CtrA potentially being polarly localized in *S. meliloti* as it has been shown
159 previously in *C. crescentus*^{24,25}. We constructed a strain expressing a C-terminal translational
160 fusion of FcrX with a yellow fluorescent protein (YFP) and further deleted the chromosomal
161 copy of *fcrX* by transduction using a $\Delta fcrX::tetR$ M12 phage lysate. This approach was used in
162 order to avoid all possible competitions of the wild-type FcrX with the tagged version and it
163 confirmed that the fusion protein retained its function. FcrX localized at the septum, as observed
164 by epifluorescence microscopy (with a less frequent localization at the cell pole, presumably
165 after cell division) (Figure 2D). Image analysis in predivisive phase cells confirmed that FcrX
166 is localized at the septum in the majority of these cells (Figure 2E). We wanted to correlate this
167 result with the subcellular localization of the interactors over the cell cycle. Therefore, we

168 constructed a C-terminal translational fusion of FtsZ1 with a cyan fluorescent protein (CFP),
169 which despite the loss of its functionality retained its native localization ²⁴. Using a similar
170 approach, we tagged FtsZ2 with CFP. CtrA localization investigation appeared more complex
171 as N- or C-terminal tags strongly stabilized its levels, which is a lethal condition in *S. meliloti*
172 ¹⁰. Therefore, we added a sequence coding for the last 15 amino acids of CtrA that constitute
173 the degradation motif, downstream of CFP to ensure the timely degradation of the fusion protein
174 by proteolysis, then deleted the chromosomal copy of *ctrA* by transduction using a $\Delta ctrA::tetR$
175 M12 phage lysate. Tagged proteins were observed under the microscope. FtsZ1 and FtsZ2
176 showed as expected a midcell localization while CtrA showed a localization at the cell pole,
177 consistently with FcrX localization (Figure S6). Together, these results indicate that FcrX may
178 interact with both CtrA and FtsZ1/2 *in vivo* but at distinct subcellular localizations.

179

180 **Transcription of *fcrX* is positively regulated by CtrA**

181 In order to define and characterize the *fcrX* promoter, we made a promoter deletion analysis by
182 introducing in *S. meliloti* wild-type cells an extra copy of *fcrX* with different putative promoter
183 lengths located on a plasmid downstream of the IPTG-regulated *lacZ* promoter (Figure S7).
184 Then, the obtained clones were transduced using a phage M12 lysate produced from the strain
185 $\Delta fcrX::tetR + Plac-fcrX$ and selected in the presence or absence of IPTG. All clones should
186 give viable colonies with IPTG while only the constructs containing a functional promoter of
187 *fcrX* should support viability without IPTG (Figure S7). Constructs with P*fcrX*-*fcrX* fragments
188 containing at least the region between -1/-287 were viable, while a shorter promoter fragment
189 (-1/-224) was not active, suggesting that the promoter region of *fcrX* resides within the -287
190 region and that the -224/-287 contains a critical promoter element.

191 To confirm these results, we used the plasmid pOT1em ²⁶, which contains genes encoding
192 mCherry and EGFP in opposite directions. Several derivatives of the *fcrX* promoter, described
193 in Figure S7, as well as the full intergenic region between *fcrX* and *ctrA*, were cloned between
194 the ATGs of mCherry and EGFP. The full intergenic region between *fcrX* and *ctrA* was able to
195 express both mCherry (*fcrX*) and EGFP (*ctrA*) (Figure 3A). Analysis of the other constructs
196 containing different versions of the upstream region of *fcrX* confirmed that only clones with at
197 least -1/-287 expressed mCherry (*fcrX*) (data not shown). The *ctrA* gene is also controlled by
198 the same intergenic region, suggesting that these two genes may share the same transcriptional
199 regulation. The analysis of the region -224/-287 revealed several interesting characteristics.
200 First, in this region the *ctrAP1* promoter ²⁷ and the estimated *pfcrX* are overlapping with RNA-

201 seq determined putative TSSs (transcriptional start site) separated only by 20/25 bp (Figure S7).
202 Second, the promoter of *fcrX* contains a CtrA binding box within the 50 bp region upstream the
203 putative TSS^{4,10}. These observations suggest that the activation of the *fcrX* promoter may
204 depend on CtrA. In order to test this hypothesis, we mutated the CtrA-binding box by replacing
205 the 5'-TTAA-3' half box with 5'-GCGC-3' in a pOT1em plasmid carrying the full intergenic
206 region between *ctrA* and *fcrX* (Figure 3A). It was shown that this mutation prevents the fixation
207 of CtrA on the box and therefore affects its transcriptional activity on the downstream gene
208 (Figure 3A) (ref). Microscopy observation showed that the strain containing the mutated CtrA-
209 binding box didn't express the mCherry fluorescence, implicating that CtrA may activate *fcrX*
210 transcription by binding to its box. On the other hand, the mutated CtrA binding box didn't
211 change the EGFP expression noticeably, excluding the implication of this box in the regulation
212 of *ctrA* transcription (Figure 3A).

213 To confirm the positive regulation of *fcrX* expression by CtrA, we tested the steady state levels
214 of FcrX upon depletion of CtrA by western blot using antibodies directed against FcrX. In the
215 absence of CtrA, we observed a significant decrease of FcrX (Figure 3B). Since CtrA is a DNA-
216 binding protein implicated in transcriptional regulation, we also performed a qRT-PCR in the
217 same conditions. Consistently, *fcrX* expression decreased upon CtrA depletion, confirming the
218 previous observation (Figure S8). These results build up a regulatory model involving a
219 negative feedback loop between CtrA and FcrX.

220 Cell cycle regulators are known to be dynamically regulated over the cell cycle, consistent with
221 the oscillatory nature of this biological phenomenon. Because FcrX is closely linked to cell
222 cycle regulation, we wanted to check whether FcrX was subject to oscillation. Therefore, a
223 synchronization of a wild type culture of *S. meliloti* was made as described before (Figure 3C)
224¹⁴. Samples were recovered every 30 minutes over a full cell cycle and the corresponding total
225 RNA was used to perform a qRT-PCR analysis while cell lysates were used for Western blot
226 experiments using antibodies directed against FcrX. Both transcription and translation of FcrX
227 increased at 90 min of the cell cycle, meaning that FcrX is not only a regulator of the cell cycle
228 but it is also subject to cell cycle oscillation. These data are consistent with previous analyses
229 on the ensemble of cell cycle regulated genes¹⁴.

