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Genetics of *Sinorhizobium*: mutants of cell cycle progression and of horizontal gene transfer

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ACKNOWLEDGEMENTS

PREFACE

Since early '90s, the laboratory of Microbial Genetics of the Department of Evolutionary Biology has been focused on the investigation of nitrogen-fixing bacteria of the genus *Sinorhizobium*. *Sinorhizobium* genus comprises several species of soil bacteria that are symbionts of leguminous plants and in particular our attention was pointed to the alpha-proteobacterium *S. meliloti* that represents the model organism to study the *Medicago-Sinorhizobium* interaction. Our lab's research has been principally focused on the study of genetic and phenotypic variability of *Sinorhizobium*, showing that high levels of polymorphism, both genetic and phenotypic, characterize these bacteria, together with a limited occurrence of horizontal gene transfer. More recently another aspect of the biology of *Sinorhizobium* was approached concerning the genetic mechanisms of cell cycle control, particularly in connection with the differentiation of the bacteroids, special bacteria forms that fix nitrogen inside the root nodules. The two research lines mentioned above, though both produced interesting results, suffered from the lack of convenient genetic manipulation techniques, particularly the methods for the construction of site directed mutations. For this reason, this Ph.D thesis is primarily focused on the acquisition of the capabilities required to study the genetics of *S. meliloti*. Several techniques have been used, in fact, for the first time in our laboratory to genetically-manipulate this bacterium, as the construction of mutants and all gene transfer methods (conjugation, transformation and transduction), that are fundamental to manipulate bacteria for many purposes. These techniques were applied, in the present Thesis, to study the two important aspects of *Sinorhizobium* biology mentioned above: cell cycle and horizontal gene transfer, that will be discussed respectively in two separated sections (chapter I, cell cycle, and chapter II, horizontal gene transfer).

During plant-bacteria interaction, plant root hairs develop in particular structures, called root nodules, where *S. meliloti* cells can fix nitrogen, after differentiation in a particular form, referred to as bacteroid. Bacteroids are morphologically diverse from the wild type cells, moreover, their viability is compromised, thus bacteroid differentiation represents an irreversible phase of the bacterium life. All these observations strongly suggest the involvement of the regulation of cell cycle progression to determinate differentiation from free-living cells to bacteroids. To investigate this hypothesis, our attention was focused on

the gene *ctrA* of *S. meliloti*. *ctrA* gene is essential and codes for an histidine kinase that represents the master regulator of cell cycle progression in the alpha-proteobacterium *C. crescentus*. Moreover, previous observations suggested CtrA functional-relation between *S. meliloti* and *C. crescentus*. Thus a *S. meliloti ctrA* conditional-mutant was produced and its morphology was analyzed.

A second important aspect of the genus *Sinorhizobium* is the complexity of the web of gene exchanges that exists in the natural environment between different strains. The two species *S. meliloti* and *S. medicae* constitute an attracting model to study aspects related to gene transfer in leguminous-associated bacteria. *S. meliloti* and *S. medicae*, in fact, are phylogenetically, genetically and ecologically closely related more than with other species, but genetic exchange between the strains of both species is rarely described and is not generalized. This observation suggests the presence of barriers to horizontal gene transfer. To evaluate this hypothesis experiments of gene exchange, between strains of both *S. meliloti* and *S. medicae*, were performed using transformation (electroporation). Moreover, a mutant for one putative restriction gene was produced and analyzed, suggesting that restriction can play an important role in the genetic isolation of strains.

GENERAL INDEX

CHAPTER I

Cell cycle progression

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A. INTRODUCTION

A.1. CELL CYCLE REGULATION IN BACTERIA

Although the cell cycle of eukaryotes has been now elucidated at molecular level, the bacterial cell cycle remains still poorly understood. Genome sequencing projects have demonstrated that the major cell cycle regulators in eukaryotes, such as cyclin-dependent kinases, are not present in bacteria. Early studies in the field of bacterial cell cycle used as model organisms the Gram negative, γ-proteobacterium *E. coli* and Gram positive bacterium *B. subtilis.* However, more recently, important advances in the comprehension of the molecular mechanism regulating bacterial cell cycle progression were achieved studying the bacterium *Caulobacter crescentus*. The α-proteobacterium *C. crescentus* is, in fact, an attractive model for examining cell cycle regulation in bacteria (McAdams and Shapiro 2003; Skerker and Laub, 2004) with peculiar features, such as asymmetric division (mother and daughter cell morphologically distinguishable), possibility to synchronize growing cells, and only one genome replication per cell cycle (see next section for more details).

