Coordination of symbiosis and cell cycle functions in Sinorhizobium meliloti

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Abstract

The symbiotic nitrogen fixing species *Sinorhizobium meliloti* represents a remarkable model system for the class *Alphaproteobacteria*, which includes genera such as *Caulobacter*, *Agrobacterium* and *Brucella*. It is capable of living free in the soil, and is also able to establish a complex symbiosis with leguminous plants, during which its cell cycle program is completely rewired presumably due, at least in part, to the action of peptides secreted by the plant. Here we will discuss how the cell cycle regulation works in *S. meliloti* and the kinds of molecular mechanisms that take place during the infection. We will focus on the complex regulation of the master regulator of the *S. meliloti* cell cycle, the response regulator CtrA, discussing its implication in symbiosis.

Keywords:

Sinorhizobium meliloti, cell cycle, symbiosis, nitrogen fixation

Sinorhizobium meliloti biology

Sinorhizobium meliloti belongs to the alpha class of the Gram-negative proteobacteria (*Alphaproteobacteria*). It has been studied for a long time for its ability to infect roots of leguminous plants, such as those of the genus *Medicago* (*M. sativa* and *M. truncatula*). By a complex mechanism (that we will describe in more details in the next sections), this bacterium is able to multiply inside plant cells, within which the bacterial cells fix atmospheric nitrogen into ammonium that can be utilized by plants. In exchange, the plant provides a nutrient rich environment in which a small part of the *S. meliloti* population can benefit. The bacterial form that is able to fix nitrogen is referred to as a bacteroid. The formation of a bacteroid involves a massive differentiation program that results in a cell unable to divide, and is therefore referred to as terminally differentiated. From an evolutionary point of view, this terminal differentiation has puzzled scientists, as it is difficult to explain what is the selective advantage for the bacterium, in the context of a beneficial symbiosis (mutual exchange of nutrients), if the majority of the population is unable to replicate. In this review, a few elements will be described in order to clarify the possible evolutionary hypotheses about the role of bacteroid differentiation.

The first contact between bacteria and plants relies on a specific exchange of molecules, Nod factors produced by bacteria and the flavonoids secreted into the rhizophere (the environment that surrounds roots) by the plants (Cooper 2007; Liu and Murray 2016). The entry of the bacteria into the plant tissue occurs following the formation of a modified radical root hair, which is specifically modulated by the bacterial Nod factors (Shaw and Long 2003; Sieberer et al. 2005). This root hair then traps a few *S. meliloti* cells, which then penetrate inside the root tissue and induce the formation of an

infection thread that is sealed after the entrance of few bacteria (Jones and Walker 2008). In this tunnel, bacteria divide and eventually reach the internal tissue that will host the future bacteroids. Although the mechanism is still poorly known, bacteria are introduced into the plant cell by invagination of the plant cell membrane, resulting in the bacterium being surrounded by a plant-derived membrane. This prokaryotic cell surrounded by the plant membrane is called a "symbiosome" (Jones et al. 2007). The presence of three membranes surrounding a bacteroid that are actively involved in secreting and importing nutrients raises important questions about mechanisms of transport, which up to now have been only partially explored.

As mentioned before, *S. meliloti* lives in the soil as free-living organism even without the presence of legumes (Carelli et al. 2000). This suggests that the capability to establish a symbiosis is not an essential function of the species, as revealed by the discovery of *S. meliloti* strains unable to infect plants. A recent discovery has highlighted that *S. meliloti* colonizes the plant as an endophyte, and can be recovered from leaves and other tissues (Pini et al. 2012). This discovery opened an interesting scenario about the bacteroid formation. In fact, if *S. meliloti* is able to colonize the whole plant, then the plant may have evolved a way to induce a terminal differentiation therefore blocking bacteria duplication and preventing uncontrolled colonization of the plant. As we will see in the next sections, induction of bacteroid differentiation is indeed under the control of plant signals, more specifically peptides, which are indeed able to induce bacteroid-like formation even in laboratory culture (Mergaert et al. 2006). As bacteroids are terminally differentiated, symbiotic peptides are indeed antimicrobial molecules (Kereszt et al. 2011).

Cell cycle, symbiotic infection and differentiation

S. meliloti division produces two different cell types (Figure 1). A "small" cell that is characterized by a smaller size and the incapacity to replicate the DNA and divide (Collier 2012). The "large" cell, on the contrary, is bigger and is able to replicate its genome once per cell division. To our knowledge, there is no exception to one single round of genome replication in *S. meliloti* in free-living cells, as the origin of replication is strictly controlled by multiple regulatory mechanisms that ensure this perfect coordination between DNA replication and cell division (Sibley et al. 2006). Its location, adjacent to *hemE*, is the same as for *oriC* in *Caulobacter crescentus*, the model organism in which the origin of replication has been characterized the most among chromosomes of alphaproteobacterial origins.