230 **FcrX is essential for the establishment of the legume symbiosis**

231 The terminal bacteroid differentiation that *S. meliloti* undergoes during its interaction with
232 *Medicago* plants involves a remodeling of the cell cycle and its regulatory network, prompting

233 us to test the involvement of FcrX in the symbiotic process. We tested the importance of FcrX
234 by inoculating *M. sativa* plants with the FcrX depletion strain and watered the plants with a
235 range of concentrations of IPTG in order to obtain different levels of FcrX expression. We also
236 inoculated plants with the wild type strain of *S. meliloti* carrying an empty plasmid as a
237 reference. At 28 days post inoculation (dpi) we checked nodule colonization using confocal
238 microscopy (Figure 4A), measured the plant dry weight (Figure 4B) and assessed bacteroid
239 differentiation by measuring the bacterial DNA content with flow cytometry (Figure S9). Plant
240 weight decreased when FcrX expression was reduced with respect to the condition with the
241 highest IPTG concentration (Figure 4B). The confocal microscopy images showed a clear
242 nodule colonization defect, with much less plant cells containing bacteroids in the plants
243 watered with 0 μ M, 10 μ M and 100 μ M IPTG compared to the plants watered with 1mM IPTG
244 and the references, implying that FcrX is essential to the establishment of a fully functional
245 symbiosis. However, the nodules from the condition watered with 1mM IPTG were still less
246 colonized and the bacteroids did not show a similar extent of terminal differentiation as
247 observed in the reference conditions, an observation that can be explained by the IPTG-
248 regulated expression of FcrX which may not mimic the natural cell cycle-regulated expression.
249 Considering our results showing that FcrX inhibits the accumulation of CtrA, FtsZ1 and FtsZ2
250 (Figure 2A), we wondered if an overexpression of FcrX may promote CtrA and FtsZ1/2
251 disappearance inside nodule cells and thereby boost the symbiotic process. To do so, we
252 inoculated plants of *M. sativa* with a *S. meliloti* strain containing a second copy of *fcrX*
253 expressed on a pSRK plasmid under the control of the *Plac* promoter, which has a weak
254 transcriptional leakage in *S. meliloti*, even in the absence of IPTG²². The upregulation of FcrX
255 resulted in a significant plant biomass gain at 28 dpi, as compared to the controls (Figure 4C).
256 However, no increase in the bacteroid differentiation level was noticed, suggesting that the
257 increase in symbiotic efficiency is likely related to the speed up of the differentiation process
258 rather than to a larger extent of differentiation (data not shown). Finally, we investigated
259 whether fully differentiated bacteroids contained FcrX by using anti-FcrX antibodies. Unlike
260 CtrA and FtsZ1/2 that are absent from mature bacteroids, FcrX remained detectable (Figure
261 4D), supporting a role of FcrX during the establishment of bacteroid differentiation and
262 maintenance.

263

264 **FcrX is a conserved factor in several species of the class *Alphaproteobacteria***

265 We further wanted to investigate the presence and functionality of FcrX in other bacteria. First,
266 the *S. meliloti* FcrX protein sequence was used to search in the Microbes Online database²⁸ for
267 similar proteins. Orthologs defined by Bidirectional Blast Hit (BBH) of FcrX were found in
268 several rhizobia (*Bradyrhizobium japonicum*, *Rhizobium leguminosarum*), the human pathogen
269 *Brucella abortus* and the phytopathogen *Agrobacterium vitis*. Except for *A. vitis* for which no
270 information is available, previous Tn-seq data on all those species revealed that the putative
271 FcrXs were all essential in the organism of origin^{29–31}. Those orthologs were tested for their
272 capacity to complement *fcrX* deletion in *S. meliloti*. We also tested a distant homolog of *fcrX*
273 from *C. crescentus*, which codes for the flagellar protein FliJ, located in the same genomic
274 context as *fcrX*. Interestingly, the *fcrX-ctrA* synteny is widely preserved among these bacteria
275 suggesting a possible conservation of *fcrX* function (Figure 5A). In order to test our hypothesis,
276 we constructed *S. meliloti* strains expressing a copy of these orthologs and we deleted by
277 transduction the chromosomal copy of *fcrX*. Results of this experiment showed that the
278 expression of the orthologs from *R. leguminosarum* and *B. abortus* were able to support *S.*
279 *meliloti* growth in a *fcrX* deletion background, implying functional conservation of FcrX. A
280 western blotting experiment was carried out confirming that the overexpression of these
281 orthologs is able to down regulate the accumulation of FtsZ and CtrA (Figure S10). However,
282 the orthologs from *B. japonicum*, *A. vitis* and the FliJ homolog from *C. crescentus* were not
283 able to complement the *fcrX* deletion. This functional complementation analysis, although still
284 limited in the number of tested species, revealed that from a functional point of view each FcrX
285 has acquired specific functions independently from the phylogenetic distance from *S. meliloti*,
286 as for example *A. vitis* (a species close to *S. meliloti*) is not able to complement while the more
287 distant *B. abortus* does. In order to have a broader view of FcrX conservation, we searched for
288 FcrX/FliJ orthologs/homologs in other alphaproteobacterial species (Figure 5B, Table S2).
289 Significantly similar sequences to either the *S. meliloti* FcrX or the *C. crescentus* FliJ, were
290 only found in the *Caulobacterales* and *Rhizobiales*. However, the two queries retrieved
291 sequences in complementary sets of species; for example, in *C. crescentus* FcrX retrieved no
292 results (Table S2). This can be consequent to a functional diversification of the same ancestral
293 gene in the *Caulobacterales* and *Rhizobiales*, which is however difficult to demonstrate because
294 of the short length and variability of these sequences, even if a phylogenetic tree of all homologs
295 found seems to suggest an orthology relationship as the topology is largely congruent with the
296 RecG tree (data not shown). In parallel we checked the distance between *fcrX/fliJ* and *ctrA*
297 genes, discovering that the *ctrA* gene is in proximity to *fcrX* orthologs (or *fliJ* in
298 *Caulobacterales*), and are often transcribed from the same intergenic region. Finally, we

299 observed that orthologs able to complement the *S. meliloti fcrX* deletion belong to the
300 phylogenetic group of *Brucella*-rhizobia (excluding bradyrhizobia). However, the functional
301 complementation of *fcrX* is not a conserved feature of specific clades, as for example *A. vitis*
302 does not complement. Finally the functional diversification between FcrX and FliJ is also
303 supported by the fact that, to the best of our investigations, FcrX has no role in the control of
304 motility.
305

306 DISCUSSION

307

308 In every organism, important functions of the cell are controlled by key factors (master
309 regulators) coordinating many related elements. This is the case, for example, of the cell cycle
310 in eukaryotes, based on cyclins ³², the sporulation process of *Bacillus subtilis*, controlled by
311 Spo0A ³³ and the cell cycle regulation in some alphaproteobacterial, such as *C. crescentus*,
312 where it is controlled by the master regulator signal transduction protein CtrA ³⁴. In this work
313 we identified in *S. meliloti* a new one key factor of cell cycle and cell division that we named
314 FcrX.

315 FcrX is a surprisingly small protein with no homology to other, so far characterized, regulators.
316 We predicted that FcrX is an alpha helix-rich protein that can oligomerize, and shares ancestry
317 with a previously characterized chaperon involved in flagellum physiology, named FliJ. In *C.*
318 *crescentus*, this small protein is specifically required for flagellum functioning through the
319 stabilization of another protein FliI, an ATPase involved in the export of flagellar subunits
320 across the membrane using a dedicated type III protein secretion system ³⁵. The analysis of
321 synteny of FcrX orthologs clearly underlined this association between FcrX and FliJ-FliI, as
322 many orthologs of FcrX are still organized in a *fliJ* operon. Another striking feature of the
323 analysis of the *fcrX* locus in many alphaproteobacteria is its proximity to the *ctrA* gene. As
324 previously hypothesized, CtrA is considered as an ancestral flagellum regulator in many
325 alphaproteobacterial species, including those in which CtrA also plays a role as cell cycle
326 regulator ^{4,36}. This conserved characteristic may suggest that FcrX evolved from FliJ, extending
327 or changing its targets from flagellar components to the divisome protein FtsZ and the cell cycle
328 regulator CtrA.

