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1                    **Cell cycle and terminal differentiation in *Sinorhizobium meliloti***

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11  
12                    **Abstract**

13                    *Sinorhizobium meliloti* of the *Alphaproteobacteria* class has a fascinating spectrum of  
14                    lifestyles, thriving as a free-living soil saprophyte, as an endophyte and as a nitrogen-fixing  
15                    legume symbiont. In symbiosis, it undergoes a striking cellular differentiation process, which  
16                    is controlled by the host plant through the activity of NCR peptides. NCRs interfere with the  
17                    cell cycle of *S. meliloti* and transform the regular cycle consisting of strict successions of single  
18                    DNA replication followed by cell division into an endoreduplication cycle of multiple genome  
19                    duplications without divisions. This cellular differentiation results in giant and polyploid  
20                    symbiotic bacterial cells that fix atmospheric nitrogen. Here we discuss the regulation of the  
21                    free-living cell cycle in *S. meliloti* and present the hypothesis that the master regulator CtrA is  
22                    the ultimate target of the NCR peptides, provoking the cell cycle switch in symbiosis.

23  
24                    **Keywords:**

- 25 *Sinorhizobium meliloti*, cell cycle, symbiosis, nitrogen fixation, terminal bacteroid
- 26 differentiation, CtrA

## 27 *Sinorhizobium meliloti* biology

28

29 The alphaproteobacterium *S. meliloti* is a free-living soil saprophyte, an endophyte and a  
30 legume endosymbiont (Poole et al. 2018). The latter lifestyle has made its renown and today it  
31 is one of the better-studied symbiotic species among all bacteria. This bacterium has the ability  
32 to colonize roots of leguminous plants of the genus *Medicago*. When it is in contact with plant  
33 roots, it induces the formation of new underground organs, called nodules (Figure 1A), in which  
34 it finds the ideal conditions to reduce atmospheric nitrogen into ammonia that is incorporated  
35 by the plant and used for its nitrogen needs in growth. In exchange, the bacterium receives  
36 nutrients and an exclusive niche inside the nodules where it can establish, starting from a single  
37 or very few bacterial cells, a very large population of several millions in the very short time  
38 span of a few days, the time it takes to form a mature and fully infected nodule. Thus, unlike  
39 the soil environment, where the rate of division is low, the nodule allows the bacteria to multiply  
40 rapidly, indicating a considerable evolutionary advantage for the symbiotic lifestyle.

41 The nodule-forming and nitrogen-fixing symbiosis is widespread in legumes, where it  
42 constitutes an ancestral trait of the family although some legume species lost their symbiotic  
43 ability (ref: Griesmann, et al 2018) . The combined nitrogen fixation activity by all legumes on earth  
44 is a key process in the biogeochemical nitrogen cycle and has therefore a tremendous impact  
45 on the ecology of our planet. Moreover, symbiotic nitrogen fixation by legumes has a  
46 considerable interest for its applications in agriculture as an alternative to chemical nitrogen  
47 fertilizer. Among the rhizobium-legume interactions, the *S. meliloti-Medicago* interaction has  
48 emerged as one of the most productive model systems for the study of the nodule-forming and  
49 nitrogen-fixing symbiosis.

50 During the establishment of the symbiotic organ, *S. meliloti* infect symbiotic plant cells and  
51 become intracellular nitrogen-fixing organelle-like structures called bacteroids. The bacteroids

52 undergo a drastic differentiation program, resulting in cells that are unable to divide and  
53 produce offspring, resulting therefore in a terminally differentiated state (Figure 1A). From an  
54 evolutionary point of view, this terminal differentiation is puzzling at first sight, because the  
55 absence of offspring seems to be incompatible with the natural selection of this process.  
56 However, since the nodule bacteria form a clonal or nearly clonal population, even if the  
57 majority of them is terminally differentiated, still a large fraction of genetically identical  
58 undifferentiated bacteria remain in the nodules and those can replenish the soil rhizobial  
59 population from senescing nodules. Moreover, it seems to be difficult to explain why a  
60 bacterium, in the context of a beneficial symbiosis, sacrifices billions of siblings for the cause  
61 of the plant. In this review, we will explain the underlying causes of the differentiation, which  
62 are derived from the plant rather than programmed by the bacterium and we will present  
63 evolutionary hypotheses about the role of bacteroid differentiation, which is probably beneficial  
64 to the plant rather than to the rhizobia. At its most basic level, the terminal differentiation of *S.*  
65 *meliloti* corresponds to a transformation of its regular cell cycle into an endoreduplication cycle  
66 with no offspring generation. In this chapter, we will present our view on how this cell cycle  
67 switch can be accomplished mechanistically.

68

## 69 **Symbiotic infection and differentiation**

70

71 The first bacterial contact with plants consists in an exchange of specific signaling molecules.  
72 Chemotaxis, particularly towards the abundant amino acids in *Medicago* exudates, guides the  
73 *S. meliloti* bacteria in the soil towards the plant roots (Compton et al. 2020). Plants also secrete  
74 flavonoids in the rhizosphere and in response to these plant molecules, rhizobia secrete  
75 lipochitooligosaccharidic signals called Nod factors (Poole et al. 2018). Interestingly flavonoids  
76 have also growth stimulating activity on the rhizobia, suggesting multiple dose-dependent  
77 ecological roles of this plant signal (Nouwen et al. 2019). Nod factors are recognized by the  
78 plant through receptors, triggering the plant program for nodule formation and infection. During  
79 the nodule formation, additional bacterial molecular patterns are monitored by the plant, in  
80 particular surface polysaccharides, such as exopolysaccharides and lipopolysaccharides.  
81 Together, these molecular keys direct the symbiotic process and are highly specific, minimizing  
82 the risk of infections by non-compatible rhizobia or opportunistic and pathogenic organisms.  
83 The entrance of *S. meliloti* in the plant tissue happens by the deformation of the normally  
84 straight growth of root hairs into a typical curled growth direction, which is specifically induced  
85 by the bacterial Nod factors. The curling root hair traps a single or very few *S. meliloti* cells,  
86 which constitutes the founding cells of what will become the complete nodule population. The  
87 entrapped rhizobia are able to penetrate the root hair cell via the formation of an infection  
88 thread, a tubular structure containing dividing bacteria. The Nod factor perception in the root  
89 hair also triggers cell divisions, at a distance, in the underlying root cortical cells. These  
90 dividing plant cells form a nodule primordium that will further develop into a nodule.  
91 Simultaneously, the infection thread that was first initiated in the root hair grows and ramifies  
92 towards the primordium, thereby conducting the rhizobia towards the newly formed cells of the  
93 incipient nodule.

94 An infection thread that has reached and penetrated a young nodule cell releases rhizobia  
95 through an endocytotic process into the plant cell. The endocytotic uptake from an infection  
96 thread in a differentiating nodule cell does not release rhizobia freely in the cytosol but inside  
97 vesicles, called symbiosomes, which have a plasmalemma-like membrane. Within the  
98 symbiosomes, the rhizobia grow and differentiate into their nitrogen-fixing forms called the  
99 bacteroids. Repeated infections and the growth of the rhizobia in symbiosomes will ultimately  
100 result in a symbiotic nodule cell that is completely packed with intracellular bacteroids (Figure  
101 1B).

102 A plant cell that has been infected does not divide anymore but switches into a differentiation  
103 path towards a nitrogen-fixing nodule cell. This differentiation includes the activation of an  
104 endoreduplication cycle, leading to polyploidy and very strong cell enlargement as well as the  
105 activation of a specific transcriptional program that will assure the maintenance and the  
106 metabolic integration of the thousands of nitrogen-fixing endosymbionts within each individual  
107 mature symbiotic nodule cell (Mergaert et al. 2020). On the other hand, a few distal cells in the  
108 incipient nodule that are not penetrated by an infection thread will constitute a nodule meristem  
109 and will continue to divide. The formation of this apical meristem in *Medicago* nodules gives  
110 the organ an indeterminate state with a continuous growth during the complete lifetime of the  
111 nodule.

112 The nitrogen-fixing *S. meliloti* bacteroids in nodule cells are in a differentiated state, which is  
113 in various ways dramatically different from its free-living state in the soil (Figure 1A). First of  
114 all, the bacteroid formation implies a switch in the bacterial physiology that is adapted to the  
115 nitrogen fixation process. This switch is made possible by a massive transcriptional activation  
116 of a large set of genes encoding nitrogen fixation and associated respiratory functions, which  
117 are completely silent in the free-living state (Roux et al. 2014a). This transcriptome switch is  
118 controlled by a regulatory cascade, composed of the FixLJ two-component regulator that senses

119 the low oxygen concentration prevailing in the nodule cells, and the downstream NifA and FixK  
120 transcription factors (Bobik et al. 2006).