The process of infection of *S. meliloti* in the plant root and multiplication inside plant cells is still poorly understood. Many functions are involved in this process, including bacterial cell cycle regulation (see next section) and specific signaling molecules produced by *Medicago* plants (Pan and Wang 2017). In this section we will briefly see how *S. meliloti* establishes this symbiosis. We will also discuss how plants control bacteroid differentiation and what possible bacterial functions may be involved.

A *S. meliloti* bacteroid is a special cell type (Figure 1) that possesses two important features: it is able to fix nitrogen, and it shows a clear, irreversible cell cycle arrest that is responsible for its inability to generate new cells once the nodule enters into a senescent state (Kereszt et al. 2011). Therefore, the bacteroid is metabolically active but indeed terminally differentiated (Barsch et al. 2006). The bacteroid metabolism is under the control of specific regulators named Fix and Nif that will not be discussed here (Jones et al. 2007). Morphologically, *S. meliloti* bacteroids are large and elongated cells (10 times bigger) with respect to the free-living cell, and therefore have a bigger

cytoplasmic volume. Bacteroids show a certain degree of branching with cells named Y-shaped having the form of the letter Y. Y-shaped cells are usually considered as bacteroids in a more mature state. A striking feature of bacteroids is the genome endoreduplication (up to 24 genome copies) (Mergaert et al. 2006), while in free-living *S. meliloti,* cells either have one copy of the genome or two right before the daughter cell separation (De Nisco et al. 2014). Finally the bacterial bacteroid membrane is highly permeable, suggesting a strong exchange of molecules with the host plant cell (Mergaert et al. 2006).

Bacteroid differentiation is controlled by peptides produced by plants, more than 600 in *M. truncatula* (Mergaert et al. 2003), called nodule-specific cysteine rich (NCR) peptides, but only a few of them have been characterized in detail. Indeed, the majority of this large family of peptides has only been predicted by bioinformatics based on the *M. truncatula* genome, while the existence of only 138 of them has been experimentally confirmed (Durgo et al. 2015). However, even for the most characterized peptides, our knowledge is still very preliminary, and the mechanism of action of those peptides on the bacterial cell is still far from understood.

One of the most investigated peptides is called NCR247, which is produced by *M. truncatula* (Van de Velde et al. 2010). Several studies have shown that this NCR peptide may affect multiple targets and functions. NCR247 is able to induce a certain degree of bacteroid differentiation in free-living bacteria cultivated in lab conditions (Van de Velde et al. 2010; Penterman et al. 2014; Farkas et al. 2014). Cysteine residues of NCR247 can be modified by di-sulfur bridge formation changing its targets with respect to the redox state (Haag et al. 2012; Shabab et al. 2016). NCR247 can penetrate the bacterial membrane and form complexes with several bacterial proteins (Farkas et al. 2014). For example this peptide interacts with FtsZ presumably inhibiting bacterial cell

division (Farkas et al. 2014). It also interacts with ribosomal proteins affecting translation and altering the proteome and the physiology of the endosymbiont. NCR247 is further able to directly interact with the chaperone GroEL, which is required for efficient infection, terminal differentiation and nitrogen fixation. Of more interesting from the perspective of this review is the link between NCR247 and the cell cycle regulators of *S. meliloti* (as we will explore in more details in the next section). In particular, sub-lethal doses of NCR247 are able to induce a cell cycle defect similar to bacteroids, by specifically affecting regulons of two master regulators of the cell cycle: GcrA and CtrA (Penterman et al. 2014). The first regulator was discovered and characterized in *C. crescentus* (Holtzendorff et al. 2006; Fioravanti et al. 2012; Murray et al. 2013; Mohapatra et al. 2014), while in *S. meliloti* its role is linked to cell cycle but its mode of action is still unknown (Robledo et al. 2015). On the contrary, CtrA has been studied in *S. meliloti* in greater detail. CtrA plays a clear role as the master regulator of cell cycle, as we will see in the next sections, suggesting that a peptide directly or indirectly acting on its regulon would influence cell differentiation (Pini et al. 2015).

Although NCR247 shows a clear negative antimicrobial effect *in vitro*, and it's presumably implicated in the differentiation of bacteroids, other peptides may actually play a protective role in the plant tissues. This is the case, for example, for NCR169 and NCR211, which were localized in the cellular space between the bacterial membrane and the plant membrane of the symbiosome (Horváth et al. 2015; Kim et al. 2015).