329 Indeed we showed here that FcrX binds directly CtrA in an unknown way and its presence plays
330 a negative role on the steady state levels of CtrA. Conversely, CtrA in addition to many cell
331 cycle genes ¹⁰ also controls *fcrX* transcription in the second half of DNA replication phase.
332 From this point of view, CtrA-FcrX forms an essential negative feedback loop contributing to
333 the oscillation of CtrA levels during cell cycle (Figure 5C). The presence of an essential genetic
334 negative feedback loop has been also demonstrated in *C. crescentus*, in which DivK and CtrA
335 are the main components of the feedback loop of this species ⁸. It is tempting to speculate that
336 although the architecture of cell cycle regulation may change between different organisms,
337 CtrA-DivK (linked by a transcriptional relationship) in *C. crescentus* and CtrA-FcrX in *S.*
338 *meliloti*, the logical principles behind the regulation remain similar.

339 In addition, this CtrA-related crucial regulatory function of FcrX is not its only role, as this
340 novel master regulator of cell cycle also negatively controls the main component of the
341 divisome, FtsZ (FtsZ1 and FtsZ2 in *S. meliloti*), by direct protein-protein interaction. However,
342 at this stage it cannot be excluded that FcrX interaction with FtsZ2 (both in BACTH and affinity
343 columns) may involve a ternary complex with FtsZ1 (either from *E. coli* in BACTH or
344 endogenous one in affinity columns) and FcrX, as FtsZ2 lacks a protein interaction domain
345 found in FtsZ1. This dual activity of FcrX makes this factor a novelty in the knowledge of cell
346 cycle regulation in alphaproteobacteria. Although cell division has been shown to be usually
347 regulated by CtrA at the transcriptional level in *S. meliloti*, but also in *C. crescentus* or *B.*
348 *abortus*³⁴, this is the first time that a negative regulator of cell cycle is able to connect directly
349 to both cell cycle regulation and the divisome itself. This dual activity of FcrX is responsible
350 of its severe depletion phenotype, leading to a block of cell cycle producing minicells that
351 contain no DNA, but also keeping a mother cell with the genome able to produce new minicells.
352 Considering potential applications that need minicells to perform specific functions^{37,38}, the
353 depletion of *fcrX* represents a miniaturized minicell factory, which may be exploited in the future
354 for biotechnological purposes.

355 Another important aspect of FcrX functionality is related to symbiosis and bacteroid
356 differentiation. It has been shown previously that bacteroids in *S. meliloti*, in order to become
357 functionally mature, must eliminate CtrA and FtsZ, leading consequently to elongated cells
358 with multiple copies of DNA. The discovery of a single regulator that is able to control
359 negatively both CtrA and FtsZ suggests that FcrX may play a role during bacteroid
360 differentiation. Indeed we have shown that FcrX is present in mature bacteroids and its function
361 is required for a correct establishment of symbiosis. Accordingly, a strain constitutively
362 expressing FcrX was able to increase plant biomass with respect to the wild-type situation,
363 suggesting that promoting CtrA and FtsZ downregulation can increase the efficiency of the
364 symbiosis, possibly by predisposing bacteria to terminal differentiation and making this process
365 take place earlier rather than increasing it to a higher level. This will open new frontiers of
366 sustainable agriculture by the use of improved bacterial inoculants based on FcrX deregulation.
367 Even more interestingly, FcrX appears as a conserved factor in several rhizobial species further
368 suggesting that this approach of plant growth amelioration may be extended to other
369 agronomically-important legumes symbionts.

370 In conclusion, FcrX is a novel global factor controlling two essential key functions of the cell,
371 regulation of cell cycle progression (CtrA) and cell division (FtsZ) (Figure 5C). This central
372 position and its integrated role in a negative feedback loop with CtrA suggests that cell

373 physiology may rely on FcrX regulation in order to perform higher levels of coordination of
374 cell cycle. In the future, the investigation should move towards exploring how FcrX is regulated
375 and what is the actual mechanism of CtrA and FtsZ1/2 inhibition by FcrX. FcrX indeed
376 represents a small protein with capacities to interact with very diverse targets that may be a tool
377 or a target for antibiotic therapies.
378

379 MATERIALS AND METHODS

380

381 Growth conditions

382 The strains used in this study are listed in the Table S3. *S. meliloti* 1021 and *E. coli* strains were
383 grown in YEB medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose,
384 0.04% MgSO₄·7H₂O, pH 7.5) at 30°C and LB medium (1% tryptone, 1% NaCl, 0.5% yeast
385 extract) at 37°C, respectively. Media were supplemented with appropriate antibiotics:
386 Kanamycin (50µg/ml), Tetracycline (10µg/ml), Gentamicin (20µg/ml) for *E. coli*,
387 Streptomycin (200µg/ml), Kanamycin (200µg/ml), Tetracycline (2µg/ml), Gentamicin
388 (20µg/ml) for *S. meliloti*. Depletion strains of *S. meliloti* were grown in a medium supplemented
389 with IPTG (100µM for *fcrX* and 1mM for *ctrA*); the depletion was accomplished by washing
390 three times the culture and resuspending it in a medium lacking IPTG at OD_{600nm} = 0.3. The
391 synchronization experiment was performed as described previously (De Nisco *et al.*, 2014).

392

393 Strain constructions

394 The two-step recombination procedure was used to perform the *fcrX* deletion using the
395 integrative plasmid pNPTS138 as previously described (Pini *et al.*, 2013). Deletions were
396 verified by PCR using primers flanking the recombination locus (see primers in Table S4).

397 To construct the fusion between the protein of interest and the fluorescent proteins (CFP or
398 YFP), the Gateway procedure (Thermo Fisher) was used. First, the gene was amplified by PCR
399 (see primers in Table S4) then introduced in the pENTR vector. Then, the vectors were mixed
400 with the destination plasmid carrying the gene coding for the fluorescent protein, to perform
401 the LR reaction as recommended by the manufacturer. The final product was amplified by PCR
402 and cloned in the pSRK vector downstream the *Plac* promoter (Khan *et al.*, 2005) and
403 electroporated in *S. meliloti* as previously described³⁹.

404 To transduce the *fcrX::tetR* deletion the phage M12 was used⁴⁰. To do so, the bacteria were
405 grown in LB containing 2.5 mM CaCl₂ and 2.5 mM MgSO₄ then mixed with the phage to give
406 a multiplicity of infection of 0.5. The mixture was incubated at room temperature for 45 min
407 and subsequently plated on LB plates with the appropriate antibiotics.

408 In order to identify the *fcrX* promoter, six different *PfcrX-fcrX* constructions were cloned into
409 the *Plac* inducible plasmid pSRK and were electroporated in *S. meliloti* wild type cells. All *S.*
410 *meliloti* clones containing an extra plasmid-encoded copy of *fcrX* with different promoter
411 lengths were transduced using a phage M12 lysate produced from the strain $\Delta fcrX::tetR + Plac-$

412 *fcxX* and selected in presence or absence of IPTG. To verify the *fcxX* promoter sequence and its
413 regulation by CtrA, the different constructions were introduced in pOTem1²⁶ vector using the
414 RF cloning procedure⁴¹.

415 *S. meliloti* 1021 (wild type) Tn-seq data of the *fcxX* gene during growth in YEB medium was
416 obtained from a previous study⁴².

