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# 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 rhizosphere (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

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



## **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

domain usually leads to dimerization (Gao and Stock 2009), creating an active dimer of the response regulator that is able to bind its consensus sequence at the promoter region of target genes, thereby regulating the genes' expression. CtrA presumably belongs to this class of response regulators, suggesting that a dimeric form of phosphorylated CtrA should interact with its palindromic consensus sequence that we can approximate to AATT(N7)AATT. This consensus sequence is conserved across alphaproteobacterial species, from *Rickettsia* to *Caulobacter*, *Sinorhizobium*, *Magnetospirillum* or *Rhodobacter* (Brassinga et al. 2002; Brilli et al. 2010; Mercer et al. 2010; Greene et al. 2012). Based on the presence of this consensus in the promoter region of genes of alphaproteobacterial genomes, the conservation of functions in all species was analyzed *in silico*, revealing that CtrA in all species belonging to this alphaproteobacterial class is usually linked to motility, which is probably the ancestral function controlled by CtrA (Greene et al. 2012; Mercer et al. 2012). In species belonging to the *Caulobacterales* (*C. crescentus*) and *Rhizobiales* (*S. meliloti*, *B. abortus* and *A. tumefaciens*, for example), CtrA potentially controls, in addition to motility, cell cycle-related functions such as cell division and DNA methylation (Brilli et al. 2010). This link to essential functions, such as cell division, explains the essential nature of the *ctrA* gene in those species, while in species in which CtrA controls only motility, the disruption of the gene only affects the flagellum biogenesis and possibly other non-essential functions (Greene et al. 2012; Mercer et al. 2012).

A combination of Chromatin Immunoprecipitation-deep sequencing (ChIPseq) and transcriptomic analysis in *S. meliloti* revealed the direct and non-direct regulons of CtrA (Pini et al. 2015). Although many genes are annotated as hypothetical, and require 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

regulation (Sibley et al. 2006; Pini et al. 2015). CtrA has been further characterized in *S. meliloti*, revealing that the *ctrA* gene is indeed essential for the growth of the bacterium (Barnett et al. 2001; Pini et al. 2015). Although the orthologous genes of the phosphorylation cascade of CtrA are present in *S. meliloti* (Brilli et al. 2010), their characterization has never been carried out. On the contrary, the role of the CtrA-inhibitor DivK, which is a single receiver domain of the two-component system protein family, similar to CheY, has been intensively investigated in *S. meliloti* together with its complex kinase/phosphatase module, composed by the kinases DivJ and CbrA and the phosphatase PleC (Lam et al. 2003; Gibson et al. 2006; Gibson et al. 2007; Sadowski et al. 2013; Pini et al. 2013; Schallies et al. 2015). DivK, in *C. crescentus*, is an essential factor for cell cycle progression as loss of function mutants of *divK* are arrested at the G1 phase (Hecht et al. 1995). DivK is also essential in *S. meliloti* and acts as the main negative regulator of CtrA (Pini et al. 2015). The absence of DivK, or an inability of DivK to be phosphorylated, results in a stable and constitutively active CtrA that in turns blocks the origin of replication.

DivK shows dynamic localization during cell cycle progression, as shown by GFP fusions (Lam et al. 2003), and its localization depends on the polarity factor PodJ1 (Fields et al. 2012). The active form of DivK, responsible for CtrA inhibition, is the phosphorylated form, DivK~P. DivK is phosphorylated by two kinases, DivJ and CbrA (Pini et al. 2013), which both contribute to the pool of DivK~P. Deletion of either of the two kinases leads to a severe cell cycle defect showing elongated and branched cells with a slow growth rate. However, the double deletion of *divJ* and *cbrA* is lethal, strongly demonstrating that phosphorylation of DivK is absolutely necessary for a proper cell cycle progression (Pini et al. 2013). Conversely, the ability at specific stages of the cell cycle to remove the phosphate group from DivK~P is also essential as the only known DivK phosphatase,