Finally, at least one membrane transporter is important for the activity of the NCR peptides, BacA (Marlow et al. 2009; Haag et al. 2011). More specifically, BacA is able to internalize several antimicrobial peptides and it's necessary to protect the cells to peptide treatments in plants (Haag et al. 2011). The presence of a transporter involved in the peptides activity suggests that the targets of the peptides should be also located in

148 the bacterial cytoplasm. Recently a genetic screening for transposon mutants resistant
149 to NCR247 revealed that tens of genes may protect cells from this peptide (Arnold et al.
150 2017). Those genes are mostly involved in membrane, peptidoglycan and cell envelope
151 physiology, but are also associated with internal functions such as regulation of
152 transcription factors or factors associated with ribosomes. These discoveries suggest
153 that the activity of each peptide may be very general acting on many levels, and possibly
154 involved in rewiring the whole physiology of the bacterial cell.
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Regulation of the cell cycle in *Sinorhizobium meliloti*

Regulation of cell cycle in alphaproteobacterial species, such as *S. meliloti*, *C. crescentus*, *Agrobacterium tumefaciens* or *Brucella abortus*, is based on several conserved factors, called master regulators, that regulate most of the genes controlling essential steps in cell cycle progression. Although our knowledge is still preliminary in many bacterial models, it is reasonable to say that the master regulators CtrA, DnaA, GcrA and CcrM are well-conserved cell cycle factors in most of the species of the class *Alphaproteobacteria* (Wright et al. 1997; Barnett et al. 2001; Brilli et al. 2010). DnaA is a conserved helicase that regulates the initiation of DNA replication in bacteria (Sibley et al. 2006; Skarstad and Katayama 2013). Removing its binding sites in the origin of replication results in a complete arrest of DNA replication (Sibley et al. 2006). As revealed by a bioinformatic analysis of alphaproteobacterial genomes, almost all factors that regulate the cell cycle in the model system *C. crescentus* are also present in *S. meliloti* (Brilli et al. 2010). The exceptions will be commented in the next paragraphs of this section. This conservation suggests a common evolution of the cell cycle program in the two organisms. However, as we will specifically discuss for *S. meliloti*, every alphaproteobacterial species appears different from the others, suggesting that the cell cycle machinery has diverged in every species in order to adapt to different life styles and physiologies (Brilli et al. 2010).

As previously mentioned, the response regulator of the family of two-component systems, named CtrA (Cell cycle Transcriptional Regulator A), plays a crucial role in the regulation of the cell cycle in alphaproteobacterial species as demonstrated for the first time in the model species *C. crescentus* (Quon et al. 1996). Response regulators are generally proteins composed by a receiver domain (REC), with a conserved aspartic residue, and an output domain, which usually binds DNA. Phosphorylation of the REC

182 domain usually leads to dimerization (Gao and Stock 2009), creating an active dimer of
183 the response regulator that is able to bind its consensus sequence at the promoter
184 region of target genes, thereby regulating the genes' expression. CtrA presumably
185 belongs to this class of response regulators, suggesting that a dimeric form of
186 phosphorylated CtrA should interact with its palindromic consensus sequence that we
187 can approximate to AATT(N7)AATT. This consensus sequence is conserved across
188 alphaproteobacterial species, from *Rickettsia* to *Caulobacter*, *Sinorhizobium*,
189 *Magnetospirillum* or *Rhodobacter* (Brassinga et al. 2002; Brilli et al. 2010; Mercer et al.
190 2010; Greene et al. 2012). Based on the presence of this consensus in the promoter
191 region of genes of alphaproteobacterial genomes, the conservation of functions in all
192 species was analyzed *in silico*, revealing that CtrA in all species belonging to this
193 alphaproteobacterial class is usually linked to motility, which is probably the ancestral
194 function controlled by CtrA (Greene et al. 2012; Mercer et al. 2012). In species belonging
195 to the *Caulobacterales* (*C. crescentus*) and *Rhizobiales* (*S. meliloti*, *B. abortus* and *A.*
196 *tumerfaciens*, for example), CtrA potentially controls, in addition to motility, cell cycle-
197 related functions such as cell division and DNA methylation (Brilli et al. 2010). This link
198 to essential functions, such as cell division, explains the essential nature of the *ctrA* gene
199 in those species, while in species in which CtrA controls only motility, the disruption of
200 the gene only affects the flagellum biogenesis and possibly other non-essential functions
201 (Greene et al. 2012; Mercer et al. 2012).
202 A combination of Chromatin Immunoprecipitation-deep sequencing (ChIPseq) and
203 transcriptomic analysis in *S. meliloti* revealed the direct and non-direct regulons of CtrA
204 (Pini et al. 2015). Although many genes are annotated as hypothetical, and require
205 further characterization, several functions appeared to be clearly controlled by CtrA.