417

418 **Nodulation assays and analysis**

419 *M. sativa* cultivar Gabès seeds were scarified with pure sulfuric acid for 8 min. After several
420 washes with distilled water, the seed surface was sterilized with bleach (150ppm) for 30 min
421 and seeds were washed again. Finally, seeds were soaked overnight under agitation in sterile
422 water and then transferred onto a Kalys agar plate for one day at 30°C in the dark to allow the
423 germination. The seedlings were planted in perlite/sand (2:1 vol/vol) in 1.5L pots in the
424 greenhouse (24°C, photoperiod 16 h of light and 8 h of dark, humidity 60%) and were
425 inoculated 7 days after planting with 50 ml per pot of the appropriate bacteria at OD_{600nm} =
426 0.05. Plants were watered every three days, alternating tap water and a commercial N-free
427 fertilizer (Plant Prod solution [N-P-K, 0-15-40; Fertil] at 1 g per liter). Plants were harvested at
428 6 weeks post inoculation (42 dpi) to analyze bacteroid colonization and nodule development by
429 confocal microscopy, level of bacteroid differentiation by flow cytometry and plant dry mass
430 measurement⁴³.

431

432 **Electron microscopy**

433 Bacteria were prefixed by adding an equal volume of fixative (2% glutaraldehyde in HEPES
434 buffer 200mM, pH 7.2) to the culture medium. After 20 min, the medium was replaced by 1%
435 glutaraldehyde in HEPES buffer for at least 1 h at 4°C. Bacteria were then washed with HEPES
436 buffer, concentrated in 2% agarose (LMP Agarose, Sigma A9414), washed again with HEPES
437 buffer and post-fixed in 1% osmium tetroxide (EMS 19150) for 1h at 4 °C. Samples were
438 washed again in distilled water and treated with 1% uranyl acetate (EMS 22400) for 1 h at 4°C
439 in the dark. Subsequently, samples were dehydrated in a graded series of acetone and embedded
440 in Epon resin. Ultrathin sections (60–90 nm) were cut, stained with uranyl acetate and lead
441 citrate and were analyzed using a Tecnai 200kV electron microscope (field electron interference
442 or FEI). Digital acquisitions were made with a numeric camera (Oneview, Gatan).

443

444 **Confocal and wide field microscopy**

445 Nodule imaging was performed on a SP8X confocal DMI 6000 CS inverted microscope (Leica)
446 equipped with hybrid and PMT detectors, a 10x dry (Plan Apo + DIC (NA: 0.4, Leica)) and a
447 63x oil immersion (Plan Apo + DIC (NA: 1.4, Leica)) objectives. For each condition, multiple
448 z-stacks were acquired (excitation: 405 nm; collection of fluorescence: 520-580 nm for
449 calcofluor excitation: 488 nm; collection of fluorescence: 520-580 nm for Syto9 and excitation:
450 561 nm; collection of fluorescence: 520-580 nm for Propidium iodide). Stacks were
451 transformed into maximum intensity projections using ImageJ software.

452 Time lapse experiments were performed on depleted cells deposited on agarose/YEB (with
453 appropriate antibiotics and inducers) and observed every 10 minutes (up to 16h) on a Nikon
454 Eclipse Ti E microscope equipped with a Yokogawa CSU-X1-A1 spinning disk system.

455

456 **qRT-PCR experiment**

457 RNA was extracted from bacterial culture samples using Maxwell® 16 LEV miRNA Tissue
458 Kit (Promega). cDNA was produced using random hexamers as primers and the GoScript™
459 Reverse Transcription kit from Promega. Amplification of 16S rRNA and *fcrX* cDNA was made
460 using SsoFast EvaGreen Supermix 2X kit (Bio-Rad, France) on a CFX96 Real-Time System
461 (Bio-Rad) instrument and the results were analyzed by Bio-Rad CFX Maestro version 1.1
462 software (Bio-Rad). For each sample, a biological duplicate was realized. Primers are listed in
463 Table S4.

464

465 **Flow cytometry**

466 Cells were heated 10 minutes at 70°C and then stained, depending on the experiment, with
467 DAPI (300µM), Syto9 (2.5nM), Propidium iodide (2.5nM) and Potomac Gold (1mM). After
468 10 minutes of incubation at room temperature, the cells were processed with Cytotflex bench-
469 top cytometer (Beckman-Coulter) and the data analyzed with CytExpert 2.5 software.

470

471 **Western blot experiment**

472 The bacterial pellets were prepared and frozen at $OD_{600nm} = 0.6$. Western blot was performed
473 as previously described (Pini et al., 2013). Anti-GroEL are commercial antibodies against *E.*
474 *coli* GroEL (Abcam). For anti-FcrX antibodies, *fcrX* was cloned into a pET derivative with N-
475 terminal His6 tag using a Gateway cloning procedure as previously described (Skerker et al.,
476 2005). His6-FcrX was purified on a nickel column and rabbits were injected using a 28 days
477 protocol (Pini et al., 2013). Purified plasma was then used for western blots.

478

479 **Protein interaction experiments**

480 For the Bacterial Two Hybrid experiment ⁴⁴, the recommendations by the supplier (Euromedex)
481 were applied. To construct recombinant proteins, vectors available from Euromedex were used.
482 These vectors enable the in frame fusion of the proteins subunits of adenylate cyclase from
483 *Bordetella pertussis* (T18, T25) at the C and N terminus. To test protein putative interactions,
484 each appropriate combination of vectors was electroporated into the *E. coli* strain β H101,
485 deleted of the gene coding for the endogenous adenylate cyclase (*cya* strain). Positive control
486 corresponds to the Tol-Pal from *E. coli* ⁴⁵.

487 For biochemical protein-protein interaction analysis, a nickel affinity column was used. Cells
488 of *E. coli* BL21 (D3A) expressing His6-FcrX and *E. coli* BL21 (D3A) with no expression vector
489 were induced 3h with 100 μ M IPTG at 30°C. Cells were harvested, sonicated as previously
490 described (Skerker et al, 2005) and soluble lysate of both strains was loaded onto prepacked
491 nickel columns. After several washes of extraction buffer (Tris 100mM, NaCl 500mM,
492 imidazole 30mM, pH 7.5), an *S. meliloti* sonicated lysate was loaded on the columns, washed
493 as previously described, followed by elution at increasing concentrations of imidazole (5%,
494 10% and 30%), collecting the eluates. Samples were loaded on SDS-PAGE gels and analyzed
495 by mass spectrometry (details in Table S1) or western blot using antibodies direct against CtrA
496 and FtsZ1 or 2 (the same polyclonal antibody is able to detect both copies).

497

498 **FcrX conservation analysis**

499 Homologs of RecG, FcrX and CtrA were identified by first blast ⁴⁶ hits, with an e-value cutoff
500 of 10⁻⁴. Proteomes for all alphaproteobacteria considered were downloaded from NCBI. Only
501 complete genomes were considered for the analysis. Genomic distances were calculated by
502 using coordinates in the corresponding “.gff” file; the distance dividing two genes was defined
503 as the minimum distance in both directions, *i.e.* taking the circularity of the genome into
504 account. Ancestral state reconstruction of distances was performed and mapped on trees with
505 function contMap from R-package phytools ⁴⁷. Alignments were performed with Muscle ⁴⁸ and
506 refined by hand in AliView ⁴⁹; maximum likelihood phylogenetic reconstructions were
507 performed with iqTree ⁵⁰, with options -nt AUTO -alrt 1000 -bb 1000, which combines
508 ModelFinder, tree search, ultrafast bootstrap and SH-aLRT test.