121 The physiological adaptation of the *S. meliloti* bacteroids is in addition accompanied with their  
122 above-mentioned remarkable terminal differentiation (Mergaert et al. 2006). This state of *S.*  
123 *meliloti* is characterized by the irreversible loss of capacity to resume growth and to reproduce.

124 The bacteroids also have a partially permeabilized membrane. However, their most striking  
125 feature is their metamorphosis into a giant, sometimes branched, bacterial cell of up to ten  
126 micrometers long. Moreover, similarly as their host cells, these bacteroids are polyploid.

127 The terminal bacteroid differentiation is a process that is determined by the host rather than  
128 being uniquely encoded in the genetic repertoire of the rhizobia. Indeed, terminal differentiation  
129 is not happening in all legumes. It is for example taking place in the Inverted Repeat Lacking  
130 Clade (IRLC) and Dalbergioid clade plants to which respectively *Medicago* and *Aeschynomene*  
131 species belong but it is not happening in the Robinioid or Millettoid clades containing the well-  
132 studied *Lotus* and *Glycine* genera, respectively (Mergaert et al. 2006; Czernic et al. 2015).

133 Broad host range rhizobia or engineered strains that have a switched host range, will form  
134 terminally differentiated bacteroids or not according to the host species in which they are found.

135 This suggests that the terminal differentiation is in the first place determined by the plant,  
136 although also the bacterial genetic repertoire contributes to the extent of the bacteroid  
137 differentiation process (Mergaert et al. 2006; Nicoud et al. 2020).

138 Based on a phylogenetic analysis of the bacteroid state in the legume family, it was proposed  
139 that the ancestral state of bacteroids is the undifferentiated type, the type that is found in the  
140 Robinioids or Millettoids (Oono et al. 2011). According to this scenario, terminal bacteroid  
141 differentiation has evolved several times in the legumes and appeared independently in for  
142 example the IRLC and Dalbergoid legumes.



## 143 **The NCR peptides, host effectors of bacteroid differentiation**

144

145 The identification of the host factors that determine the terminal bacteroid differentiation was  
146 based on genomic and transcriptomic comparisons of legumes that display the feature or not  
147 (Mergaert et al. 2003; Alunni et al. 2007; Van de Velde et al. 2010). These analyses correlated  
148 the formation of terminally differentiated bacteroids with the expression in nodules of a  
149 particular family of genes encoding peptides which were called the NCRs for nodule-specific  
150 and cysteine-rich peptides. In *M. truncatula*, these peptides are very specifically produced in  
151 nodules, in the infected symbiotic cells, and nowhere else in the plant (Guefrachi et al. 2014).  
152 Remarkably, many legume species produce a large diversity of them, sometimes over several  
153 hundred different ones. *M. truncatula* for example expresses over 600 different *NCR* genes in  
154 nodules (Montiel et al. 2017). NCR peptides are small secretory peptides characterized by a  
155 pattern of conserved cysteine residues. Importantly, the NCRs are related to antimicrobial  
156 peptides, which are innate immunity effectors that are used by eukaryotic hosts, namely plants  
157 and animals, to attack and eliminate invading microbes (Mergaert 2018).

158 All tested species of the IRLC legumes produce NCR peptides in their nodules, which are of  
159 the same phylogenetic family and have thus a common ancestor. On the other hand, the  
160 *Aeschynomene* legumes of the Dalbergoid clade produce in their nodules NCRs of an unrelated  
161 family with distinct sequences and cysteine patterns (Czernic et al. 2015; Gully et al. 2018;  
162 Quilbé et al. 2020). The use of different NCR families is in agreement with the independent  
163 evolution of bacteroid differentiation in these two clades (Oono et al. 2011).

164 Several arguments have confirmed the initial phylogenomic correlation between the production  
165 of NCRs in the symbiotic nodule cells and the terminal differentiation of bacteroids (Figure  
166 1A). A recent analysis for example showed that the degree of differentiation of bacteroids in  
167 species of the IRLC correlates with the amount of NCRs expressed in nodules and also with the

168 type of peptides they produce. The higher the diversity of peptides and the more cationic the  
169 NCR peptides are, the stronger the morphological change of the bacteroids (Montiel et al.  
170 2017).

171 The majority of the NCR peptides, if not all, are transported to the bacteroids indicating that  
172 the endosymbionts are their target. Indeed, the localization of many individual peptides in the  
173 bacteroids or symbiosomes have been demonstrated by immunolocalization, by expressing  
174 NCR fusion proteins with fluorescent markers as well as by cell fractionations of nodule  
175 extracts and purifications of bacteroids followed by western analysis or proteomics (Van de  
176 Velde et al. 2010; Haag et al. 2011; Durgo et al. 2015; Czernic et al. 2015; Horváth et al.  
177 2015; Kim et al. 2015; Wang et al. 2017; Yang et al. 2017).

178 *In vitro* experiments have shown that pure (synthetic?) NCRs induce features on free-living *S.*  
179 *meliloti* that mimic the terminal bacteroids such as cell elongation and polyploidy (Van de  
180 Velde et al. 2010; Haag et al. 2011; Penterman et al. 2014; Montiel et al. 2017). Moreover,  
181 transferring *NCR* genes to *Lotus japonicus*, a legume that does not have them and that makes  
182 normally reversible bacteroids, leads to new bacteroid features similar to terminal bacteroids  
183 (Van de Velde et al. 2010).

184 Complementary to these “gain-of-function” methods, also loss-of-function experiments are  
185 confirming the key role of NCR peptides in bacteroid differentiation. NCR peptides are  
186 secretory peptides, which depend on their signal peptide to be taken in charge by the secretory  
187 pathway for trafficking to their cellular destination. In the *M. truncatula* mutant of the secretory  
188 pathway *dnf1*, NCR transport to the bacteroids is blocked. Thus, NCRs are stuck in the  
189 endoplasmic reticulum in the infected nodule cells and this prevents terminal bacteroid  
190 differentiation (Van de Velde et al. 2010; Wang et al. 2010). Similarly, downregulation of the  
191 orthologous secretory pathway gene *DNF1* in *Aeschynomene* nodules by RNAi blocks  
192 bacteroid differentiation (Czernic et al. 2015). More recently, several mutants or allelic

193 variations in particular *NCR* genes were identified in *M. truncatula* that affect the bacteroid  
194 differentiation and persistence (Horváth et al. 2015; Kim et al. 2015; Wang et al. 2017; Yang  
195 et al. 2017; Wang et al. 2018). These are very surprising findings in light of the high number  
196 of *NCR* genes in *M. truncatula*, which would intuitively led us to suppose a very high level of  
197 redundancy, but they provide very strong support for the key role of the peptides in the bacteroid  
198 formation.

199 From the bacterial side, several factors were identified that are involved in the response of  
200 bacteroids to this assault of NCR peptides (Mergaert 2018). They can be divided in three broad  
201 categories of functions. First, the NCR peptides interfere with several metabolic processes  
202 including protein synthesis, energy household and nitrogen fixation itself (Farkas et al. 2014).  
203 Second, as mentioned above NCRs are similar to antimicrobial peptides and they indeed have  
204 antimicrobial activity. *S. meliloti* bacteroids use several mechanisms to protect themselves  
205 against this harmful activity of the NCRs (Haag et al. 2011; Montiel et al. 2017; Arnold et al.  
206 2018; Nicoud et al. 2020). The most notable among them is mediated by the peptide transporter  
207 BacA. Finally, the polyploid state of the bacteroids implies that the terminal bacteroid  
208 differentiation is driven by a switch in the bacterial cell cycle whereby the regular cycle  
209 composed of sequential steps of a single genome replication followed by cell division is  
210 transformed into a process of repeated genome replications without cell divisions (Mergaert et  
211 al. 2006). In the next sections, we first discuss the state of the art of our knowledge of the regular  
212 cell cycle control in *S. meliloti* and then we will analyze the available data that highlight how  
213 the NCR peptides can interfere with the cell cycle to promote the bacteroid differentiation.