A.1.1. The bacterial model organism *Caulobacter crescentus*

The dimorphic and intrinsically asymmetric α-proteobacterium *C. crescentus* has became an important model organism for the study the bacterial cell cycle, cell polarity, and polar differentiation. Members of the genus *Caulobacter* are dimorphic, stalked bacteria and inhabit almost all water bodies on Earth, where they play an important role in global carbon cycling by mineralization of dissolved organic material (Poindexter, 1981). One important feature of these bacteria is dimorphism. In *Caulobacter* dimorphism is maintained by obligate asymmetric cell division at each reproductive cycle, giving rise to two genetically identical, but morphologically different daughter cells: a sessile cell equipped with an adhesive stalk and a motile flagellated swarmer cell (Brun and Janakiraman, 2000) (Figure 1). The two daughter cells inherit a different developmental program. Stalked cell, immediately after cell division, reenters in a new cell cycle starting replication. On the other hand, the swarmer cell lives a first period with obligate motile life phase and both DNA replication and cell division are inhibited. After this period the swarmer cell can differentiate in a stalked cell and the process involves ejection of the flagellum, retraction of the pili, and generation of a stalk at the pole previously occupied by the flagellum and pili. During these differentiation

events the new stalked cell becomes actively reproductive, initiating a new cell cycle. The motile G1 phase, typical of the swarmer cell cycle, is presumed to give the opportunity to search for nutrients and to disperse the population to minimize competition for resources.
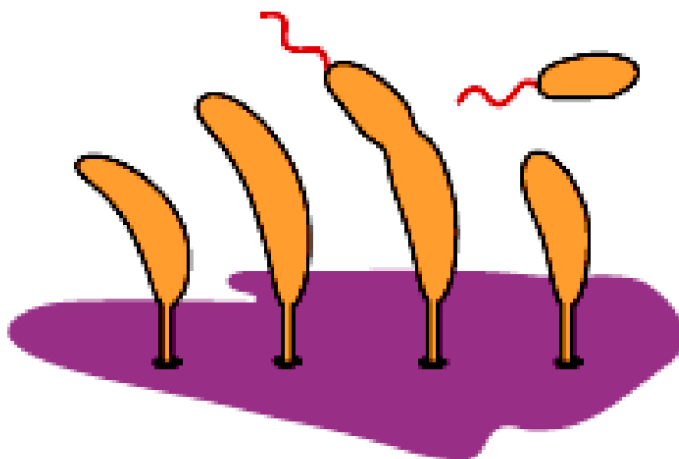
Figure 1. The *Caulobacter crescentus* cell cycle. Cells attached to a surface by the adhesive holdfast at the end of the stalk produce motile swarmer daughter cells at each cell division (image from Laub *et al.*, 2007).

A.2. REGULATION OF CELL CYCLE PROGRESSION

A.2.1. Cell cycle progression involves changes in global profiles of gene and protein expression.

A unique strength of the *Caulobacter* system is the ease to obtain synchronized cell populations with a density gradient centrifugation that separates swarmer cells from stalked cells (Evinger and Agabian, 1977). Moreover, its genome has been completely sequenced and annotated (Nierman *et al.*, 2001). The small size of the *Caulobacter* genome and the ease of obtaining synchronized cell populations have opened the door to genome and proteome wide studies to investigate differentiation and cell cycle processes. A DNA microarray analysis of 90% of all predicted ORFs showed that 19% of the genes significantly change their expression at the mRNA level as a function of the cell cycle (Laub *et al.*, 2000). This global analysis revealed an overall temporal correlation between the time of gene expression and the time when the corresponding gene product is needed. Genes involved in the initiation of chromosome replication, DNA methylation, chromosome segregation, cell division, and membrane and peptidoglycan synthesis were expressed in accordance with the time of their expected function (Laub *et al.*, 2000). Similarly, genes encoding proteins participating in the assembly of polar organelles, such as the flagellum and pili, were