509

510

511 **FIGURE LEGENDS**

512

513 **Figure 1: *fcrX* is an essential gene in *S. meliloti*.**

514 (A) Scheme representing the cell cycle progression of *S. meliloti* in free-living condition
515 and in symbiosis with legume plants. The green color refers to CtrA concentration,
516 which is low at the beginning of S-phase and after cell division in large cells. During
517 bacteroid differentiation CtrA is removed in order to induce morphological changes.

518 (B) Transmission contrast (T) and electron microscopy (EM) of a depletion strain of *fcrX* in
519 presence and absence of the inducer (IPTG). In particular, in no IPTG conditions, the
520 depletion of *fcrX* is causing the formation of small cells (red asterisks).

521 (C) Viability test on *S. meliloti* containing empty plasmid and a depletion strain of *fcrX*.
522 Cells of *fcrX* depletion strain or wild type strain carrying an empty plasmid were grown
523 with IPTG and then washed before plating. From left to right, non-diluted to 1/10⁶
524 diluted cell suspensions were spotted on an agar plate with or without IPTG.

525 (D) Electron microscopy of a depletion strain of *fcrX* (EM) and overlay of transmission
526 contrast (T) and epifluorescence microscopy of a depletion strain of *fcrX* labeled with
527 Syto9.

528 (E) Time lapse microscopy on a depletion strain of *fcrX*. Orange dots represent the mother
529 cells and the blue dots represent the daughter cells. Only daughter cells are able to
530 produce abnormal mini cells. Scale bar corresponds to 2 μ m.

531

532 **Figure 2: FcrX down regulates and interacts directly with the master regulator CtrA and**
533 **the Z ring proteins (FtsZ1, FtsZ2).**

534 (A) Western blot using Anti- FtsZ, CtrA, FcrX and GroEL on the *fcrX* depletion strain in
535 comparison with wild type (WT) and a strain containing the empty vector used in the
536 *fcrX* depletion strain (Empty). For the depletion strain of *fcrX*, a depleted culture was
537 reincubated with IPTG for 2h (IPTG 2h) or without for 2h (2h) or 3h (3h).

538 (B) Affinity column western blot using CtrA, FcrX and FtsZ antibodies. Percentages
539 represent imidazole concentration (see Materials and Methods for details). Membrane
540 stained with Ponceau Red shows His6-FcrX.

541 (C) Bacterial Two hybrid (BACTH) testing FcrX interaction with FtsZ1, FtsZ2, CtrA and
542 itself. Upper right box shows negative and positive controls provided by the supplier.
543 25 and 18 are the two subunits of adenylate cyclase of the BACTH (see Materials and
544 Methods).

545 (D) Functional FcrX-YFP C-terminal fusion observed by transmission (T) and
546 epifluorescence microscopy (EM) in a *fcrX* deletion genetic background (see text for
547 details). Bar corresponds to 1 μ m. Cells showing mid-cell localization are marked with
548 an orange asterisks, while the cell with polar localization is marked with a blue asterisk.
549 (E) Heatmap of YFP-FcrX subcellular localization in a synchronized cell population
550 (predivisional phase, 150 minutes). Analysis performed on >300 cells (56 with foci).

551

552 **Figure 3: FcrX is cell cycle regulated and its transcription depends on CtrA.**

553 (A) *S. meliloti* strain containing the intergenic region between *fcrX* and *ctrA* fused with
554 mCherry and EGFP, respectively. Lower panels correspond to the same intergenic
555 region mutated in the CtrA box (see text for details). This mutation doesn't affect the
556 expression of *ctrA* but it completely abolishes the expression of *fcrX*.
557 (B) Western blot using Anti FcrX antibodies using FcrX depletion and CtrA depletion
558 samples. First lane is purified His6-FcrX. M = Marker (sizes are reported).
559 (C) qRT-PCR of *fcrX* and Western blot using anti-FcrX antibodies on a synchronized
560 population of *S. meliloti*. Bottom part represents a timeline of cell cycle phases.

561

562 **Figure 4: FcrX is important for symbiosis.**

563 (A) Nodules of 42 dpi plants infected by wild type (left row) and the depletion strain of *fcrX*,
564 in different IPTG-watering conditions, were photographed (upper panels) and then
565 sectioned and stained with Calcofluor, Syto9 and IP as explained in materials and
566 methods (lower panels at high magnification levels).
567 (B) Dry biomass per plant (left panel) and aspect (right panel) of 42 dpi *M. sativa* infected
568 by wild type and the depletion strain of *fcrX* at different IPTG concentrations. Asterisks
569 correspond to significant differences (More than 20 plants for each condition, less than
570 $P < 0,05$, Kruskal Wallis test). NI = Non inoculated.
571 (C) Dry biomass per plant (left panel) and aspect (right panel) of 42 dpi *M. sativa* infected
572 by wild type strain containing an empty vector (empty), wild type and a strain
573 expressing an extra copy of *fcrX* (FcrX+). Asterisks correspond to significant
574 differences (More than 20 plants for each condition, less than $P < 0,05$, Kruskal Wallis
575 test). NI = Non inoculated.
576 (D) Western blot using FcrX, FtsZ, CtrA and GroEL antibodies on free living and bacteroid
577 cells.

578

579 **Figure 5: FcrX is conserved among alphaproteobacteria**

580 (A) Typical organization of *fcrX* genomic loci in model alphaproteobacteria. The presence
581 and the relationship between locations of *fcrX* and *ctrA* genes in those
582 alphaproteobacterial species is highlighted with a blue asterisk in figure 5B.

583 (B) Phylogenetic tree of FcrX orthologs-containing species (as described in Materials and
584 Methods). The color code of the tree marks the distance between the *fcrX* and *ctrA* genes
585 (values are bp). Species used in Figure 5A are marked with a blue asterisk, tree based
586 on RecG sequences.

587 (C) Model of FcrX role in cell cycle regulation with respect to main functions of cell cycle
588 and CtrA/DivK/ClpXP essential regulators.