214

215 **The cell cycle in *Sinorhizobium meliloti***

216

217 *S. meliloti* division is asymmetrical and always produces two different cell types, a “small” type  
218 and a “large” type (Figure 2). The large cell is able to replicate its genome and to produce new  
219 small and large cells. The small cell, on the contrary, does not have the capacity to replicate its  
220 DNA and to divide immediately. It must first differentiate into a large cell before initiating a  
221 new cell cycle (De Nisco et al. 2014). This morphological asymmetry imposes thus a  
222 continuous asynchrony between the subsequent cell cycles of the daughter cells after division.  
223 To our knowledge, there is no exception in *S. meliloti* to the rule of one single round of genome  
224 replication per cell division as the origin of replication is strictly controlled by multiple  
225 regulatory mechanisms that ensure this perfect coordination between DNA replication and cell  
226 division (De Nisco et al. 2014; Pini et al. 2015).

227 The expression of almost 500 genes varies as a function of progression in cell cycle in *S.*  
228 *meliloti*, and these genes show peak expression corresponding to the timing of their cellular  
229 function (De Nisco et al. 2014). This time-regulated expression of genes, which are required  
230 for specific functions, was analyzed by developing a new method of synchronization of *S.*  
231 *meliloti* bacterial cultures. The method is based on the induction of the stringent response  
232 (induced by carbon and nitrogen starvation) able to block cells in G1 phase by Rel-dependent  
233 ppGpp accumulation (De Nisco et al. 2014). G1-blocked cells are then able to proceed through  
234 a complete and synchronized cell cycle with only one DNA replication round, ultimately  
235 leading to an asymmetrical cell division. DNA replication was analyzed further by tracking the  
236 origin of replication of the different replicons of *S. meliloti* (Frage et al. 2016a). This bacterium  
237 possesses three large replicons: a 4 mega-bases circular chromosome with a single DnaA-  
238 dependent origin of replication, a second smaller replicon (1.9 mega-bases), named pSymB,  
239 that contains several essential genes and many genes involved in the adaptation to

240 environmental niches and finally a dispensable smaller mega plasmid (1,5 mega-bases), named  
241 pSymA, carrying genes mostly associated to symbiosis (Galibert et al. 2001; Capela et al. 2001;  
242 Finan et al. 2001). Surprisingly, the initiation of replication of the three origins of replication is  
243 temporally and spatially separated in the cell, with the chromosome being the first to be  
244 replicated with the newly-replicated origins located very close to the polar regions of the cell.  
245 The mega plasmid pSymB follows the chromosome replication with its origin located in  
246 proximity of the pole but shifted towards the center of the cell. Finally, pSymA replication starts  
247 after pSymB and its origin at the beginning of its replication is localized almost at mid-cell  
248 (Frage et al. 2016a). This remarkable spatial and temporal organization suggests that DNA  
249 replication in *S. meliloti* is highly organized by mechanisms that are still unknown.

250

251 **Cell cycle regulation in *Sinorhizobium meliloti***

252

253 ***The conserved architecture of the master cell cycle regulatory circuit***

254 As revealed by the bioinformatic analysis of alphaproteobacterial genomes, almost all factors  
255 that regulate the cell cycle in the model system *Caulobacter crescentus*, are also present in *S.*  
256 *meliloti* (Brilli et al. 2010). The conservation obviously suggests the evolution of the cell cycle  
257 program from a common ancestor of the two organisms. However, as we will specifically  
258 discuss here for *S. meliloti*, every alphaproteobacterial species appears different from the others  
259 by displaying variations on the common theme, suggesting that the cell cycle machinery has  
260 subsequently diverged in order to adapt to different lifestyles and physiologies. The adaptation  
261 to intracellular life or life in host tissues in the case of alphaproteobacterial species interacting  
262 with eukaryotes (rhizobia, *Brucella*, *Agrobacterium* etc.) involves the formation of specific  
263 infecting cell types that may have required the evolution of particular cell cycle regulators.  
264 Regulation of cell cycle in *S. meliloti* and other alphaproteobacterial species is based on a small  
265 number of conserved master regulators of the cell cycle. These master regulators coordinate  
266 most of the genes controlling essential steps in cell cycle progression and together constitute  
267 the master regulatory circuit of the cell cycle. Although our knowledge is still preliminary in  
268 many bacterial models, it is reasonable to assume that the master regulators DnaA, GcrA, CtrA  
269 and CcrM are well-conserved cell cycle factors in most of the species of the class  
270 *Alphaproteobacteria* (Wright et al. 1997; Barnett et al. 2001; Brilli et al. 2010). The four master  
271 regulators, at least in *C. crescentus*, where they have been studied since the early 90s, are  
272 synthesized in succession to drive sequential steps of the cell cycle but also to directly activate  
273 the downstream master regulator (Figure 3). DnaA activates *gcrA*, GcrA activates *ctrA*, CtrA  
274 activates *ccrM*, and finally, the DNA methylase CcrM resets the cycle by completely  
275 methylating the chromosome. DnaA is a protein that activates the initiation of DNA replication

276 in bacteria by opening the double helix at the origin of replication and facilitating the action of  
277 the helicase DnaB (Sibley et al. 2006a; Skarstad and Katayama 2013). However, DnaA has a  
278 dual role and is also involved in the transcriptional regulation of the next master regulator gene  
279 *gcrA* although the mechanism by which DnaA is able to activate gene expression remains still  
280 elusive. GcrA promotes on its turn *ctrA* transcription, most likely indirectly since it is probably  
281 not a transcription factor. CtrA (Cell cycle Transcriptional Regulator A) is a DNA-binding  
282 response regulator, member of the two-component signal transduction family. CtrA is the most  
283 interconnected regulator of the four master regulators. CtrA controls transcription of the  
284 downstream master regulator *ccrM* but it also inhibits *gcrA* transcription, promotes its own  
285 transcription and inhibits DnaA-mediated DNA replication through binding to the replication  
286 origin. Finally, the methylase CcrM exerts a negative epigenetic regulation on its own  
287 expression and expression of *ctrA* and a positive epigenetic regulation on *dnaA* expression. In  
288 addition, it methylates the origin of replication possibly making it competent for a new round  
289 of replication, although its precise role with respect to the initiation of DNA replication is not  
290 yet uncovered. A second regulatory circuit is integrated in this loop of master regulators at the  
291 level of CtrA and is discussed in detail in the following paragraph.

292

### 293 ***CtrA: evolution from control of motility to a cell cycle regulator***

294 CtrA can be considered as the most important cell cycle regulator in *S. meliloti*. The crucial role  
295 in the regulation of the cell cycle by the CtrA response regulator was demonstrated for the first  
296 time in the model species *C. crescentus* (Quon et al. 1996). Response regulators belong to the  
297 family of Two-Component systems (TCS) and are generally proteins composed by a receiver  
298 domain (REC) with a conserved aspartic residue and an output domain, which usually binds  
299 DNA. Phosphorylation of the REC domain leads to dimerization (Gao and Stock 2009),  
300 creating an active dimer of the response regulator that is able to bind its consensus sequence (a

301 palindromic sequence composed by two half sites) located in the promoter region of target  
302 genes and activate their expression. CtrA presumably belongs to this class of response  
303 regulators suggesting that a dimeric form of phosphorylated CtrA interacts with its palindromic  
304 consensus sequence that we can approximate to the sequence AATT(N<sub>7</sub>)AATT. This consensus  
305 sequence is conserved across alphaproteobacterial genera, spanning from *Rickettsia* to  
306 *Caulobacter*, *Sinorhizobium*, *Magnetospirillum* or *Rhodobacter* (Brassinga et al. 2002; Brillì et  
307 al. 2010; Mercer et al. 2010; Greene et al. 2012). Based on the presence of this consensus in the  
308 promoter region of genes of alphaproteobacterial genomes, the conservation of functions in  
309 alphaproteobacterial species was analyzed *in silico*, revealing that regulation by CtrA is usually  
310 linked to motility, which is probably the ancestral function controlled by CtrA (Brillì et al.  
311 2010; Greene et al. 2012; Mercer et al. 2012). In species belonging to the *Caulobacteriales*  
312 (including *C. crescentus*) and *Rhizobiales* (*S. meliloti*, *B. abortus* and *A. tumefaciens*, for  
313 example), CtrA controls in addition to motility also cell cycle-related functions (Brillì et al.  
314 2010). This recruitment of CtrA to the essential function of cell division is obviously associated  
315 to the essentiality of the gene *ctrA* in these species (Quon et al. 1996; Barnett et al. 2001; Pini  
316 et al. 2015). Conversely, in species in which CtrA controls only motility, CtrA is not essential  
317 for bacterial viability and its disruption only affects the flagellum biogenesis and possibly some  
318 other non-essential functions (Greene et al. 2012; Mercer et al. 2012).