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

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

273 The genome of *S. meliloti* consists of three replicons: a 4 mega-bases circular  
274 chromosome with a single DnaA-dependent origin of replication, a replicon, named  
275 pSymB, that contains two essential genes and many genes involved in the adaptation to  
276 environmental niches, and a dispensable megaplasmid, named pSymA, mostly  
277 associated with symbiosis (Galibert et al. 2001; Capela et al. 2001; Finan et al. 2001).  
278 DNA replication in this organism was analyzed further by looking at the origin of  
279 replication of the three large replicons of *S. meliloti* (Frage et al. 2016). Surprisingly the  
280 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  $\Delta cpdR$  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 investigation 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



351 loss of function strongly suggest that inactivation of CtrA is an essential step in the  
352 development of bacteroids. Recent evidences have also pointed that peptides, such as  
353 NCR247, may be targeting directly or indirectly CtrA and its complex regulatory  
354 apparatus. Research should focus now on revealing this molecular link between NCR  
355 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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393

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397

- 401 1. Arnold MFF, Shabab M, Penterman J, Boehme KL, Griffiths JS, Walker GC (2017)  
402 Genome-Wide Sensitivity Analysis of the Microsymbiont *Sinorhizobium meliloti*  
403 to Symbiotically Important, Defensin-Like Host Peptides. *mBio* 8: . doi:  
404 10.1128/mBio.01060-17
- 405 2. Barnett MJ, Hung DY, Reisenauer A, Shapiro L, Long SR (2001) A homolog of the  
406 CtrA cell cycle regulator is present and essential in *Sinorhizobium meliloti*. *J*  
407 *Bacteriol* 183:3204–3210 . doi: 10.1128/JB.183.10.3204-3210.2001
- 408 3. Barsch A, Tellström V, Patschkowski T, Küster H, Niehaus K (2006) Metabolite  
409 profiles of nodulated alfalfa plants indicate that distinct stages of nodule  
410 organogenesis are accompanied by global physiological adaptations. *Mol Plant-*  
411 *Microbe Interact MPMI* 19:998–1013 . doi: 10.1094/MPMI-19-0998
- 412 4. Biondi EG, Reisinger SJ, Skerker JM, Arif M, Perchuk BS, Ryan KR, Laub MT (2006)  
413 Regulation of the bacterial cell cycle by an integrated genetic circuit. *Nature*  
414 444:899–904 . doi: 10.1038/nature05321
- 415 5. Brassinga AKC, Siam R, McSween W, Winkler H, Wood D, Marczyński GT (2002)  
416 Conserved response regulator CtrA and IHF binding sites in the alpha-  
417 proteobacteria *Caulobacter crescentus* and *Rickettsia prowazekii* chromosomal  
418 replication origins. *J Bacteriol* 184:5789–5799
- 419 6. Brilli M, Fondi M, Fani R, Mengoni A, Ferri L, Bazzicalupo M, Biondi EG (2010)  
420 The diversity and evolution of cell cycle regulation in alpha-proteobacteria: a  
421 comparative genomic analysis. *BMC Syst Biol* 4:52 . doi: 10.1186/1752-0509-4-  
422 52
- 423 7. Capela D, Barloy-Hubler F, Gouzy J, Bothe G, Ampe F, Batut J, Boistard P, Becker A,  
424 Boutry M, Cadieu E, Dréano S, Gloux S, Godrie T, Goffeau A, Kahn D, Kiss E,  
425 Lelaure V, Masuy D, Pohl T, Portetelle D, Pühler A, Purnelle B, Ramsperger U,  
426 Renard C, Thébault P, Vandenbol M, Weidner S, Galibert F (2001) Analysis of the  
427 chromosome sequence of the legume symbiont *Sinorhizobium meliloti* strain  
428 1021. *Proc Natl Acad Sci U S A* 98:9877–9882 . doi: 10.1073/pnas.161294398
- 429 8. Carelli M, Gnocchi S, Fancelli S, Mengoni A, Paffetti D, Scotti C, Bazzicalupo M  
430 (2000) Genetic diversity and dynamics of *Sinorhizobium meliloti* populations  
431 nodulating different alfalfa cultivars in Italian soils. *Appl Environ Microbiol*  
432 66:4785–4789
- 433 9. Collier J (2012) Regulation of chromosomal replication in *Caulobacter*  
434 *crescentus*. *Plasmid* 67:76–87 . doi: 10.1016/j.plasmid.2011.12.007
- 435 10. Cooper JE (2007) Early interactions between legumes and rhizobia: disclosing  
436 complexity in a molecular dialogue. *J Appl Microbiol* 103:1355–1365 . doi:  
437 10.1111/j.1365-2672.2007.03366.x
- 438 11. De Nisco NJ, Abo RP, Wu CM, Penterman J, Walker GC (2014) Global analysis of  
439 cell cycle gene expression of the legume symbiont *Sinorhizobium meliloti*. *Proc*  
440 *Natl Acad Sci U S A*. doi: 10.1073/pnas.1400421111
- 441 12. Durgó H, Klement E, Hunyadi-Gulyás E, Szucs A, Kereszt A, Medzihradszky KF,  
442 Kondorosi E (2015) Identification of nodule-specific cysteine-rich plant peptides  
443 in endosymbiotic bacteria. *Proteomics*. doi: 10.1002/pmic.201400385
- 444 13. Farkas A, Maróti G, Durgó H, Györgypál Z, Lima RM, Medzihradszky KF, Kereszt A,