expressed in regulatory cascades, reflecting the order of assembly of their gene products (Laub *et al.*, 2000). Thus, transcriptional control clearly plays a crucial role in the temporal regulation of polar morphogenesis and the cell cycle. Moreover, another interesting observation was that a large part of the general metabolism (e.g. oxidative respiration) and other cellular housekeeping activities (ribosomal genes) might be under cell cycle control (Laub *et al.*, 2000).

Grunenfelder *et al.* (2001) complemented the genome-wide gene expression data examining the protein expression profiles of synchronized cell populations during the course of the cell cycle. In agreement with the microarray data, a large portion of detected proteins (15%), including many metabolic proteins, were differentially synthesized during the cell cycle. An important finding was that, proteins with a cell cycle–regulated expression were more likely to be unstable relative to the length of the cell cycle than proteins constitutively expressed during the cell cycle. This indicates that rapid and targeted degradation of proteins is an important mechanism to generate periodic changes in their abundance during the cell cycle, suggesting a global role of proteolysis in the regulation of the bacterial cell cycle.

A.2.2. A master response regulator controls global regulation of cell cycle

Transcriptional control of gene expression plays a critical role in determining the temporal occurrence of events during the cell cycle. What are the factors involved in transcriptional regulation? Because the polar morphogenetic events in *Caulobacter* invariably happen in coordination with cell cycle progression, it was originally proposed that the cell cycle acts as a biological clock that provides cues for the timing of events involved in morphological differentiation (Huguenel and Newton, 1982). One important early observation was that chromosome replication was required for flagellum formation (Sheffery and Newton, 1981), suggesting that a common regulator controls both the transcription of early flagellar genes and the initiation of DNA replication. Owing to the latter activity, this gene was expected to be essential for viability and a genetic screen was designed to isolate temperature-sensitive (ts) mutants that were defective in the regulation of an early flagellar gene, *fliF*, at 28°C (which is the normal growth temperature used in laboratory) and had a lethality defect at 37°C (Quon *et al.*, 1996). Performing this screen and another one with a similar logic (Jacobs et al., 1999) yielded the identification of two essential signaling proteins, the CtrA response regulator and the CckA histidine kinase (Jacobs et al., 1999; Quon *et al.*, 1996). Recently,

Biondi *et al.* (2006) found a third signaling protein, ChpT, that is also essential for viability and is strongly related to CtrA and CckA (for details see A.2.6. and figure 3). CtrA, CckA and ChpT belong to the superfamily of two-component signal transduction proteins (see Box 1) which play a major role in signal transduction in bacteria (Stock *et al.*, 2000).