319 In cell cycle regulation, CtrA controls DNA replication and cell division. Genome replication  
320 is affected in a negative way. In *C. crescentus*, this inhibition is direct by binding of  
321 phosphorylated CtrA (CtrA~P) to several CtrA boxes present in the origin of replication,  
322 preventing DnaA to initiate replication (Quon et al. 1998). In contrast, in *S. meliloti* this control  
323 cannot be direct as there are no CtrA boxes in its origin of replication (Sibley et al. 2006a; Pini  
324 et al. 2015). However, upon depletion of CtrA, the cell fails to block the reinitiation of DNA  
325 replication, resulting in cells with multiple DNA copies. This observation suggests that also in



326 *S. meliloti* some CtrA-dependent mechanism for inhibition of DNA replication exists. Thus  
327 even if the molecular mechanisms are different between *C. crescentus* and *S. meliloti*, in both  
328 strains the function of CtrA to inhibit replication is conserved. Cell division, on the contrary, is  
329 positively regulated by CtrA (Quon et al. 1996; Pini et al. 2015). Although the gene sets  
330 regulated by CtrA are strikingly different in the *Alphaproteobacteria*, among them are motility  
331 and chemotaxis functions, DNA methylation and cell division (Laub et al. 2002; De Nisco et  
332 al. 2014; Pini et al. 2015). For example, in *S. meliloti*, CtrA represses the Min system, which  
333 inhibits septum formation and division by preventing FtsZ polymerization and Z-ring  
334 formation, while in *C. crescentus*, which lacks the Min system, *ftsZ* transcription is positively  
335 regulated by CtrA.

336 The dual and opposite activity on replication and division places CtrA at the center of the strict  
337 cell cycle control in *Alphaproteobacteria*. It further suggests that CtrA levels must change  
338 during the cell cycle: at the onset of DNA replication, CtrA must be inactive in order to activate  
339 DNA replication, while in the pre-divisional step CtrA must be present in order to activate  
340 crucial division functions. This observation implies that CtrA activity must be highly regulated.  
341 In the next section, we will review these CtrA regulatory mechanisms (Figure 3).

342

### 343 ***The CtrA regulatory circuit: multiple levels of regulation of CtrA***

344 CtrA has to be phosphorylated to be active and this is mediated by the DivL, CckA and ChpT  
345 phosphorelay cascade in *C. crescentus* (Figure 3) (Xue and Biondi 2019). Although the  
346 orthologous genes of this cascade are present in *S. meliloti* (Brilli et al. 2010), their  
347 characterization has not yet been carried out.

348 On the contrary, the module of the CtrA-inhibitor DivK, a single receiver domain of the two-  
349 component system protein family, similar to CheY, has been intensively investigated in *S.*  
350 *meliloti*, together with its complex kinase/phosphatase module, composed of the kinases DivJ

351 and CbrA and the phosphatase PleC (Figure 3) (Lam et al. 2003; Gibson et al. 2006; Gibson et  
352 al. 2007; Sadowski et al. 2013; Pini et al. 2013; Schallies et al. 2015a). In *C. crescentus*  
353 phosphorylated DivK blocks, by protein-protein interactions, the DivL, CckA and ChpT  
354 ~~phosphorelay cascade~~ and thereby prevents CtrA phosphorylation and activation. DivK in *C.*  
355 *crescentus* is an essential factor for cell cycle progression as loss of function mutants of *divK*  
356 are arrested at the G1 phase (Hecht et al. 1995). DivK is also essential in *S. meliloti* acting as  
357 the main negative regulator of CtrA (Pini et al. 2013; Pini et al. 2015). The absence of DivK or  
358 its inability to be phosphorylated, results in a stable and constitutively active CtrA that on its  
359 turn blocks the origin of replication.

360 In *S. meliloti* the active form of DivK, responsible for CtrA inhibition, is phosphorylated by  
361 two kinases, DivJ and CbrA (Pini et al. 2013), which both contribute to the pool of DivK~P.  
362 Deletion of either of the two kinases leads to a severe cell cycle defect showing elongated and  
363 branched cells with a slow growth rate. The double deletion of *divJ* and *cbrA* is lethal,  
364 unambiguously demonstrating that phosphorylation of DivK is absolutely necessary for a  
365 proper cell cycle progression (Pini et al. 2013). Conversely, the ability to remove the phosphate  
366 group from DivK~P at specific stages of the cell cycle is also essential as the only known DivK  
367 phosphatase, PleC, is equally indispensable in *S. meliloti* (Fields et al. 2012; Pini et al. 2013).  
368 Surprisingly, in *C. crescentus* the deletion of *divJ*, a gene encoding the only known DivK  
369 kinase, the deletion of the phosphatase-encoding *pleC* are possible, including the double  
370 deletion, while the mutation of the phosphorylation site in DivK is not tolerated by *C.*  
371 *crescentus* cells. This observation suggests an unknown redundant function that may  
372 compensate the absence of DivK phosphorylation or an alternative pathway that phosphorylates  
373 DivK. The CbrA alternative kinase of DivK in *S. meliloti* does not exist in *C. crescentus*,  
374 illustrating the evolution of unique architectural features of the cell cycle network in different  
375 species.

376 CtrA protein levels in *S. meliloti* are modulated during cell cycle progression with a minimum  
377 at the G1-S transition (initiation of the chromosome replication) (Pini et al. 2015). Presumably,  
378 this decrease of CtrA levels depends on a mechanism of active degradation of the protein by  
379 the protease ClpXP, assisted by several other proteins that are present and well characterized in  
380 *C. crescentus* (Figure 3). Specifically, the single receiver domain protein CpdR, active in the  
381 non-phosphorylated form, is required for CtrA degradation (Kobayashi et al. 2009; Pini et al.  
382 2015; Schallies et al. 2015a). CpdR is itself phosphorylated and inactivated by ChpT of the  
383 above-mentioned DivL, CckA and ChpT phosphorelay cascade (Figure 3) (Schallies et al.  
384 2015b). Moreover the protein RcdA is essential in *S. meliloti* and it is required for CtrA  
385 degradation, as a conditional mutant of *rcdA* showed high levels of CtrA and a lethal block of  
386 the cell cycle (Pini et al. 2015).

387 Besides the post-translational control by phosphorylation and protein degradation of the CtrA  
388 protein activity, the *ctrA* gene also has a complex transcriptional regulation (Figure 3). Its  
389 transcription is driven by a compound promoter region with at least two different promoters,  
390 named P1 and P2 (Barnett et al. 2001). The *ctrA* promoter in *C. crescentus* has two equivalent  
391 P1 and P2 promoters. The upstream master regulator GcrA activates the P1 promoter but  
392 indirectly. The subsequently produced phosphorylated CtrA blocks then the P1 promoter but  
393 activates transcription from the P2 promoter. In *S. meliloti* however, CtrA has only a mild  
394 positive regulation of its own promoter at P1 (Pini et al. 2015). Thus other factors than those  
395 operating in *C. crescentus* are probably involved in the transcriptional regulation of *S. meliloti*  
396 *ctrA*. The role of *S. meliloti* GcrA in the activation of *ctrA* expression has not been studied yet  
397 but the phenotype of the genetic depletion of GcrA is compatible with such an activity (Robledo  
398 et al. 2015). Furthermore, the *ctrA* gene in *C. crescentus* is also epigenetically regulated by  
399 CcrM methylation. In *S. meliloti*, this mechanism needs to be examined. In addition, two small  
400 non-coding RNAs (sRNA) were recently identified that may post-transcriptionally repress the

401 expression of the *ctrA* gene. The sRNA EcpR1 was predicted to target multiple cell cycle genes  
402 in *S. meliloti*, including *ctrA*. However, further experimental validation with a GFP reporter  
403 assay, involving wild-type and mutant sRNA and mRNA pairs, did confirm regulation by  
404 EcpR1 only for *dnaA* and *gcrA* but not for *ctrA* (Robledo et al. 2015). Another sRNA, named  
405 GspR, was confirmed with the GFP reporter assay to post-transcriptionally down-regulated *ctrA*  
406 expression (Robledo et al. 2018). Thus, sRNAs may directly or indirectly through *gcrA* fine-  
407 tune *ctrA* expression and modulate the cell cycle regulation, potentially in response to external  
408 factors.