- Mergaert P, Kondorosi E (2014) *Medicago truncatula* symbiotic peptide NCR247 contributes to bacteroid differentiation through multiple mechanisms. *Proc Natl Acad Sci U S A* 111:5183–5188 . doi: 10.1073/pnas.1404169111
14. Fields AT, Navarrete CS, Zare AZ, Huang Z, Mostafavi M, Lewis JC, Rezaeihaighi Y, Brezler BJ, Ray S, Rizzacasa AL, Barnett MJ, Long SR, Chen EJ, Chen JC (2012) The conserved polarity factor podJ1 impacts multiple cell envelope-associated functions in *Sinorhizobium meliloti*. *Mol Microbiol* 84:892–920 . doi: 10.1111/j.1365-2958.2012.08064.x
15. Finan TM, Weidner S, Wong K, Buhrmester J, Chain P, Vorhölter FJ, Hernandez-Lucas I, Becker A, Cowie A, Gouzy J, Golding B, Pühler A (2001) The complete sequence of the 1,683-kb pSymB megaplasmid from the N<sub>2</sub>-fixing endosymbiont *Sinorhizobium meliloti*. *Proc Natl Acad Sci U S A* 98:9889–9894 . doi: 10.1073/pnas.161294698
16. Fioravanti A, Clantin B, Dewitte F, Lens Z, Verger A, Biondi EG, Villeret V (2012) Structural insights into ChpT, an essential dimeric histidine phosphotransferase regulating the cell cycle in *Caulobacter crescentus*. *Acta Crystallograph Sect F Struct Biol Cryst Commun* 68:1025–1029 . doi: 10.1107/S1744309112033064
17. Frage B, Döhlemann J, Robledo M, Lucena D, Sobetzko P, Graumann PL, Becker A (2016) Spatiotemporal choreography of chromosome and megaplasmids in the *Sinorhizobium meliloti* cell cycle. *Mol Microbiol* 100:808–823 . doi: 10.1111/mmi.13351
18. Galibert F, Finan TM, Long SR, Puhler A, Abola P, Ampe F, Barloy-Hubler F, Barnett MJ, Becker A, Boistard P, Bothe G, Boutry M, Bowser L, Buhrmester J, Cadieu E, Capela D, Chain P, Cowie A, Davis RW, Dreano S, Federspiel NA, Fisher RF, Gloux S, Godrie T, Goffeau A, Golding B, Gouzy J, Gurjal M, Hernandez-Lucas I, Hong A, Huizar L, Hyman RW, Jones T, Kahn D, Kahn ML, Kalman S, Keating DH, Kiss E, Komp C, Lelaure V, Masuy D, Palm C, Peck MC, Pohl TM, Portetelle D, Purnelle B, Ramsperger U, Surzycki R, Thebault P, Vandenbol M, Vorholter FJ, Weidner S, Wells DH, Wong K, Yeh KC, Batut J (2001) The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* 293:668–672 . doi: 10.1126/science.1060966
19. Gao R, Stock AM (2009) Biological insights from structures of two-component proteins. *Annu Rev Microbiol* 63:133–154 . doi: 10.1146/annurev.micro.091208.073214
20. Gibson KE, Barnett MJ, Toman CJ, Long SR, Walker GC (2007) The symbiosis regulator CbrA modulates a complex regulatory network affecting the flagellar apparatus and cell envelope proteins. *J Bacteriol* 189:3591–3602 . doi: 10.1128/JB.01834-06
21. Gibson KE, Campbell GR, Lloret J, Walker GC (2006) CbrA is a stationary-phase regulator of cell surface physiology and legume symbiosis in *Sinorhizobium meliloti*. *J Bacteriol* 188:4508–4521 . doi: 10.1128/JB.01923-05
22. Greene SE, Brilli M, Biondi EG, Komeili A (2012) Analysis of the CtrA pathway in *Magnetospirillum* reveals an ancestral role in motility in alphaproteobacteria. *J Bacteriol* 194:2973–2986 . doi: 10.1128/JB.00170-12
23. Haag AF, Balaban M, Sani M, Kerscher B, Pierre O, Farkas A, Longhi R, Boncompagni E, Hérouart D, Dall’angelo S, Kondorosi E, Zanda M, Mergaert P, Ferguson GP (2011) Protection of *Sinorhizobium* against host cysteine-rich antimicrobial peptides is critical for symbiosis. *PLoS Biol* 9:e1001169 . doi: 10.1371/journal.pbio.1001169