206 Several motility and chemotaxis genes are indirect targets of CtrA, such as genes
207 encoding the flagellum apparatus of *S. meliloti* (i.e. *flgBCDH* and *fliEFIL*).
208 Among the important cell cycle regulators that will be introduced in the next
209 paragraphs, the genes *sciP* and *divJ* are directly controlled by CtrA in *S. meliloti*. Unlike in
210 *C. crescentus*, CtrA indirectly regulates *divK* transcription in *S. meliloti*, while CtrA also
211 regulates the second DivK-kinase encoding *cbrA* expression in *S. meliloti* but not in *C.*
212 *crescentus*, where *cbrA* is not present. So this alternative architecture may give a
213 differential degree of control in the negative feedback loop regulating CtrA functions in
214 *S. meliloti*.
215 Genes *minC* and *minD* are the only characterized cell division-related genes directly
216 repressed by CtrA in *S. meliloti*. In *S. meliloti*, as with many other bacteria, MinC and
217 MinD repress cell division by inhibiting FtsZ polymerization and Z-ring formation in the
218 polar regions (Shih and Zheng 2013).
219 In *C. crescentus* and *S. meliloti,* CtrA indeed controls DNA replication and cell division;
220 DNA replication is negatively regulated by CtrA, while cell division genes are directly
221 and positively activated by CtrA (Pini et al. 2015). This dual and opposite activity
222 suggests that CtrA levels and activity must change during the cell cycle; at the onset of
223 DNA replication, CtrA must be inactive in order to activate DNA replication, while in the
224 following steps, CtrA must be present in order to activate crucial functions. This
225 observation implies that CtrA activity should be highly regulated. In this section we will
226 also review all those CtrA regulatory mechanisms.
227 In *C. crescentus* the negative control of DNA replication is dependent on the presence of
228 CtrA binding sites at the origin of replication (Quon et al. 1998). In contrast, no CtrA
229 binding sites have been found in the DnaA-dependent DNA replication origin of *S.*
230 *meliloti*, suggesting either an alternative negative control or possibly the absence of this

231 regulation (Sibley et al. 2006; Pini et al. 2015). CtrA has been further characterized in *S.*
232 *meliloti*, revealing that the *ctrA* gene is indeed essential for the growth of the bacterium
233 (Barnett et al. 2001; Pini et al. 2015). Although the orthologous genes of the
234 phosphorylation cascade of CtrA are present in *S. meliloti* (Brilli et al. 2010), their
235 characterization has never been carried out. On the contrary, the role of the CtrA-
236 inhibitor DivK, which is a single receiver domain of the two-component system protein
237 family, similar to CheY, has been intensively investigated in *S. meliloti* together with its
238 complex kinase/phosphatase module, composed by the kinases DivJ and CbrA and the
239 phosphatase PleC (Lam et al. 2003; Gibson et al. 2006; Gibson et al. 2007; Sadowski et al.
240 2013; Pini et al. 2013; Schallies et al. 2015). DivK, in *C. crescentus,* is an essential factor
241 for cell cycle progression as loss of function mutants of *divK* are arrested at the G1 phase
242 (Hecht et al. 1995). DivK is also essential in *S. meliloti* and acts as the main negative
243 regulator of CtrA (Pini et al. 2015). The absence of DivK, or an inability of DivK to be
244 phosphorylated, results in a stable and constitutively active CtrA that in turns blocks the
245 origin of replication.
246 DivK shows dynamic localization during cell cycle progression, as shown by GFP fusions
247 (Lam et al. 2003), and its localization depends on the polarity factor PodJ1 (Fields et al.
248 2012). The active form of DivK, responsible for CtrA inhibition, is the phosphorylated
249 form, DivK~P. DivK is phosphorylated by two kinases, DivJ and CbrA (Pini et al. 2013),
250 which both contribute to the pool of DivK~P. Deletion of either of the two kinases leads
251 to a severe cell cycle defect showing elongated and branched cells with a slow growth
252 rate. However, the double deletion of *divJ* and *cbrA* is lethal, strongly demonstrating that
253 phosphorylation of DivK is absolutely necessary for a proper cell cycle progression (Pini
254 et al. 2013). Conversely, the ability at specific stages of the cell cycle to remove the
255 phosphate group from DivK~P is also essential as the only known DivK phosphatase,

PleC, is indispensable in *S. meliloti* (Fields et al. 2012; Pini et al. 2013). Surprisingly, in *C. crescentus* deletion of DivJ, the only known DivK kinase in this species, or deletion of the DivK phosphatase PleC are possible, as is the double deletion, while mutation of the phosphorylation site in DivK is not tolerated by *C. crescentus* cells. This observation suggests a redundant function that may compensate for the absence of DivK phosphorylation (Lori et al. 2015), or an alternative phosphorylation pathway. In *S. meliloti*, this redundancy is observed, arguing that different species have evolved a unique architecture of the cell cycle network.