Box 1. Two component signal transduction proteins

In the two-component paradigm, after receiving a signal on its sensor domain, the histidine kinase autophosphorylates on a conserved histidine residue of its transmitter domain (Wolanin *et al.*, 2002). Signal transduction is achieved by the transfer of the phosphoryl group to a conserved aspartate residue in the receiver domain of the cognate response regulator. Phosphorylation of the response regulator results in execution of the output response, which often is transcriptional activation or repression of target genes (Stock *et al.*, 2000). A variation of the two-component system is the multicomponent phosphorelay signal transduction system, in which a receiver domain resembling those found in response regulators and a histidine phosphotransferase domain participate in a phosphorelay that culminates in the phosphorylation of the response regulator that mediates the output response. (Image from www.user.gwdg.de/~genmibio/mascher/research1.html).
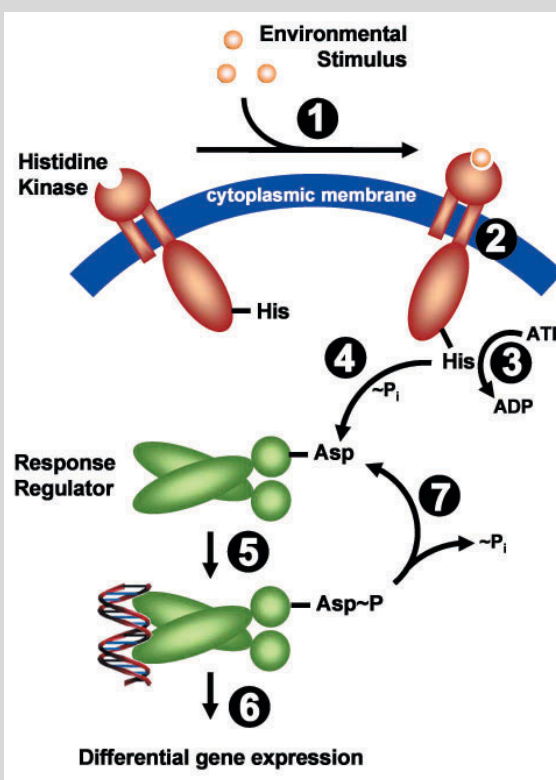
A.2.3. The master regulator of cell cycle progression and polar morphogenesis: CtrA

CtrA is a response regulator with a conventional structure consisting of a conserved N-terminal receiver domain and a C-terminal DNA binding output domain (Quon *et al.*, 1996). Phosphorylation at the conserved aspartate residue (Asp51) of the receiver domain enhances the binding activity of CtrA for its target DNA sequences (Reisenauer *et al.*, 1999; Siam and Marczynski, 2000). Accordingly, phosphorylation of CtrA (CtrA~P) is essential for its activity and therefore for cell viability (Quon *et al.*, 1996). Interestingly, already before the discovery of CtrA, a conserved 9-mer sequence motif had been detected in the promoter regions of many cell cycle–regulated genes, including several of the early flagellar genes

(Stephens and Shapiro, 1993; Zhuang and Shapiro, 1995), the essential DNA methyltransferase encoding gene *ccrM* (Stephens *et al.*, 1995), and the *hemE* Ps promoter that resides within the chromosomal origin of replication (Marczynski *et al.*, 1995; Marczynski and Shapiro, 1992). DNA footprinting and genome-wide location experiments have shown that CtrA~P binds to this conserved 9-mer sequence motif (Boyd and Gober, 2001; Laub *et al.*, 2002; Mohr et al., 1998; Ouimet and Marczynski, 2000; Quon *et al.*, 1998). CtrA controls both polar morphogenesis and essential cell cycle processes. For instance, expression of *ftsZ* encoding the essential cell division protein FtsZ is directly controlled by CtrA~P (Kelly *et al.*, 1998; Laub *et al.*, 2002). Transcriptional control of *ftsZ* is an important mechanism by which to control the abundance of FtsZ in the cell, thereby regulating the initiation of cell division (Kelly *et al.*, 1998; Quardokus *et al.*, 1996; Quardokus *et al.*, 2001). CtrA also controls the expression of *ccrM*, a gene encoding an essential DNA methyltransferase that is involved in cell cycle control (Laub *et al.*, 2002; Reisenauer and Shapiro 2002; Stephens *et al.*, 1996; Zweiger *et al.*, 1994). Similarly, CtrA controls the expression of many genes involved in flagellar synthesis, pili assembly, and chemotaxis (Boyd and Gober, 2001; Jones *et al.*, 2001; Leclerc *et al.*, 1998; Mohr *et al.*, 1998; Ouimet and Marczynski, 2000; Skerker and Shapiro, 2000). Comparative expression microarray experiments performed on a wild-type *Caulobacter* strain and a derivative strain harboring a *ctrA* ts loss-of-function allele indicated that a third of the cell cycle–regulated genes are directly or indirectly under the control of CtrA (Laub *et al.*, 2000).