24. Haag AF, Kerscher B, Dall'Angelo S, Sani M, Longhi R, Baloban M, Wilson HM, Mergaert P, Zanda M, Ferguson GP (2012) Role of cysteine residues and disulfide bonds in the activity of a legume root nodule-specific, cysteine-rich peptide. *J Biol Chem* 287:10791–10798 . doi: 10.1074/jbc.M111.311316
25. Hecht GB, Lane T, Ohta N, Sommer JM, Newton A (1995) An essential single domain response regulator required for normal cell division and differentiation in *Caulobacter crescentus*. *EMBO J* 14:3915–3924
26. Holtzendorff J, Reinhardt J, Viollier PH (2006) Cell cycle control by oscillating regulatory proteins in *Caulobacter crescentus*. *BioEssays News Rev Mol Cell Dev Biol* 28:355–361 . doi: 10.1002/bies.20384
27. Horváth B, Domonkos Á, Kereszt A, Szűcs A, Ábrahám E, Ayaydin F, Bóka K, Chen Y, Chen R, Murray JD, Udvardi MK, Kondorosi É, Kaló P (2015) Loss of the nodule-specific cysteine rich peptide, NCR169, abolishes symbiotic nitrogen fixation in the *Medicago truncatula* dnf7 mutant. *Proc Natl Acad Sci U S A* 112:15232–15237 . doi: 10.1073/pnas.1500777112
28. Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat Rev Microbiol* 5:619–633 . doi: 10.1038/nrmicro1705
29. Jones KM, Walker GC (2008) Responses of the model legume *Medicago truncatula* to the rhizobial exopolysaccharide succinoglycan. *Plant Signal Behav* 3:888–890
30. Kereszt A, Mergaert P, Kondorosi E (2011) Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims? *Mol Plant-Microbe Interact MPMI* 24:1300–1309 . doi: 10.1094/MPMI-06-11-0152
31. Kim M, Chen Y, Xi J, Waters C, Chen R, Wang D (2015) An antimicrobial peptide essential for bacterial survival in the nitrogen-fixing symbiosis. *Proc Natl Acad Sci U S A* 112:15238–15243 . doi: 10.1073/pnas.1500123112
32. Kobayashi H, De Nisco NJ, Chien P, Simmons LA, Walker GC (2009) *Sinorhizobium meliloti* CpdR1 is critical for co-ordinating cell cycle progression and the symbiotic chronic infection. *Mol Microbiol* 73:586–600 . doi: 10.1111/j.1365-2958.2009.06794.x
33. Lam H, Matroule J-Y, Jacobs-Wagner C (2003) The asymmetric spatial distribution of bacterial signal transduction proteins coordinates cell cycle events. *Dev Cell* 5:149–159
34. Liu C-W, Murray JD (2016) The Role of Flavonoids in Nodulation Host-Range Specificity: An Update. *Plants Basel Switz* 5: . doi: 10.3390/plants5030033
35. Lori C, Ozaki S, Steiner S, Böhm R, Abel S, Dubey BN, Schirmer T, Hiller S, Jenal U (2015) Cyclic di-GMP acts as a cell cycle oscillator to drive chromosome replication. *Nature* 523:236–239 . doi: 10.1038/nature14473
36. Marlow VL, Haag AF, Kobayashi H, Fletcher V, Scocchi M, Walker GC, Ferguson GP (2009) Essential role for the BacA protein in the uptake of a truncated eukaryotic peptide in *Sinorhizobium meliloti*. *J Bacteriol* 191:1519–1527 . doi: 10.1128/JB.01661-08
37. Mercer RG, Callister SJ, Lipton MS, Pasa-Tolic L, Strnad H, Paces V, Beatty JT, Lang AS (2010) Loss of the response regulator CtrA causes pleiotropic effects on gene expression but does not affect growth phase regulation in *Rhodobacter capsulatus*. *J Bacteriol* 192:2701–2710 . doi: 10.1128/JB.00160-10
38. Mercer RG, Quinlan M, Rose AR, Noll S, Beatty JT, Lang AS (2012) Regulatory systems controlling motility and gene transfer agent production and release in *Rhodobacter capsulatus*. *FEMS Microbiol Lett* 331:53–62 . doi: 10.1111/j.1574-