The expression of almost 500 genes varies as a function of the cell cycle in *S. meliloti* (De Nisco et al. 2014). As in *C. crescentus,* many genes show peak expression corresponding with the timing of their cellular function (De Nisco et al. 2014). This time-regulated expression of genes, which are required for specific functions, was analyzed by developing a new method of synchronization for *S. meliloti*, based on the induction of the stringent response (carbon and nitrogen starvation) able to induce G1-blocked cells by Rel-dependent ppGpp accumulation (De Nisco et al. 2014). G1-blocked cells were then able to proceed through a complete and synchronized cell cycle with only one DNA replication cycle, ultimately leading to an asymmetrical cell division.

The genome of *S. meliloti* consists of three replicons: a 4 mega-bases circular chromosome with a single DnaA-dependent origin of replication, a replicon, named pSymB, that contains two essential genes and many genes involved in the adaptation to environmental niches, and a dispensable megaplasmid, named pSymA, mostly associated with symbiosis (Galibert et al. 2001; Capela et al. 2001; Finan et al. 2001). DNA replication in this organism was analyzed further by looking at the origin of replication of the three large replicons of *S. meliloti* (Frage et al. 2016). Surprisingly the three origins of replication are temporally and spatially separated in the cell, with the

chromosome being the first to be replicated with its origin located very close to the polar regions. The megaplasmid pSymA follows the chromosome replication with its origin located in proximity of the pole but shifted towards the center of the cell. Finally pSymB replication starts after pSymA and its origin localization at the beginning of its replication is almost at mid-cell (Frage et al. 2016). This remarkable organization suggests that DNA replication in *S. meliloti* is highly organized with replicons that are kept in the right subcellular localization by mechanisms that are still unknown.

CtrA encoding gene transcription is driven by a complex promoter region with at least two different promoters, named P1 and P2 (Barnett et al. 2001). As in *C. crescentus*, CtrA protein levels change as a function of cell cycle, with the protein levels at a minimum during the G1-S transition (initiation of the chromosome replication) (Pini et al. 2015). Presumably this decrease of CtrA levels depends on a mechanism of active degradation of the protein, which depends on the protease ClpXP and several alphaproteobacterial proteins that are present also in *C. crescentus*. Specifically the single receiver domain protein CpdR, active in the non-phosphorylated form, is required for CtrA degradation and symbiosis (Kobayashi et al. 2009; Pini et al. 2015; Schallies et al. 2015). Moreover the protein RcdA is not dispensable in *S. meliloti* and it's required, as in *C. crescentus,* for CtrA degradation, as a conditional mutant of *rcdA* shows high levels of CtrA and a lethal block of cell cycle (Pini et al. 2015).

Symbiosis and the cell cycle

Surprisingly, the phenotype of *ctrA* depletion resembles the morphology of bacteroids with elongated and enlarged cells that sometimes showing a Y shaped form (Pini et al. 2015). Moreover *ctrA*-depleted cells also show an increase in genome ploidy as bacteroids with all replicons increasing equally their copy number (Pini et al. 2015).

306 This phenotype is consistent with the absence of the CtrA protein in bacteroids
307 extracted from nodules (Pini et al. 2013), and the observation that *ctrA* is barely
308 expressed in the zone of differentiation, while the DNA replication initiation factor DnaA
309 is highly expressed (Roux et al. 2014). These results are also consistent with the results
310 of plants inoculated with a *cpdR* deletion mutant, a protein required for CtrA proteolysis.
311 The nodules Δ*cpdR* inoculated plants are unable to fix nitrogen and contain bacteria that
312 are not differentiated into bacteroids, consistent with a model in which cells with a
313 stable CtrA are unable to differentiate in bacteroids (Kobayashi et al. 2009). As said
314 before, NCR247-treated cells experience a down-regulation of the CtrA-controlled genes,
315 consistent with a mechanism in which bacteroid differentiation depends on CtrA
316 depletion. This results is further reinforced by the observation of a symbiotic defect of a
317 *divJ* deletion mutant that shows cells arrested in the intracellular infection (Pini et al.
318 2013).
319 The link between morphology of bacteroids and nitrogen fixation is not clear yet. For
320 example, the shape and membrane surface/volume ratio of bacteroids may influence the
321 nitrogen fixing performance. On the contrary, the plant's ability to induce terminal
322 differentiation could be instead linked to the necessity to produce bacterial farms,
323 unable to divide but efficiently fixing nitrogen. The latter explanation could suggest that
324 bacteroid formation is required by plants in order to avoid a dangerous multiplication of
325 bacteria inside the plant tissues.