The direct gene targets of CtrA were later determined in a genome-wide location study (Laub *et al.*, 2002). This study showed that CtrA directly bound as many as 55 promoters, controlling 95 genes (some of which were in fact organized in operons). In addition to its role as a global transcriptional regulator, CtrA~P represses the initiation of chromosome replication by directly binding to five sites within the chromosomal origin of replication (Quon *et al.*, 1998, Siam and Marczynski, 2000). These CtrA binding sites overlap an essential DnaA box and a promoter in the origin of replication, both of which are essential for initiation of chromosome replication (Marczynski *et al.*, 1995).

By controlling polar morphogenesis on the one hand and the initiation of chromosome replication and cell division on the other, CtrA orchestrates the coordinated progression of these distinct events. Thus, the control of CtrA activity, which determines when these cell cycle events occur, is critical for the cell.

A.2.4. CtrA controls gene expression throughout the cell cycle

CtrA does not exert its regulation only at one specific cell cycle stage. Instead, CtrA-dependent events occur throughout the cell cycle. This was illustrated observing that the 55 promoters that are directly controlled by CtrA~P are regulated at different times during the cell cycle (Laub *et al.*, 2002, Laub *et al.*, 2000). CtrA~P has the ability to either activate or repress gene transcription. Genes repressed by CtrA~P are typically expressed during the G1-S cell transition when CtrA activity is cleared from the cells, whereas genes activated by CtrA~P are maximally expressed in the predivisional cells when CtrA is present and phosphorylated at the highest level (Laub *et al.*, 2002). Another mechanism governing temporal regulation of gene expression relies on the binding affinity of CtrA~P for the promoter region and the regulation of CtrA~P levels during the cell cycle. The levels of CtrA~P are significantly higher in predivisional cells than in swarmer cells (Domian *et al.*, 1997; Jacobs *et al.*, 2003) (Figure 2), and this difference may be sufficient to differentially regulate the temporal expression of different genes. In this context, low levels of CtrA~P in swarmer cells bind only to high-affinity CtrA binding sequences, such as those in the chromosomal origin of replication (Laub *et al.*, 2002, Quon *et al.*, 1998; Siam and Marczynski, 2000). Later, the combined action of dephosphorylation and degradation of CtrA~P during the G1-S cell transition results in initiation of DNA replication. In early predivisional cells CtrA is resynthesized and immediately phosphorylated to ensure that no extra round of initiation of DNA replication occurs. As CtrA~P levels rise in predivisional cells, CtrA~P binds and activates gene promoters in the reverse order of their binding affinity for CtrA~P. For example, early flagellar genes and the *ccrM* gene are expressed in the early and late predivisional cell stages, respectively (Reisenauer *et al.*, 1999). Thus, the careful control of CtrA phosphorylation levels during the entire course of the cell cycle play an important role in orchestrating the orderly sequence of cell cycle events.

A.2.5. Mechanisms regulating CtrA activity

CtrA activity is regulated by multiple spatial and temporal mechanisms (Figure 2). As described previously, CtrA activity is controlled temporally at three levels: transcription,

proteolysis, and phosphorylation. The transcription of *ctrA* is cell cycle regulated, with a peak expression in the predivisional cell stage (Domian *et al.*, 1999; Laub *et al.*, 2000; Quon *et al.*, 1996).

Expression of *ctrA* is under the control of two promoters, P1 and P2, that are active at different times during the predivisional stage and are directly regulated by CtrA itself (Domian *et al.*, 1999). The weaker P1 promoter is active in the early predivisional cell and is repressed by CtrA, whereas the stronger P2 promoter is activated later during the predivisional stage and is positively regulated by CtrA. Thus, the absence of active CtrA in the stalked cells due to proteolysis and dephosphorylation (as explained below) presumably triggers the expression of CtrA from the weaker P1 promoter in the early predivisional cells. Accumulation of CtrA (and subsequent phosphorylation of it) causes the repression of the P1 promoter and the activation of the stronger P2 promoter, resulting in elevated levels of the CtrA protein in the late predivisional cell (Domian *et al.*, 1999).