409 **Symbiosis and the *S. meliloti* cell cycle**

410

411 The polyploidy of the *S. meliloti* bacteroids is a deviation of the single round of genome  
412 replication per cell division rule, which is governed as discussed above by CtrA and other  
413 master regulators (Figure 3). Thus, the cell cycle switch underlying the bacteroid differentiation  
414 should perturb this cascade. Most tellingly, the cell cycle switch is clearly observable by  
415 analyzing inside nodule tissues the expression of an extended set of *S. meliloti* cell cycle  
416 regulators (Figure 4). The meristem of *M. truncatula* nodules continuously generates new cells,  
417 which become infected and in which bacteria differentiate. This differentiation takes place  
418 gradually along the longitudinal axis of the nodule. The expression of plant and bacterial genes  
419 was analyzed by combining laser-capture microdissection of nodule tissues along this  
420 longitudinal axis with RNA-seq (Roux et al. 2014b). A uniform expression of the cell cycle  
421 genes in all tissues would be expected in case the cell cycle stays unaffected during bacteroid  
422 differentiation. However, this is not what is observed (Figure 4). Rather, the relative expression  
423 of genes greatly varies in the different tissues and thus according to the stage of bacterial  
424 differentiation. This modulation of expression is gene- and cell cycle- dependent. For example,  
425 the expression of *ctrA* as well as of many CtrA-regulated genes or genes encoding CtrA  
426 phosphorylation regulators are very rapidly downregulated when differentiation starts.  
427 Accordingly, Western blot analysis confirmed the absence of the CtrA and FtsZ proteins from  
428 bacteroids extracted from *Medicago* nodules (Pini et al. 2013; Farkas et al. 2014). The DNA  
429 replication associated genes *dnaA*, *dnaN* and *hdaA* on the other hand show the strongest relative  
430 expression in the nodule tissues where differentiation and genome amplification is taking place.  
431 The cell cycle regulatory cascade is a robust machinery that allows the strict respect of the  
432 cycle: haploid state – replication – diploid state – division, characterizing wild type *S. meliloti*  
433 growth. However, genetic or pharmacological interference with this cascade can disrupt the

434 regular cell cycle and induce bacteroid-like cells, which are strongly enlarged and branched and  
435 have a multiplied genome while at the same time further growth and cell divisions are blocked.  
436 For example, the depletion of *ctrA* has such an effect (Pini et al. 2015). Also depletion of the  
437 *ctrA*-transcriptional regulators *gcrA* and *ntrX* or overexpression of *ccrM* or the sRNA EcpR1  
438 provoke the same phenomenon (Wright et al. 1997; Robledo et al. 2015; Xing et al. 2020).  
439 Furthermore, the overexpression of *divJ*, depletion of *pleC* or mutation of *podJ1* lead to the  
440 accumulation of phosphorylated DivK which blocks ~~which encode negative regulators of CtrA~~  
441 phosphorylation. This prevents CtrA ~~and thus~~ activation and results in bacteroid-like cells, ~~has~~  
442 ~~a similar effect~~ (Fields et al. 2012; Pini et al. 2015). ~~as well as the~~ The expression of a  
443 constitutively active form of CpdR1 that stimulates CtrA degradation has a similar effect  
444 (Kobayashi et al. 2009). ~~Furthermore~~ Moreover, mutation or overexpression of the septum-  
445 formation controlling genes *ftsZ* and *minCDE* or pharmacological inhibition of septum  
446 formation again provoke the same cell elongation and branching effect (Latch and Margolin  
447 1997; Cheng et al. 2007). Finally, overexpression of the *dnaA* and *hdaA* genes, encoding the  
448 replication machinery, have also such a cellular effect, although in that case the balance  
449 between the three replicons, the chromosome, pSymA and pSymB, is not maintained in the  
450 amplified genomes (Sibley et al. 2006b; Frage et al. 2016b). Taken together, perturbing the  
451 CtrA pathway leads thus systematically to cellular changes that mimic partially or strongly the  
452 bacteroid state.

453 Thus, CtrA, because of its key position in the cell cycle regulation, is a likely target for the  
454 NCR peptides in bacteroid differentiation: its elimination would be compatible with the  
455 inhibition of cell division and the continued DNA replication (Figure 3). As suggested by the  
456 above-cited genetic studies, CtrA could be directly targeted or it could be inactivated and  
457 eliminated via its transcriptional or post-translational regulators. In agreement with the key  
458 position of CtrA for bacteroid differentiation, *S. meliloti* mutants in the genes encoding

459 negative regulators of CtrA, such as *cbrA*, *divJ* and *cpdR* genes, make non-functional nodules  
460 without bacteroid differentiation (Gibson et al. 2006; Kobayashi et al. 2009; Pini et al. 2013).  
461 Another strong argument in favor of CtrA being the ultimate target of the NCR peptides to  
462 trigger the bacteroid differentiation is the demonstration that in NCR247-treated synchronized  
463 *S. meliloti* cells the expression of the CtrA-controlled genes are not properly activated during  
464 the progression of the cell cycle (De Nisco et al. 2014).

465

466 **Benefits of terminal bacteroid differentiation**

467

468 *S. meliloti* lives in the soil as a free-living saprophyte even without the presence of legumes  
469 (Carelli et al. 2000). This suggests that the capability to establish a symbiosis is not an essential  
470 function of the species, as it is further revealed by the discovery of *S. meliloti* strains unable to  
471 induce and infect nodules. A recent study has highlighted that *S. meliloti* colonizes the plant as  
472 an endophyte, being recovered from leaves and other tissues from *Medicago* plants (Pini et al.  
473 2012). This observation suggests an interesting scenario about the evolutionary origin of  
474 bacteroid formation. The plant may have evolved a way to prevent uncontrolled infection of  
475 the endophyte, by blocking bacterial duplication and inducing a “terminal differentiation”.  
476 Possibly, the NCR peptides that are now only active in nodules are derived from immune  
477 peptides that were originally employed to control endophytic bacteria including *S. meliloti*  
478 strains.

479 The multiple independent origins of terminal bacteroid differentiation in the legume family is  
480 a strong suggestion that the process provides benefits to the host plant (Oono et al. 2011). The  
481 benefits should be indeed on the plant side because it is the host that imposes the process by the  
482 production of NCR peptides and the process limits very strongly the bacterial reproduction.  
483 Several studies have provided experimental confirmations that the terminal bacteroid  
484 differentiation improves the efficiency of the symbiosis by increasing the plant biomass  
485 production per investment in the symbiosis. These analyses were comparative studies in which  
486 either a particular rhizobium strain was compared on two host plants, or alternatively, one host  
487 plant nodulated with different rhizobium strains. When different hosts were compared, one host  
488 induced terminal bacteroid differentiation and the other host undifferentiated bacteroids (Sen  
489 and Weaver 1981; Sen and Weaver 1984; Oono et al. 2011) or the two hosts induced both  
490 terminal bacteroid differentiation but to different levels (Lamouche et al. 2019a; Lamouche et



491 al. 2019b). In the opposite type of comparison, *Medicago* hosts were nodulated with a panel of  
492 strains displaying contrasted bacteroid differentiation levels (different levels of genome  
493 amplification and cell enlargement in the bacteroids), correlating well with the efficiency of the  
494 interaction (Kazmierczak et al. 2017). However, these comparisons although consistent with  
495 what we predicted, can be criticized as comparing apples with oranges because it is not possible  
496 to determine how much of the differences is due to bacteroid differentiation, and how much is  
497 due to other differences between the compared plant species or bacterial species. Moreover,  
498 they only show correlations, which do not mean causality. To go beyond these correlations, an  
499 experimental system would be required that uses one particular host in interaction with one  
500 rhizobium strain and in which the bacteroid differentiation can be manipulated. Possibly, the *in*  
501 *planta* modification of the expression of cell cycle regulators, as discussed above in *in vitro*  
502 studies, can offer such opportunities.

503 Such an approach could provide a firm proof for the improvement of the symbiotic functioning  
504 of the bacteroids when terminally differentiated. However, this would still not explain why this  
505 type of bacteroids is performing better. This is at present an unresolved question but we can  
506 speculate about some of the consequences of the bacteroid differentiation that could impact the  
507 functioning of the bacteroids. The first possibility is that the advantage is linked to the cell size  
508 and that the cell enlargement makes bacteroids better nitrogen-fixing machines. Could larger  
509 bacteroids be more energy-efficient than small non-differentiated ones? This is not self-evident.  
510 Larger bacterial cells means a higher volume-to-surface ratio but since respiratory energy  
511 production is a membrane process, an increased volume-to-surface ratio is energetically not  
512 advantageous. On the other hand, if energy production would not be rate limiting for nitrogen  
513 fixation in bacteroids, a larger volume could favor protein synthesis (for example for the  
514 massive production of the nitrogenase complex and its metal cofactors) by reducing its cost.  
515 Cell enlargement could also be viewed as a form of cell compartmentalization that physically

516 separates the oxygen-requiring respiratory complexes from the oxygen-sensitive nitrogenase  
517 machinery. In a large cell, the nitrogenase could be located in the center of the cell, far away  
518 (on an atomic scale) from respiration on the cell membranes.