589

590

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592

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604

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FIGURE 1

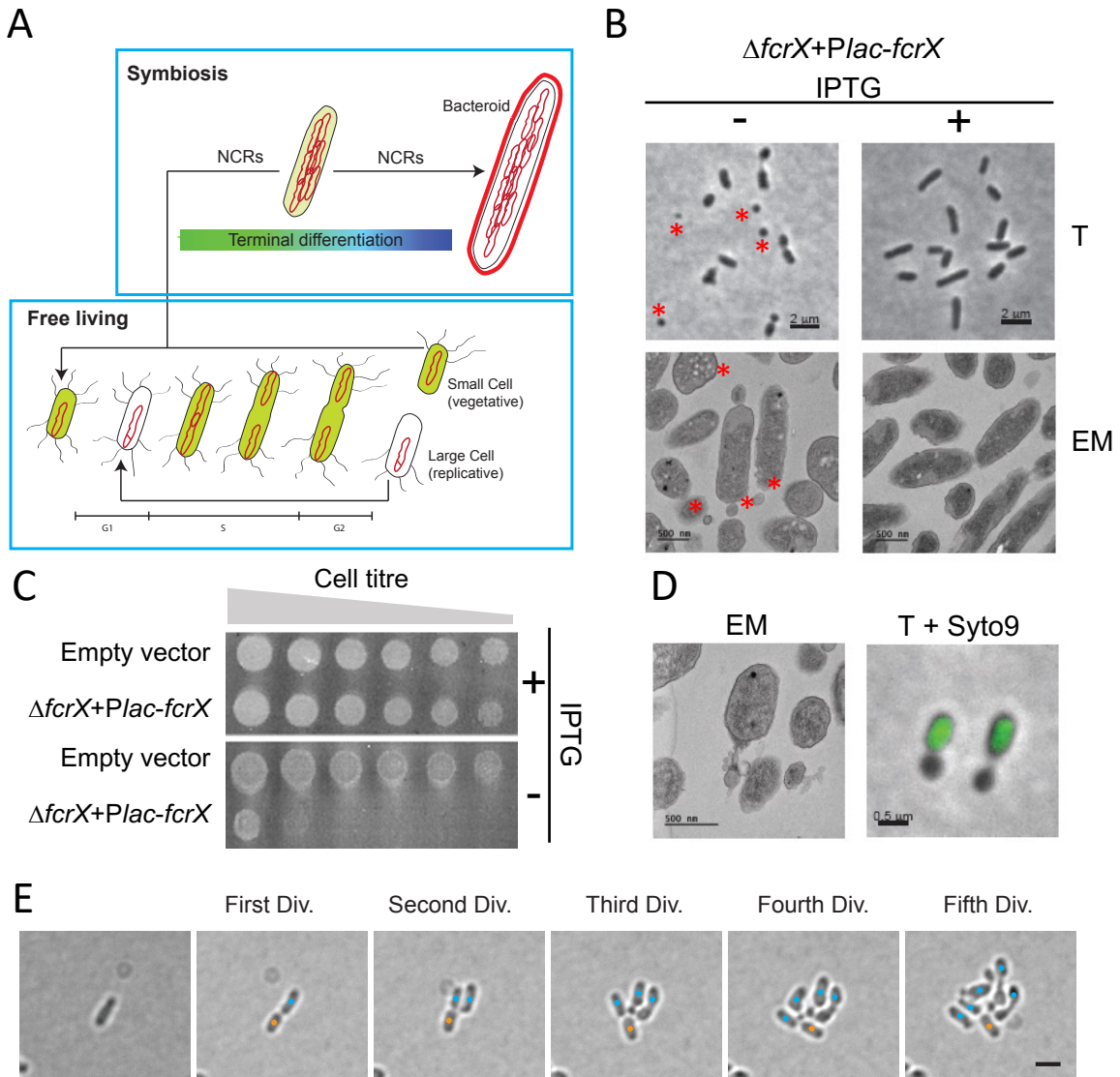


Figure 1: *fcrX* is an essential gene in *S. meliloti*.

- (A) Scheme representing the cell cycle progression of *S. meliloti* in free-living condition and in symbiosis with legume plants. The green color refers to CtrA concentration, which is low at the beginning of S-phase and after cell division in large cells. During bacteroid differentiation CtrA is removed in order to induce morphological changes.
- (B) Transmission contrast (T) and electron microscopy (EM) of a depletion strain of *fcrX* in presence and absence of the inducer (IPTG). In particular, in no IPTG conditions, the depletion of *fcrX* is causing the formation of small cells (red asterisks).
- (C) Viability test on *S. meliloti* containing empty plasmid and a depletion strain of *fcrX*. Cells of *fcrX* depletion strain or wild type strain carrying an empty plasmid were grown with IPTG and then washed before plating. From left to right, non-diluted to 1/10⁶ diluted cell suspensions were spotted on an agar plate with or without IPTG.
- (D) Electron microscopy of a depletion strain of *fcrX* (EM) and overlay of transmission contrast (T) and epifluorescence microscopy of a depletion strain of *fcrX* labeled with Syto9.
- (E) Time lapse microscopy on a depletion strain of *fcrX*. Orange dots represent the mother cells and the blue dots represent the daughter cells. Only daughter cells are able to produce abnormal mini cells. Scale bar corresponds to 2 μ m.

FIGURE 2

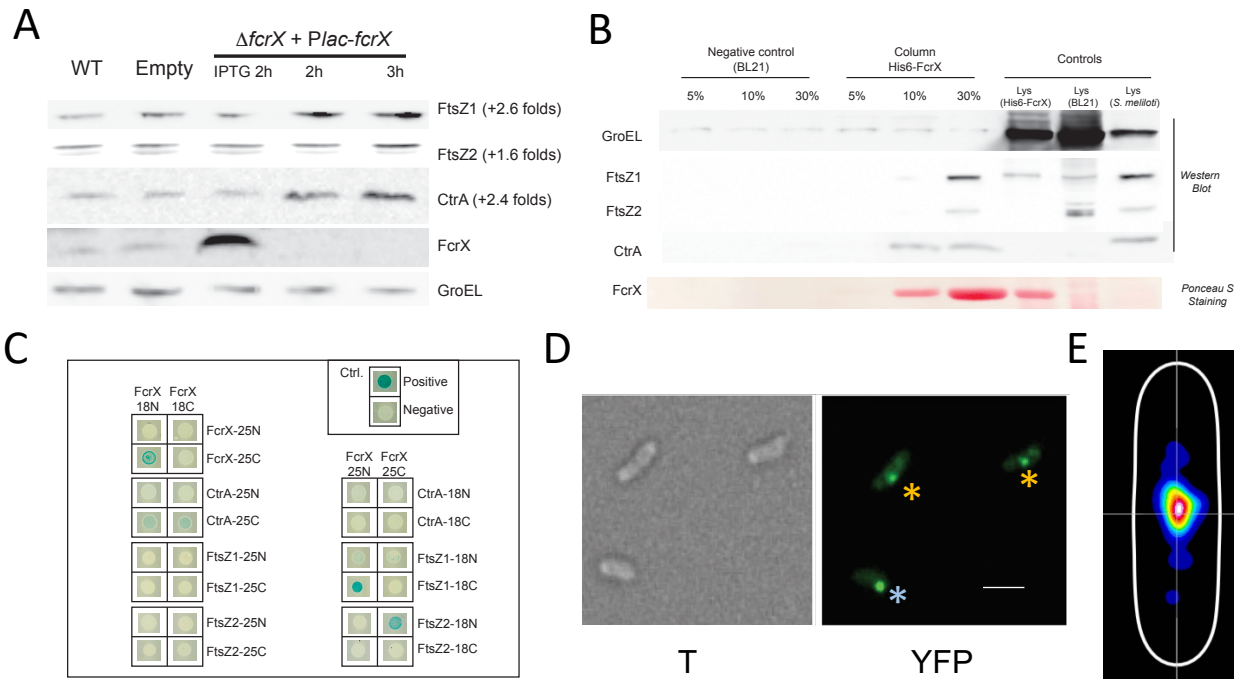


Figure 2: FcrX down regulates and interacts directly with the master regulator CtrA and the Z ring proteins (FtsZ1, FtsZ2).