- 6968.2012.02553.x
39. Mergaert P, Nikovics K, Kelemen Z, Maunoury N, Vaubert D, Kondorosi A, Kondorosi E (2003) A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. *Plant Physiol* 132:161–173 . doi: 10.1104/pp.102.018192
40. Mergaert P, Uchiumi T, Alunni B, Evanno G, Cheron A, Catrice O, Mausset A-E, Barloy-Hubler F, Galibert F, Kondorosi A, Kondorosi E (2006) Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium-legume* symbiosis. *Proc Natl Acad Sci U S A* 103:5230–5235 . doi: 10.1073/pnas.0600912103
41. Mohapatra SS, Fioravanti A, Biondi EG (2014) DNA methylation in *Caulobacter* and other Alphaproteobacteria during cell cycle progression. *Trends Microbiol* 22:528–535 . doi: 10.1016/j.tim.2014.05.003
42. Murray SM, Panis G, Fumeaux C, Viollier PH, Howard M (2013) Computational and genetic reduction of a cell cycle to its simplest, primordial components. *PLoS Biol* 11:e1001749 . doi: 10.1371/journal.pbio.1001749
43. Pan H, Wang D (2017) Nodule cysteine-rich peptides maintain a working balance during nitrogen-fixing symbiosis. *Nat Plants* 3:17048 . doi: 10.1038/nplants.2017.48
44. Penterman J, Abo RP, De Nisco NJ, Arnold MFF, Longhi R, Zanda M, Walker GC (2014) Host plant peptides elicit a transcriptional response to control the *Sinorhizobium meliloti* cell cycle during symbiosis. *Proc Natl Acad Sci U S A* 111:3561–3566 . doi: 10.1073/pnas.1400450111
45. Pini F, De Nisco NJ, Ferri L, Penterman J, Fioravanti A, Brilli M, Mengoni A, Bazzicalupo M, Viollier PH, Walker GC, Biondi EG (2015) Cell Cycle Control by the Master Regulator CtrA in *Sinorhizobium meliloti*. *PLoS Genet* 11:e1005232 . doi: 10.1371/journal.pgen.1005232
46. Pini F, Frage B, Ferri L, De Nisco NJ, Mohapatra SS, Taddei L, Fioravanti A, Dewitte F, Galardini M, Brilli M, Villeret V, Bazzicalupo M, Mengoni A, Walker GC, Becker A, Biondi EG (2013) The DivJ, CbrA and PleC system controls DivK phosphorylation and symbiosis in *Sinorhizobium meliloti*. *Mol Microbiol* 90:54–71 . doi: 10.1111/mmi.12347
47. Pini F, Frascella A, Santopolo L, Bazzicalupo M, Biondi EG, Scotti C, Mengoni A (2012) Exploring the plant-associated bacterial communities in *Medicago sativa* L. *BMC Microbiol* 12:78 . doi: 10.1186/1471-2180-12-78
48. Quon KC, Marczyński GT, Shapiro L (1996) Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* 84:83–93
49. Quon KC, Yang B, Domian IJ, Shapiro L, Marczyński GT (1998) Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc Natl Acad Sci U S A* 95:120–125
50. Robledo M, Frage B, Wright PR, Becker A (2015) A stress-induced small RNA modulates alpha-rhizobial cell cycle progression. *PLoS Genet* 11:e1005153 . doi: 10.1371/journal.pgen.1005153
51. Roux B, Rodde N, Jardinaud M-F, Timmers T, Sauviac L, Cottret L, Carrère S, Sallet E, Courcelle E, Moreau S, Debelle F, Capela D, de Carvalho-Niebel F, Gouzy J, Bruand C, Gamas P (2014) An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *Plant J Cell Mol Biol* 77:817–837 . doi: 10.1111/tpj.12442