519 Additionally, large cells could dampen functional heterogeneity between bacteroids as recently  
520 demonstrated. Indeed, many bacterial genes show cell-to-cell fluctuations due to noise in gene  
521 expression, leading to phenotypic diversity between cells (Ackermann 2015). Heterogeneity in  
522 a cell population can be advantageous to bacteria in certain circumstances, by providing  
523 adaptability to unpredictably changing environments. In the nodule however, it could be  
524 detrimental for the symbiosis and be associated with suboptimal performance of a  
525 subpopulation of bacteroids. In large cells such as terminally differentiated bacteroids, gene  
526 expression noise could be reduced by effectively averaging cell contents, as has been shown in  
527 polyploid division-blocked *Bacillus subtilis* mutants, resulting in a decreased functional  
528 heterogeneity between cells (Süel et al. 2007).

529 Another possibility is that the polyploidy state may provide the improvement of bacteroid  
530 functioning. The respiration of bacteroids and the nitrogen fixation process itself by the  
531 nitrogenase are inevitable sources of reactive oxygen species (Matamoros et al. 2003). Reactive  
532 oxygen species may induce deleterious mutations, which in the long term may affect the  
533 functioning of the bacteroids. Polyploid bacteroids could be less sensitive to DNA damage  
534 because they have multiple gene copies. Thus, the polyploid state of bacteroids could increase  
535 their longevity, which would imply a delayed senescence. The polyploid chromosomes in  
536 bacteroids could bring along also a benefit at another level. More condensed than the  
537 chromosomes in free-growing rhizobia (Mergaert et al. 2006), the polyploid chromosomes in  
538 bacteroids could function differently. Their compaction could have an epigenetic impact on for  
539 example gene expression.

540 On the other hand, the cell cycle switch with the ensuing polyploidy and cell enlargement could  
541 be only side effects of another important function of the NCRs on the bacteroids. The NCR  
542 peptides, as many other antimicrobial peptides, disturb the membrane integrity of bacteria (Van  
543 de Velde et al. 2010; Mikuláss et al. 2016) and this correlates with the known enhanced  
544 membrane permeability in terminally differentiated bacteroids (Mergaert et al. 2006). The  
545 membrane permeabilization of the bacteroids could enhance the metabolic exchanges between  
546 the symbionts thereby favoring optimally the nitrogen-fixation metabolism of the bacteroids  
547 with the metabolism of the host cell (Mergaert et al. 2017). Moreover, metabolite exchange can  
548 also be favored in the terminally differentiated bacteroids because they are individually  
549 enclosed in a symbiosome and have a much closer contact with the symbiosome membrane  
550 than undifferentiated bacteroids. In the last case a single symbiosome harbours multiple bacteria  
551 and thus the direct contact of the bacteria with the symbiosome membrane is reduced.  
552 Furthermore, NCR peptides were reported to interact directly with several metabolic enzymes,  
553 including the ribosomes, chaperones, enzymes of the energy metabolism and the nitrogenase  
554 (Farkas et al. 2014). Thus, the primary effect of the NCRs could be the manipulation of the  
555 metabolism of the endosymbiont in order to mold the bacterial metabolism for optimal nitrogen  
556 fixation (Kereszt et al. 2011; Farkas et al. 2014). For example, to maintain a redox balance  
557 during nitrogen fixation, bacteroids channel part of their carbon sources in lipid and  
558 polyhydroxybutyrate electron sinks (Terpolilli et al. 2016). From the plant perspective, this  
559 accumulation of carbon by the bacteroids is a net loss of resources. It is striking that  
560 undifferentiated bacteroids accumulate much larger amounts of these storage compounds than  
561 terminally differentiated bacteroids (Lodwig et al. 2005).

562 A final hypothesis is related to the terminally differentiated state of the bacteroids. The terminal  
563 differentiation could limit the release of rhizobia from senescing nodules thereby moderating  
564 the impact of the symbiosis on the rhizosphere and endophyte microbiota. Moreover, the plant

565 recovers during senescence the bacterial biomass from terminally differentiated bacteroids,  
566 which are entirely digested during nodule senescence (Van de Velde et al. 2006) whereas  
567 undifferentiated bacteroids largely survive nodule senescence (Müller et al. 2001).

568

569 **Conclusions**

570 The regulation of the cell cycle in *S. meliloti* can be largely modeled on *C. crescentus*, which  
571 has been extensively studied in recent decades. At the same time, the study of the *S. meliloti*  
572 cell cycle has revealed that despite the large conservation, many *Sinorhizobium* specificities do  
573 exist and those might be very important because they are likely specific adaptations to the  
574 particularities of the lifestyle of this bacterium. One of the most striking of these particularities  
575 is the cell cycle (de)regulation that happens during the terminal bacteroid differentiation.

576 As argued here, the CtrA master cell cycle regulator is the preferred suspect to be targeted by  
577 the NCR peptides and to direct this major cell cycle event. CtrA has a complex regulation on  
578 its own implementing transcriptional control, small RNA post-transcriptional regulation,  
579 epigenetic mechanisms by DNA methylation, and posttranslational regulation by  
580 phosphorylation and by targeted proteolysis. In principle, NCRs could interfere with any of  
581 these regulations (Fig. 3). Future studies will have to decipher at which regulatory stage this  
582 cascade is affected. A complication in this challenge is the large number and diversity of NCR  
583 peptides that are produced by the plant. It is very well possible that not a single peptide does  
584 the full job but that several peptides act synergistically, sequentially or redundantly. Moreover,  
585 the NCRs could be interacting directly with these intracellular regulators but they could also  
586 act indirectly at the level of the bacterial membrane and interfere with the cell cycle via a signal  
587 transduction process.

588 We can furthermore hope that deepening our understanding of this particular cell cycle  
589 operating in bacteroid differentiation will uncover novel aspects of the regular cell cycle in *S.*  
590 *meliloti* and by extension in other members of the class *Alphaproteobacteria*.

591

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596

597 **Legends**

598

599 **Figure 1: The symbiosis is a close relationship between two different organisms.** (Panel A)

600 The symbiosis between the alphaproteobacterium *Sinorhizobium meliloti* and the legume  
601 *Medicago sativa* (on the left) produces a new organ called nodule, where an exchange of  
602 metabolites occurs: the bacterium reduces atmospheric nitrogen into ammonia that is absorbed  
603 by the plant; in return this latter provides carbon sources and protection to the bacterium. On  
604 the top panel, a zoom on the structure of a nodule and, indicated with colored bars, the  
605 expression of various NCR (Nodule Cysteine rich) peptides are shown. Once in the infection  
606 zone, the bacterium begins a dramatic differentiation process under the influence of the NCR  
607 peptides leading to a bacteroid cell. A symbiosome (a zoom is represented in the bottom central  
608 part) is composed by a layer of vegetal membrane containing a bacteroid cell, characterized by  
609 (a) multiple copies of DNA, (b) a cell enlargement (ten times bigger than the free-living cell  
610 also showed here), (c) an inability to divide and (d) a higher permeability, as shown by a dotted  
611 envelope. (Panel B) Image of a symbiotic plant cell stained with comassie (vegetal cell wall in  
612 blue) full of bacteroids (blue); plant nucleus is white (Peter Mergaert, unpublished). Black bar  
613 corresponds to 10  $\mu\text{m}$ .

614

615 **Figure 2. The cell cycle in *Sinorhizobium meliloti*.** *S. meliloti* is a rod-shaped bacterium  
616 belonging to the *Alphaproteobacteria* class. The bacterium contains three replicons (circles of

617 different colors) that duplicate only once per cell cycle. DNA replication is followed by an  
618 asymmetrical cell division producing a large and a small cell. From the left to the right: during  
619 the G1 phase (one copy of each replicon) a small cell differentiates into a large cell, this latter  
620 begins the S phase (DNA replication), then in G2 phase the pre-divisional cell divides  
621 asymmetrically producing a new small cell, unable to replicate, and a large cell that is able to  
622 immediately initiate a new round of DNA replication.