- (A) Western blot using Anti- FtsZ, CtrA, FcrX and GroEL on the *fcrX* depletion strain in comparison with wild type (WT) and a strain containing the empty vector used in the *fcrX* depletion strain (Empty). For the depletion strain of *fcrX*, a depleted culture was reincubated with IPTG for 2h (IPTG 2h) or without for 2h (2h) or 3h (3h).
- (B) Affinity column western blot using CtrA, FcrX and FtsZ antibodies. Percentages represent imidazole concentration (see Materials and Methods for details). Membrane stained with Ponceau Red shows His6-FcrX.
- (C) Bacterial Two hybrid (BACTH) testing FcrX interaction with FtsZ1, FtsZ2, CtrA and itself. Upper right box shows negative and positive controls provided by the supplier. 25 and 18 are the two subunits of adenylate cyclase of the BACTH (see Materials and Methods).
- (D) Functional FcrX-YFP C-terminal fusion observed by transmission (T) and epifluorescence microscopy (EM) in a *fcrX* deletion genetic background (see text for details). Bar corresponds to 1 μ m. Cells showing mid-cell localization are marked with an orange asterisks, while the cell with polar localization is marked with a blue asterisk.
- (E) Heatmap of YFP-FcrX subcellular localization in a synchronized cell population (predivisional phase, 150 minutes). Analysis performed on >300 cells (56 with foci).

FIGURE 3

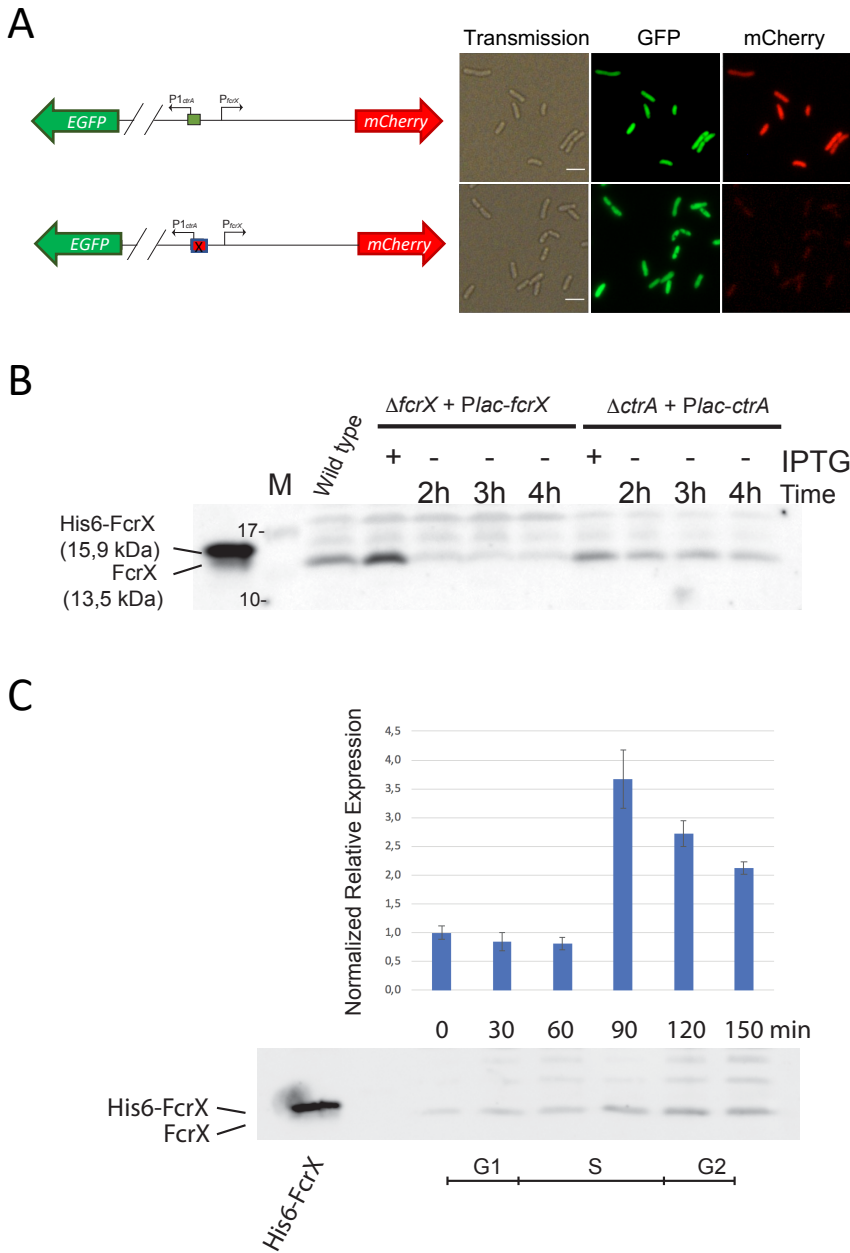


Figure 3: FcrX is cell cycle regulated and its transcription depends on CtrA.

- (A) *S. meliloti* strain containing the intergenic region between *fcrX* and *ctrA* fused with mCherry and EGFP, respectively. Lower panels correspond to the same intergenic region mutated in the CtrA box (see text for details). This mutation doesn't affect the expression of *ctrA* but it completely abolishes the expression of *fcrX*.
- (B) Western blot using Anti FcrX antibodies using FcrX depletion and CtrA depletion samples. First lane is purified His6-FcrX. M = Marker (sizes are reported).
- (C) qRT-PCR of *fcrX* and Western blot using anti-FcrX antibodies on a synchronized population of *S. meliloti*. Bottom part represents a timeline of cell cycle phases.