Conclusions

Regulators of bacterial cell cycle are undoubtedly involved in the bacteroid differentiation program of *S. meliloti*. The regulatory network that coordinates DNA replication, cell division and presumably bacteroid differentiation relies on the activity of a master regulator of cell cycle named CtrA, whose role in cell cycle regulation is conserved across alphaproteobacterial species, such as *C. crescentus, B. abortus* and *A. tumefaciens* (Brilli et al. 2010). Among alphaproteobacteria, *C. crescentus* is one of the best models in which cell cycle regulation has been intensively investigated. More recently other bacterial species, such as *S. meliloti,* have also been analyzed in more detail, revealing that although factors are conserved, every species has a unique behavior with differences that may reflect the adaptation to specific life-styles.

In *S. meliloti*, CtrA is essential for viability and controls essential functions such as cell division, DNA replication and DNA methylation. Moreover it controls motility and its regulation by direct activation of the expression of cell cycle regulators. For example, CtrA controls its activity by regulating the DivK module directly (DivJ and CbrA) and indirectly (DivK itself). This negative feedback from CtrA to DivK, the inhibitor of CtrA activity, is also present in *C. crescentus*; however, in *C. crescentus*, this essential transcriptional feedback is directly acting on the *divK* gene (Biondi et al. 2006). From a systems biology point of view, the two different architectures in *C. crescentus* and *S. meliloti,* although similar, may underline a different response to, for example, environmental variations or stresses. Further investaigation on the mechanistic properties of *S. meliloti* should reveal important features of this architecture.

An increasing body of evidence suggests that CtrA may be a crucial factor during bacteroid differentiation. Its absence in mature bacteroids and the phenotype of a CtrA

loss of function strongly suggest that inactivation of CtrA is an essential step in the development of bacteroids. Recent evidences have also pointed that peptides, such as NCR247, may be targeting directly or indirectly CtrA and its complex regulatory apparatus. Research should focus now on revealing this molecular link between NCR peptides and the cell cycle machinery.

Legends

Figure 1. Schematics of *S. meliloti* cell cycle. Cells are rod-shaped and contain three replicons, here represented with three different colors. The chromosome, in red, is the biggest, pSymB is in green, and the smallest is pSymA in blue. Every cell division, two different cell types are formed: a large cell and a small cell, each containing a copy of replicon. The large cell is able to immediately initiate a new round of DNA replication (S phase), while the small cell (G1) must first differentiate into a large cell. Replicons do not replicate at the same time; the chromosome is the first replicon to initiate its replication, followed by pSymA and then pSymB. Moreover, the single origins of replication of each replicon are spatially localized. The chromosome origin has a polar localization, the pSymA origin is proximal to the polar regions, while pSymB possesses almost a mid cell localization. Molecular determinants responsible for this spatial organization of the chromosome are still poorly known. Due to the secretion of NCR peptides by leguminous plants such as *M. sativa*, *S. meliloti* undergoes differentiation becoming larger and longer and accumulating all three replicons up to 24 copies. The dotted line suggests that the connection of bacteroid differentiation and the free-living cell cycle is still unknown. Bacteroid differentiation is defined “terminal” as, to our knowledge, there is no possibility of cell division by bacteroids. Bacteroids are surrounded by a plant membrane (orange line).

Figure 2. Cell cycle regulation network in *S. meliloti.* The circuit is centered on the response regulator CtrA that regulates multiple general functions, such as motility, pilus biogenesis and chemotaxis. More specifically, phosphorylated CtrA (CtrA-P) activates the expression of *ccrM*, an essential methyl-transferase that regulates the cell cycle, *sciP*

encoding the homolog of the *C. crescentus* inhibitor of CtrA activity, and finally DivJ and CbrA, the two kinases of DivK. On the contrary, PleC functions as a phosphatase, removing the phosphate from DivK-P. CtrA directly represses (solid red lines) the Min system, which in turn has an inhibitory activity on FtsZ. Although the molecular link is still unclear, CtrA (dotted red lines) plays a positive role on *divK* transcription and presumably is essential for the coordination of DNA replication, as the absence of CtrA leads to an accumulation of chromosomes. Phosphorylation of CtrA presumably requires DivL, CckA and ChpT and it is inhibited by phosphorylated DivK (DivK-P). Finally CtrA (and/or CtrA-P) is degraded by ClpPX-dependent proteolysis that requires two adapter proteins, named RcdA and CpdR1.