To be active CtrA has to be phosphorylated (Jacobs *et al.*, 2003; Quon *et al.*, 1996). In fact the levels of CtrA~P change dynamically during the course of cell cycle (Domian *et al.*, 1997; Jacobs *et al.*, 2003). CtrA is present and phosphorylated in swarmer cells. The level of CtrA~P drops during the swarmer-to-stalked (G1-S) cell transition. When CtrA is resynthesized by transcriptional activation in early predivisional cells, the CtrA~P level rises to reach the highest level in predivisional cells (Figure 2). Because CtrA~P represses initiation of DNA replication by directly binding to the origin of replication (Quon *et al.*, 1998), the disappearance of CtrA~P during the G1-S cell transition relieves repression and thereby allows initiation of DNA replication to occur. Two redundant mechanisms are responsible for removing CtrA~P from the cells at the G1-S cell transition. One mechanism is the targeted proteolysis of CtrA by the ClpXP protease complex (Domian *et al.*, 1997; Jenal and Fuchs, 1998). An another mechanism is dephosphorylation (Domian *et al.*, 1997). Thus, CtrA phosphorylation is also cell cycle regulated (Figure 2).
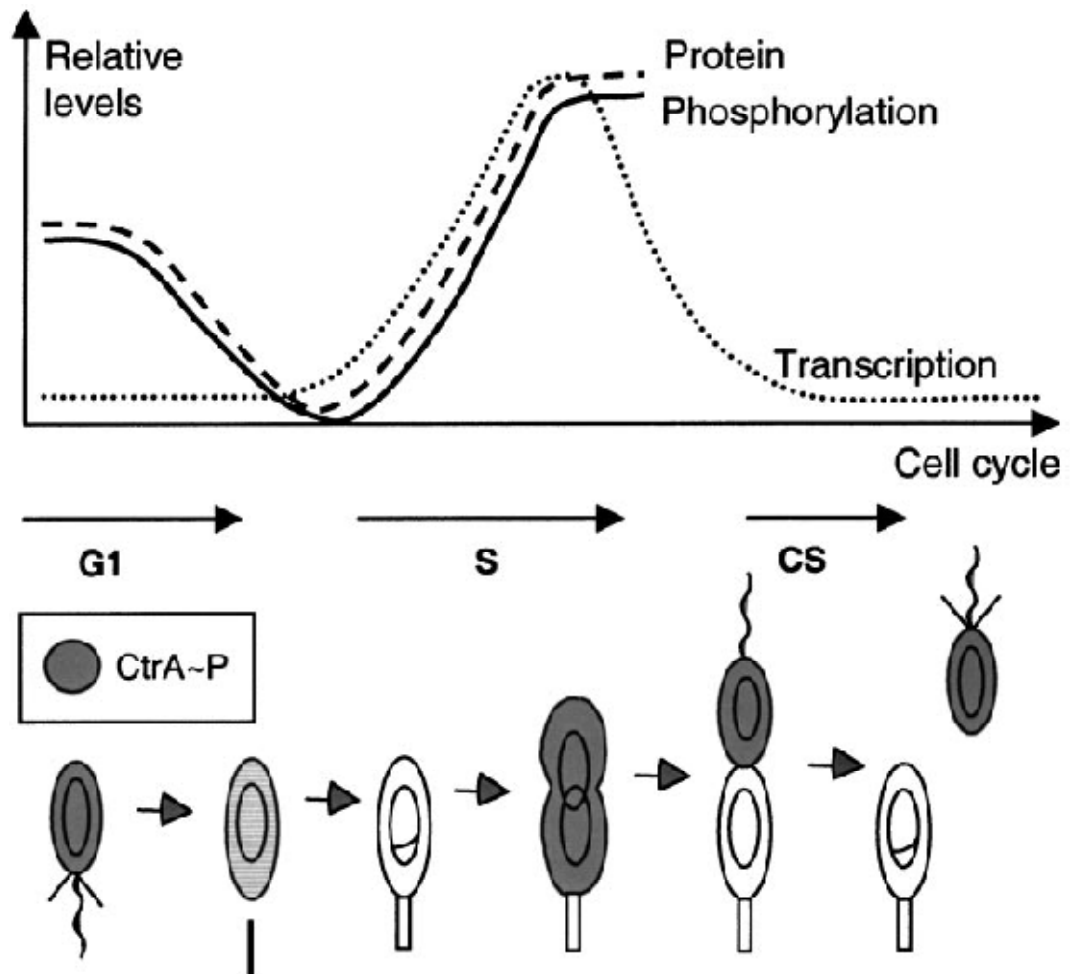
Figure 2. Schematic representation of the regulatory mechanisms that modulate the level of CtrA~P (in gray) during the cell cycle (from Ausmees and Jacobs-Wagner, 2003).