- 592 52. Sadowski C, Wilson D, Schallies K, Walker G, Gibson KE (2013) The  
593 Sinorhizobium meliloti sensor histidine kinase CbrA contributes to free-living cell  
594 cycle regulation. Microbiol Read Engl. doi: 10.1099/mic.0.067504-0
- 595 53. Schallies KB, Sadowski C, Meng J, Chien P, Gibson KE (2015) Sinorhizobium  
596 meliloti CtrA Stability Is Regulated in a CbrA-Dependent Manner That Is  
597 Influenced by CpdR1. J Bacteriol 197:2139–2149 . doi: 10.1128/JB.02593-14
- 598 54. Shabab M, Arnold MFF, Penterman J, Wommack AJ, Bocker HT, Price PA, Griffiths  
599 JS, Nolan EM, Walker GC (2016) Disulfide cross-linking influences symbiotic  
600 activities of nodule peptide NCR247. Proc Natl Acad Sci U S A 113:10157–10162 .  
601 doi: 10.1073/pnas.1610724113
- 602 55. Shaw SL, Long SR (2003) Nod factor elicits two separable calcium responses in  
603 Medicago truncatula root hair cells. Plant Physiol 131:976–984 . doi:  
604 10.1104/pp.005546
- 605 56. Shih Y-L, Zheng M (2013) Spatial control of the cell division site by the Min  
606 system in Escherichia coli. Environ Microbiol 15:3229–3239 . doi: 10.1111/1462-  
607 2920.12119
- 608 57. Sibley CD, MacLellan SR, Finan T (2006) The Sinorhizobium meliloti  
609 chromosomal origin of replication. Microbiol Read Engl 152:443–455 . doi:  
610 10.1099/mic.0.28455-0
- 611 58. Sieberer BJ, Timmers ACJ, Emons AMC (2005) Nod factors alter the microtubule  
612 cytoskeleton in Medicago truncatula root hairs to allow root hair reorientation.  
613 Mol Plant-Microbe Interact MPMI 18:1195–1204 . doi: 10.1094/MPMI-18-1195
- 614 59. Skarstad K, Katayama T (2013) Regulating DNA replication in bacteria. Cold  
615 Spring Harb Perspect Biol 5:a012922 . doi: 10.1101/cshperspect.a012922
- 616 60. Van de Velde W, Zehirov G, Szatmari A, Debreczeny M, Ishihara H, Kevei Z, Farkas  
617 A, Mikulass K, Nagy A, Tiricz H, Satiat-Jeunemaître B, Alunni B, Bourge M, Kucho  
618 K, Abe M, Kereszt A, Maroti G, Uchiumi T, Kondorosi E, Mergaert P (2010) Plant  
619 peptides govern terminal differentiation of bacteria in symbiosis. Science  
620 327:1122–1126 . doi: 10.1126/science.1184057
- 621 61. Wright R, Stephens C, Shapiro L (1997) The CcrM DNA methyltransferase is  
622 widespread in the alpha subdivision of proteobacteria, and its essential functions  
623 are conserved in Rhizobium meliloti and Caulobacter crescentus. J Bacteriol  
624 179:5869–5877