623

624 **Figure 3: Schematics of the complex regulation network of the cell cycle in *S. meliloti* and**  
625 **the involvement of NCR peptides during the symbiosis process.** (A) CtrA is the master  
626 regulator of the cell cycle; its active form is phosphorylated and it directly inhibits the DNA  
627 replication and indirectly activates the cell division by inhibiting the expression of Min system,  
628 an inhibitor of the cell division. CtrA is strictly regulated to ensure a normal cell cycle  
629 progression. This regulation occurs at different levels, including a post translation level, by  
630 phosphorylation (yellow box) and proteolysis (orange box), as well as the transcription level  
631 through potentially GcrA and CcrM. The potential targets cell cycle of NCRs peptides are  
632 indicated with blue arrows. (Panel B) The free-living *S. meliloti* replicates its genome only once  
633 per cell cycle leading each time to two cell daughters (blue box). However, during the symbiotic  
634 process (pink box) the bacterium is targeted by NCRs which lead to a drop of CtrA levels; as a  
635 result of this differentiation process the bacterium becomes a bacteroid characterized by  
636 multiple copies of DNA (nC) and a cell division stop.

637

638 **Figure 4: The gene expression of cell cycle regulators during the differentiation.** Under  
639 the impact of NCRs peptides, the bacterium differentiates into a bacteroid (upper left  
640 schematics). This differentiation consists of an endoreduplication and an absence of division  
641 resulting in a larger cell with, up to 24 16-32 copies of DNA copies. Transcriptomic data of the

642 cell cycle regulators genes in different nodule zones (on the right), shows a variation of the  
643 expression of cell cycle genes compared to the free-living growth condition. In particular  
644 DnaA/GcrA and CtrA/CcrM patterns are indicated in the lower left part of the figure.

645  
646  
647



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649

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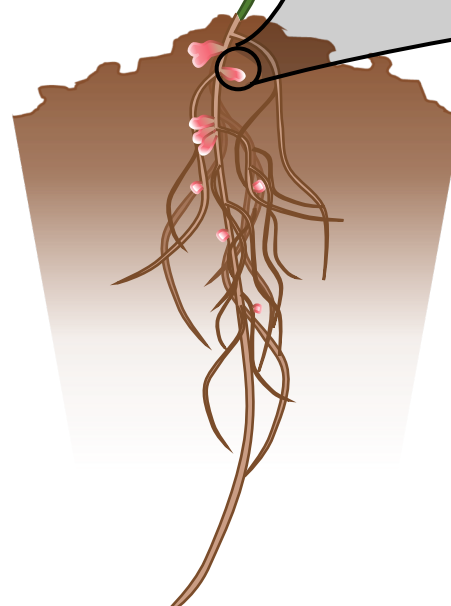
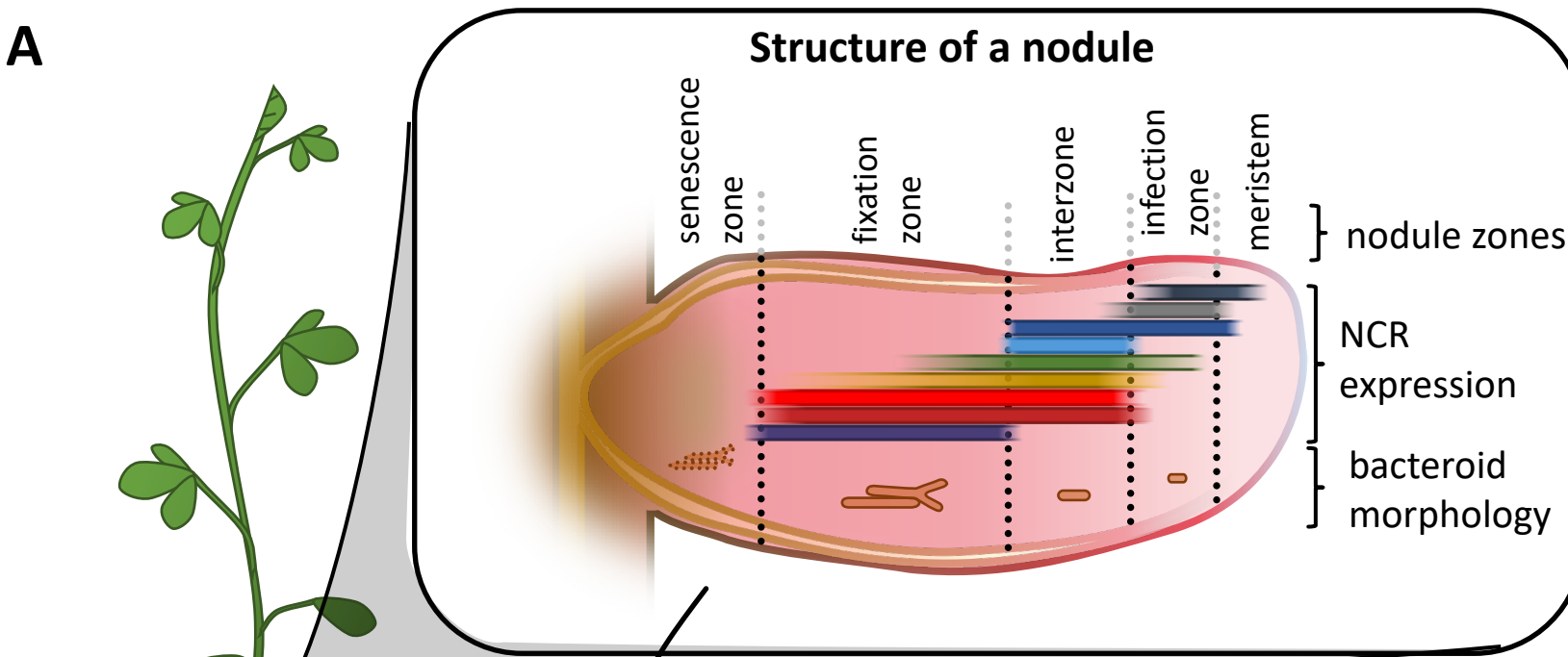
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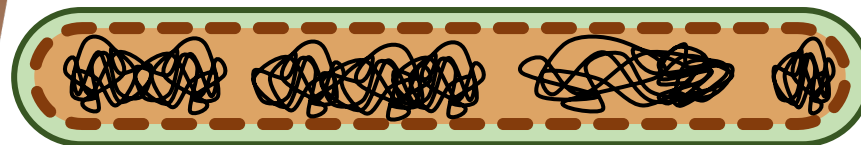
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950

Figure 1

A



**Zoom on a bacteroid**



Bacteroid (up to 10  $\mu\text{m}$ )



Free bacterium  
(approx. 1-2  $\mu\text{m}$ )