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Figure 1

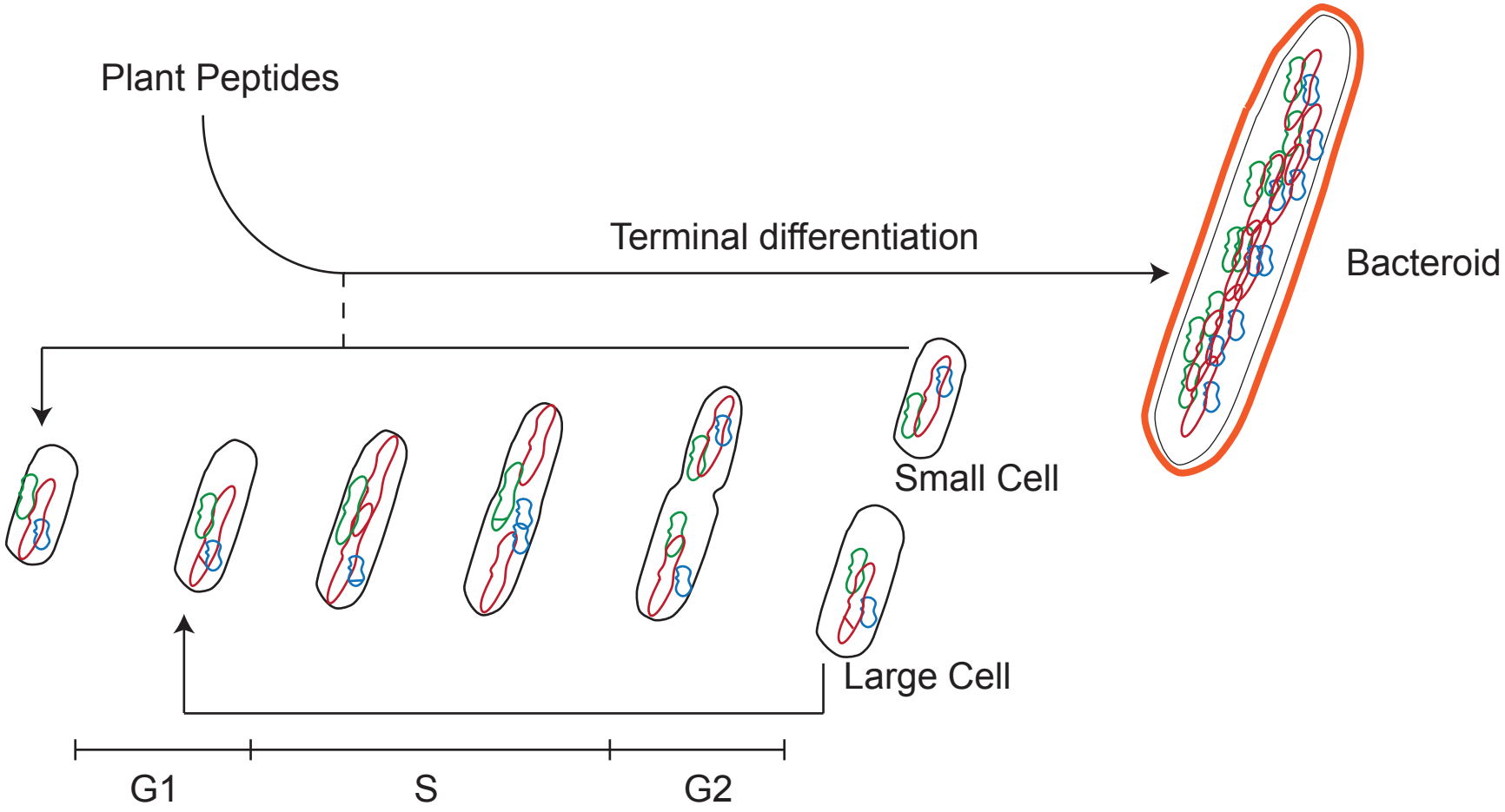
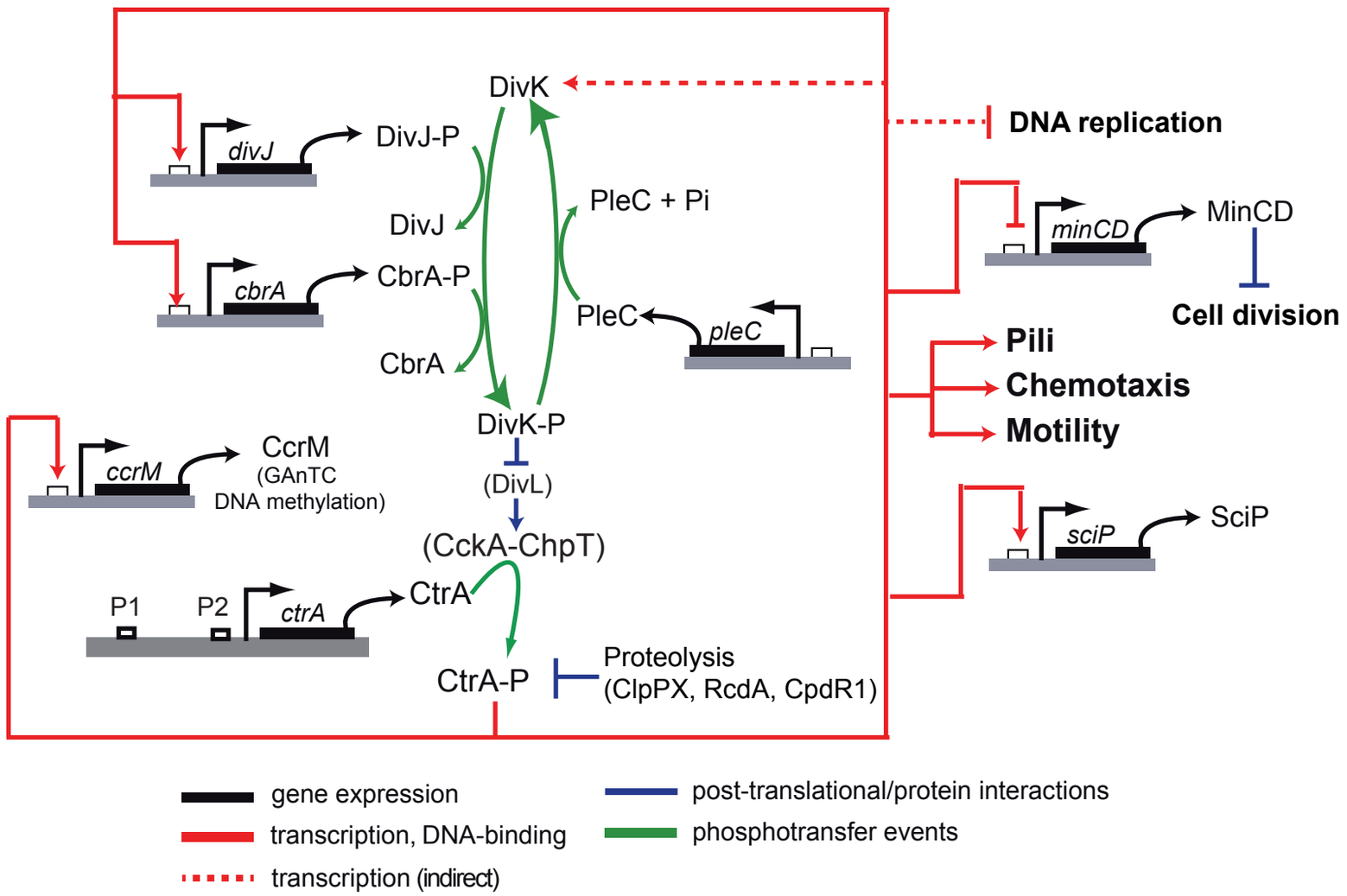

Plant Peptides
Terminal differentiation
Bacteroid
Small Cell
Large Cell
G1
S
G2

Figure 2


DivK
DivJ-P
divJ
DivJ
PleC + Pi
CbrA-P
cbrA
PleC
pleC
CbrA
DivK-P
(DivL)
(CckA-ChpT)
CtrA
P1
P2
ctrA
CtrA-P
Proteolysis
(ClpPX, RcdA, CpdR1)
ccrM
CcrM
(GAnTC
DNA methylation)
DNA replication
minCD
MinCD
Cell division
Pili
Chemotaxis
Motility
sciP
SciP
gene expression
transcription, DNA-binding
transcription (indirect)
post-translational/protein interactions
phosphotransfer events