FIGURE 4

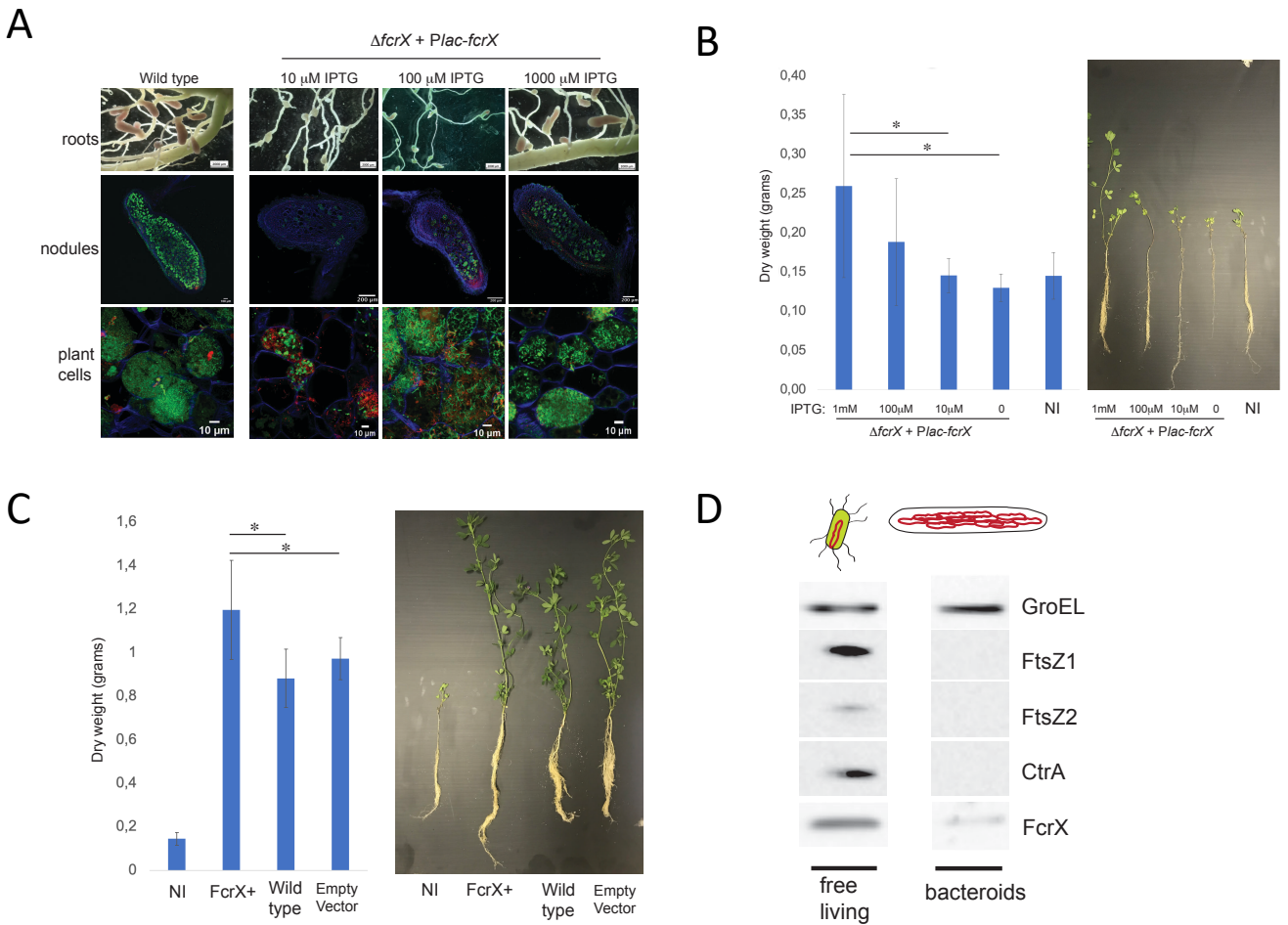
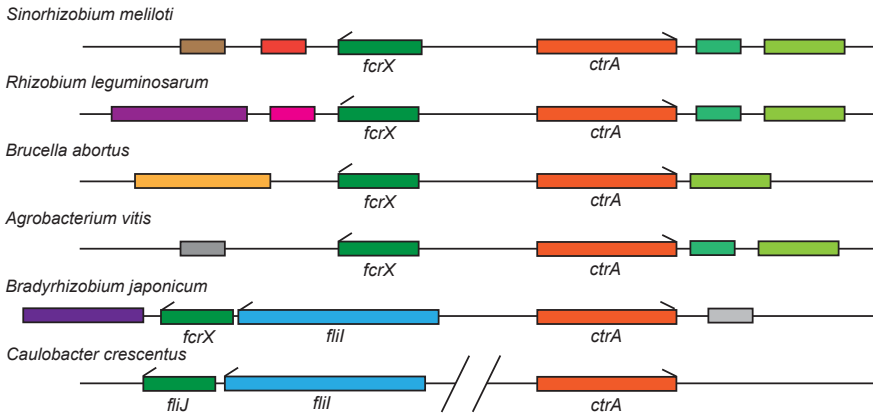


Figure 4: FcrX is important for symbiosis.

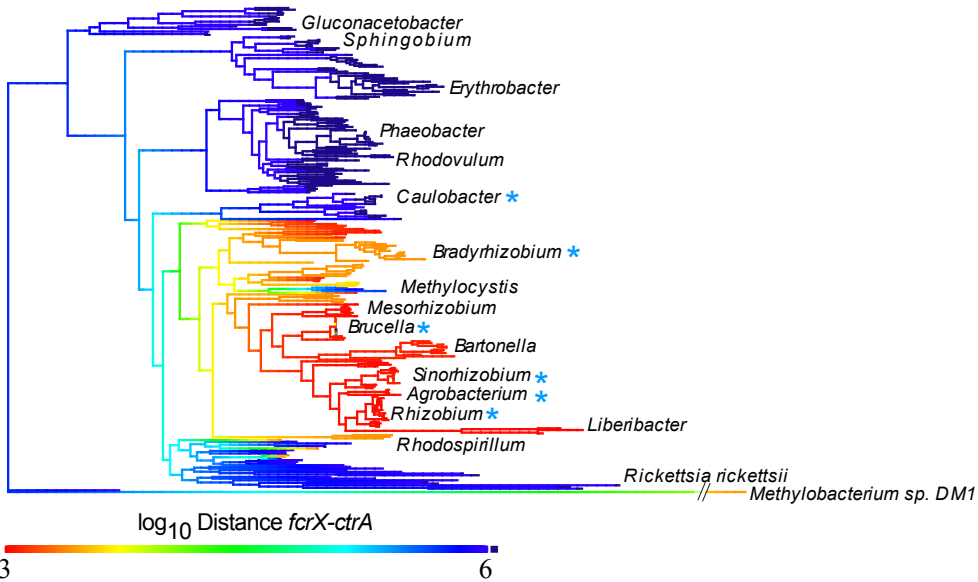
- (A) Nodules of 42 dpi plants infected by wild type (left row) and the depletion strain of *fcrX*, in different IPTG-watering conditions, were photographed (upper panels) and then sectioned and stained with Calcofluor, Syto9 and IP as explained in materials and methods (lower panels at high magnification levels).
- (B) Dry biomass per plant (left panel) and aspect (right panel) of 42 dpi *M. sativa* infected by wild type and the depletion strain of *fcrX* at different IPTG concentrations. Asterisks correspond to significant differences (More than 20 plants for each condition, less than $P < 0,05$, Kruskal Wallis test). NI = Non inoculated.
- (C) Dry biomass per plant (left panel) and aspect (right panel) of 42 dpi *M. sativa* infected by wild type strain containing an empty vector (empty), wild type and a strain expressing an extra copy of *fcrX* (FcrX+). Asterisks correspond to significant differences (More than 20 plants for each condition, less than $P < 0,05$, Kruskal Wallis test). NI = Non inoculated.
- (D) Western blot using FcrX, FtsZ, CtrA and GroEL antibodies on free living and bacteroid cells.

FIGURE 5

A



B



C

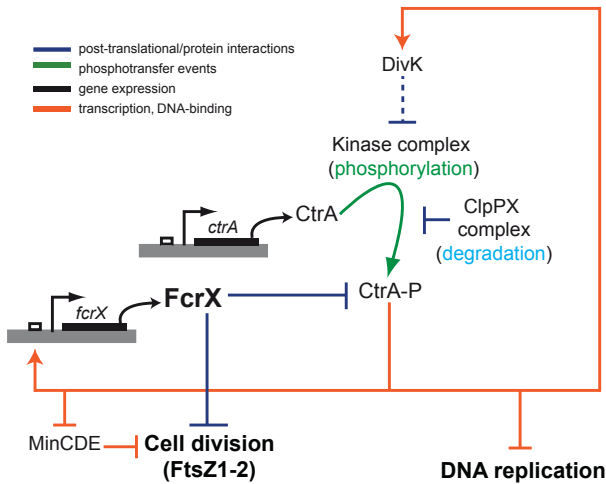


Figure 5: FcrX is conserved among alphaproteobacteria

- (A) Typical organization of *fcrX* genomic loci in model alphaproteobacteria. The presence and the relationship between locations of *fcrX* and *ctrA* genes in those alphaproteobacterial species is highlighted with a blue asterisk in figure 5B.
- (B) Phylogenetic tree of FcrX orthologs-containing species (as described in Materials and Methods). The color code of the tree marks the distance between the *fcrX* and *ctrA* genes (values are bp). Species used in Figure 5A are marked with a blue asterisk, tree based on RecG sequences.
- (C) Model of FcrX role in cell cycle regulation with respect to main functions of cell cycle and CtrA/DivK/ClpXP essential regulators.