B

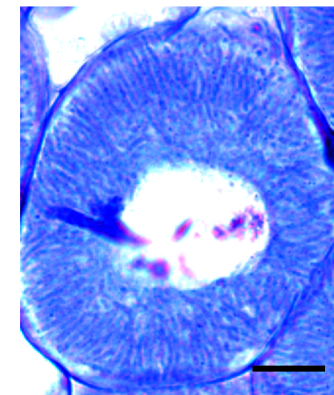




Figure 2

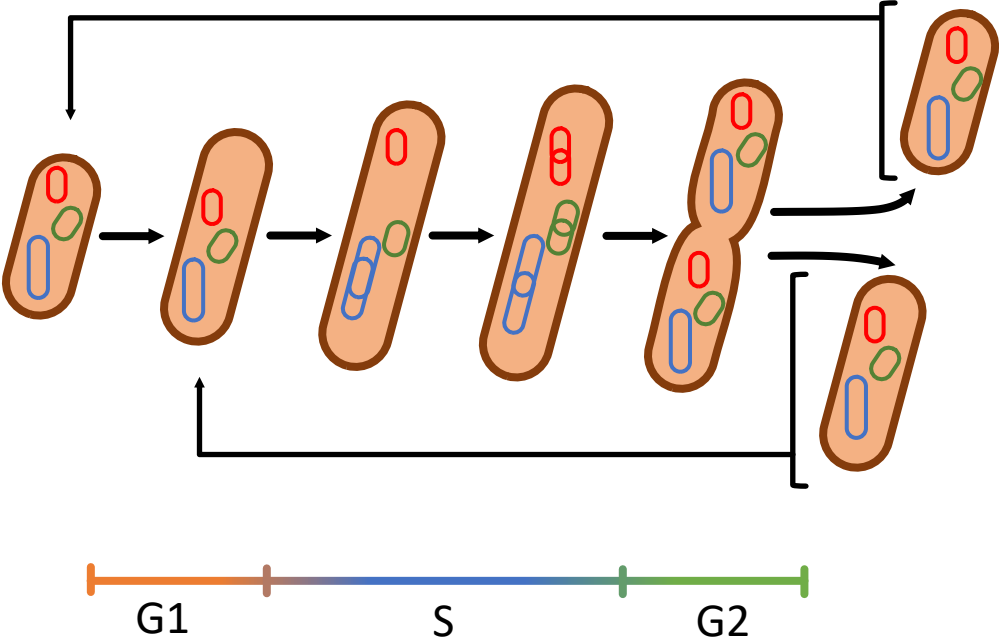


Figure 3

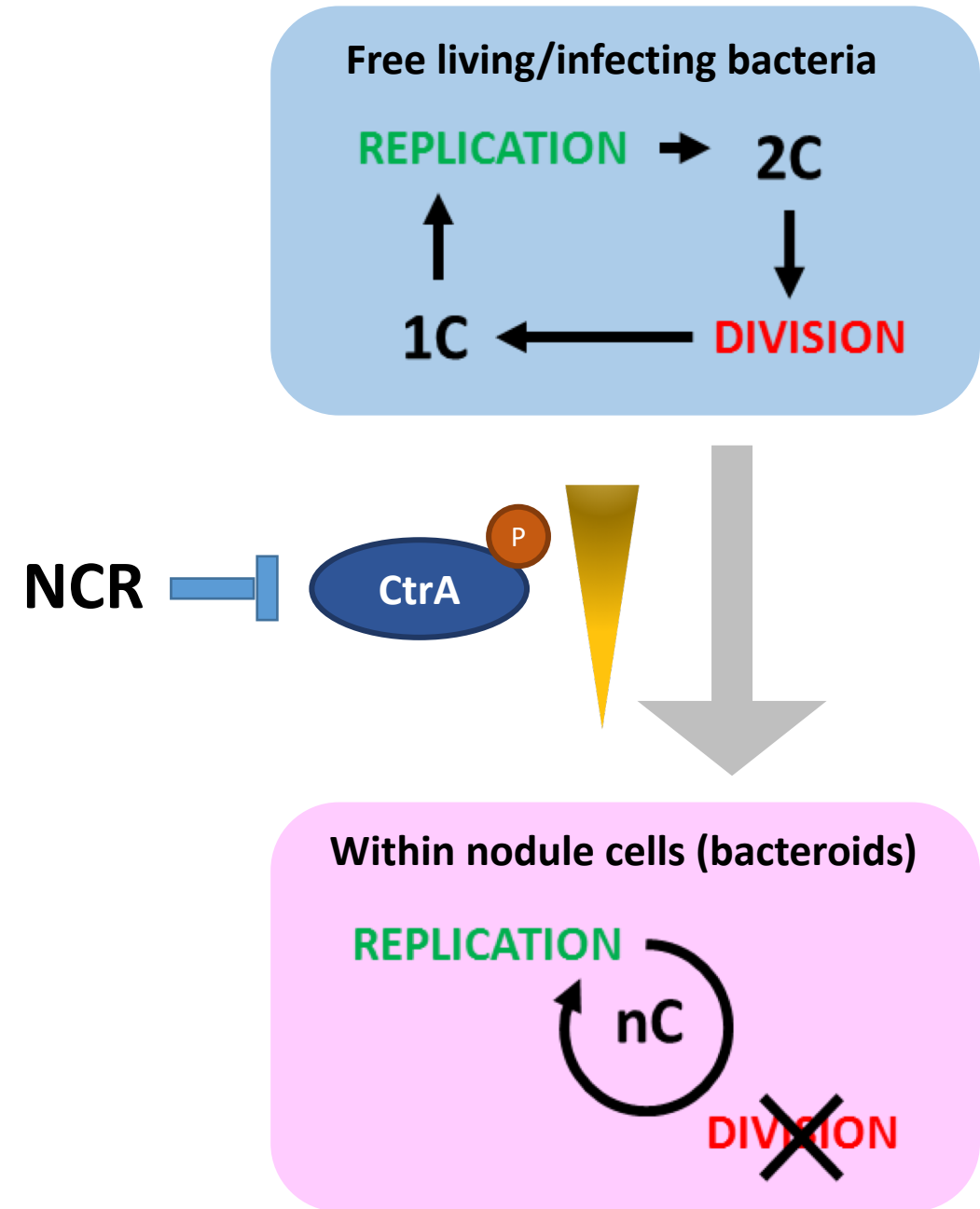
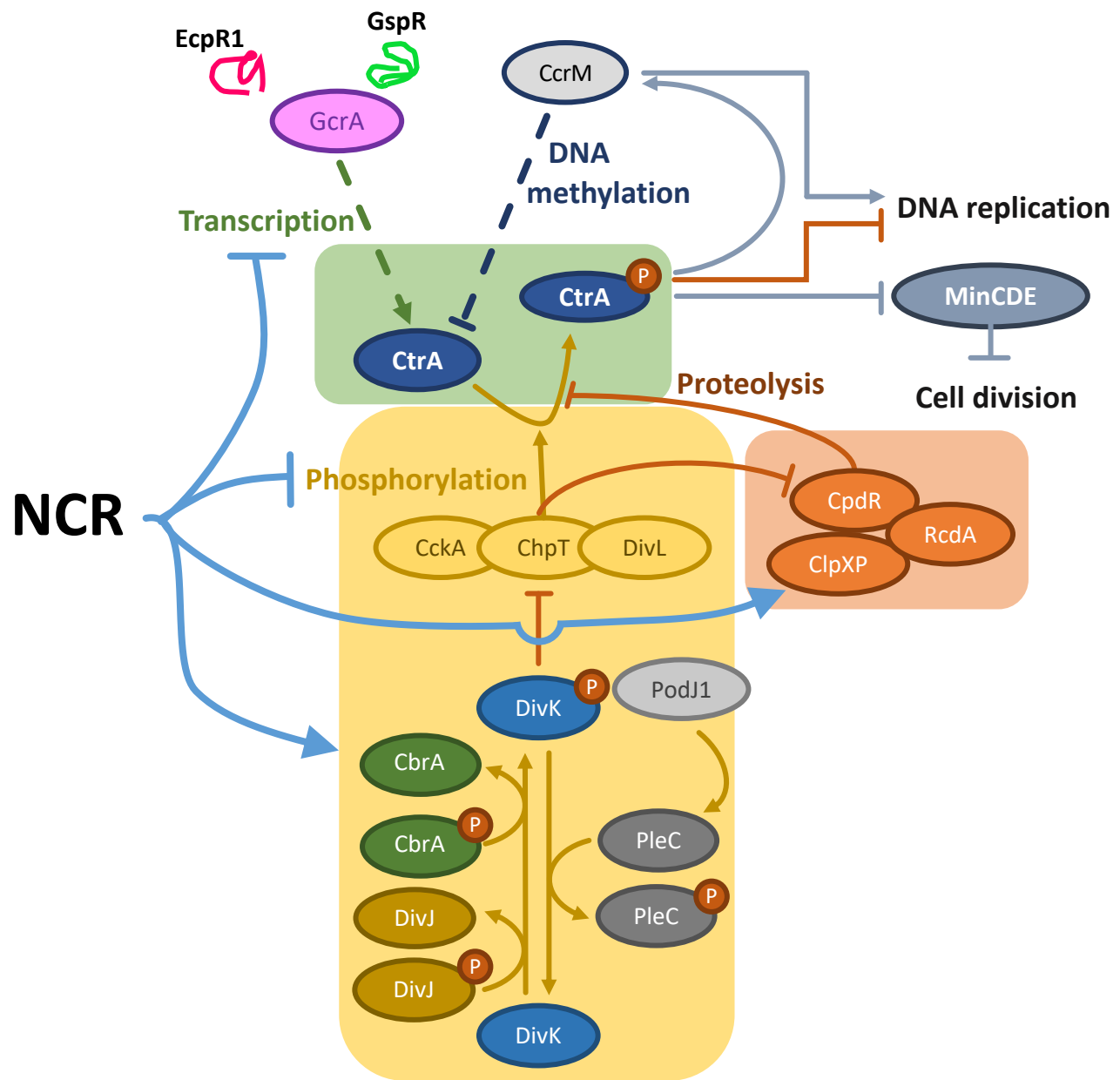


Figure 4