Figure 1

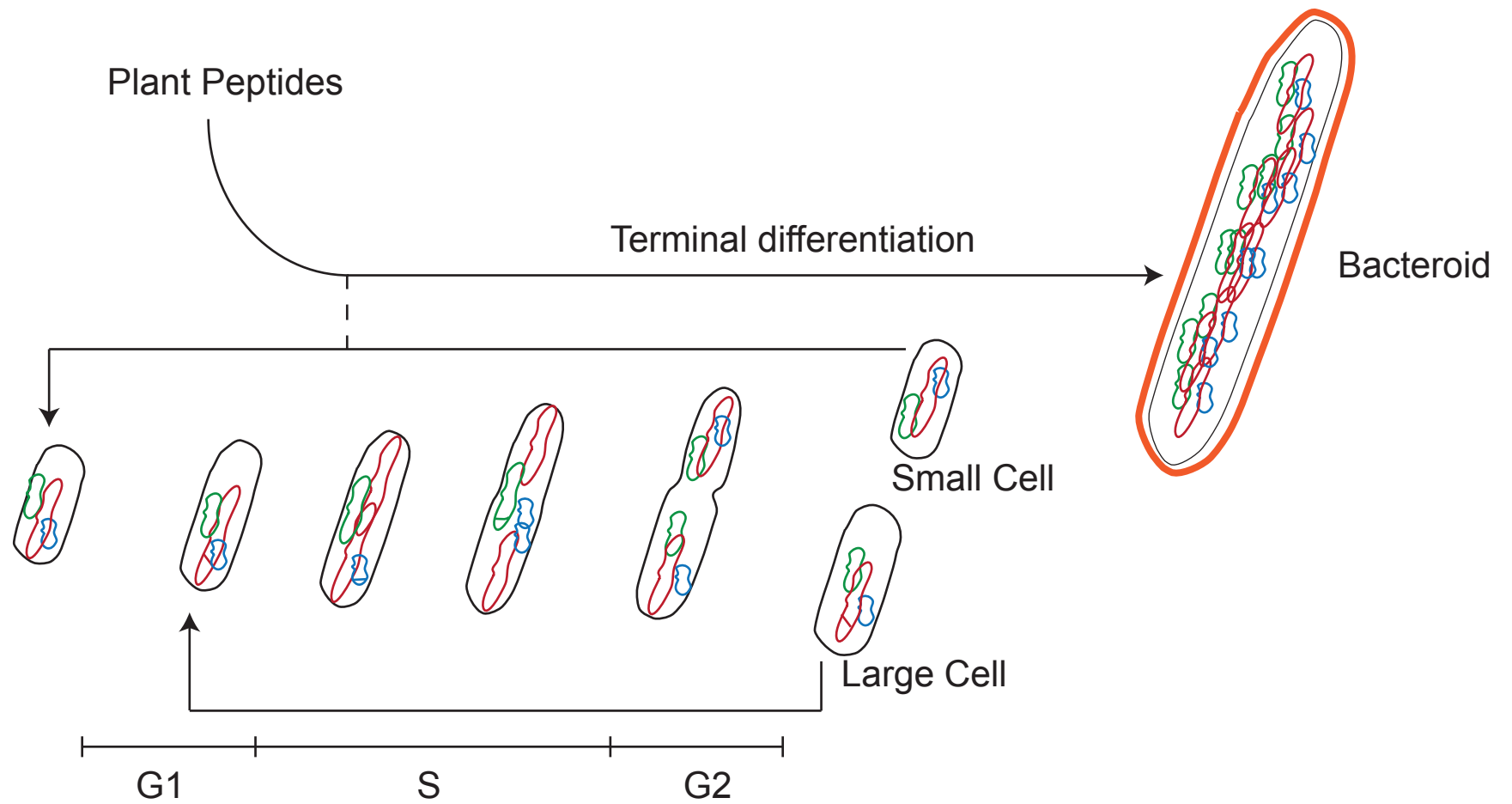


Figure 2