Upon cell division the asymmetry in CtrA~P levels in the late predivisional cell generates a swarmer daughter cell with CtrA activity and a stalked daughter cell without CtrA activity. This segregation in CtrA activity results in a different competence in DNA replication and in a different program of gene expression between the two daughter cells. Thus, CtrA~P acts as a cell fate determinant.

A.2.6. Signal transduction pathways upstream CtrA: an integrated genetic circuit regulates the bacterial cell cycle

Given its importance, the cellular level of CtrA~P must be precisely controlled throughout the cell cycle. Biondi *et al.* (2006b) proposed for the first time an integrated molecular-level model of a regulatory network that accounts for *Caulobacter* cell cycle oscillations and the

ability of a single cell to produce daughter cells committed to different cell cycle phases. A multicomponent signal transduction pathway controls CtrA phosphorylation (Figure 3), involving the CckA→ChpT (CtrA, CpdR) pathway and the PleC-DivJ→DivK patway (Biondi *et al.*, 2006a, Biondi *et al.*, 2006b). This multipathway signaling network leads to the precise and dynamic control of CtrA activity during the cell cycle. In stalked and predivisional cells the histidine-kinase CckA is active and localized to both cell poles. CckA leads to CtrA phosphorylation and its protection from proteolysis. As CtrA~P accumulates, it triggers the expression of several genes that are required for late stages of the cell cycle, including *divK* and the essential cell division genes *ftsQ* and *ftsA* (Laub *et al.*, 2002; Hung and Shapiro 2002; Wortinger *et al.*, 2000). The primary DivK kinase (DivJ) and phosphatase (PleC) are located at opposite ends of the predivisional cell such that daughter cells inherit one or the other (Wheeler and Shapiro, 1999). The stalked cell inherits DivJ, but not PleC, and can therefore accumulate phosphorylated DivK. This DivK~P triggers the delocalization and downregulation of CckA, thereby preventing the phosphorylation of CtrA and CpdR. Consequently, CtrA is dephosphorylated and degraded, permitting another round of DNA replication. This eventually triggers new synthesis of CtrA and, hence, resets the cell cycle (Holtzendorff *et al.*, 2004). By contrast, the swarmer cell inherits PleC and dephosphorylates its pool of DivK. The lower levels of DivK~P allow CckA to remain localized and active, which stabilizes CtrA~P levels and blocks DNA replication initiation. As the swarmer cell develops, DivJ replaces PleC at the newly formed stalked pole (Wheeler and Shapiro, 1999; Sciochetti *et al.*, 2002), DivK~P accumulates and CckA is delocalized and inactivated. As with the stalked cell, these steps allow the initiation of DNA replication, the expression of *gcrA* and *ccrM* and resetting of the cell cycle. *cckA*, *chpT*, *ctrA* and *divK* are each essential for cell cycle progression, *divJ* and *divJ-pleC* mutants are viable, albeit with severe phenotypes (Biondi *et al.*, 2006b), indicating that other factors might regulate DivK. This integrated network forms the basis of an oscillatory circuit that underlies cell cycle progression in *Caulobacter* (Figure 3). Thus, the core oscillating machinery involves the master regulator CtrA, whose activity accumulates during the cell cycle. CtrA triggers its own destruction by inducing *divK* transcription and cell division, which ultimately enable DivK~P to feedback and inhibit CckA and CtrA (Biondi *et al.*, 2006b).
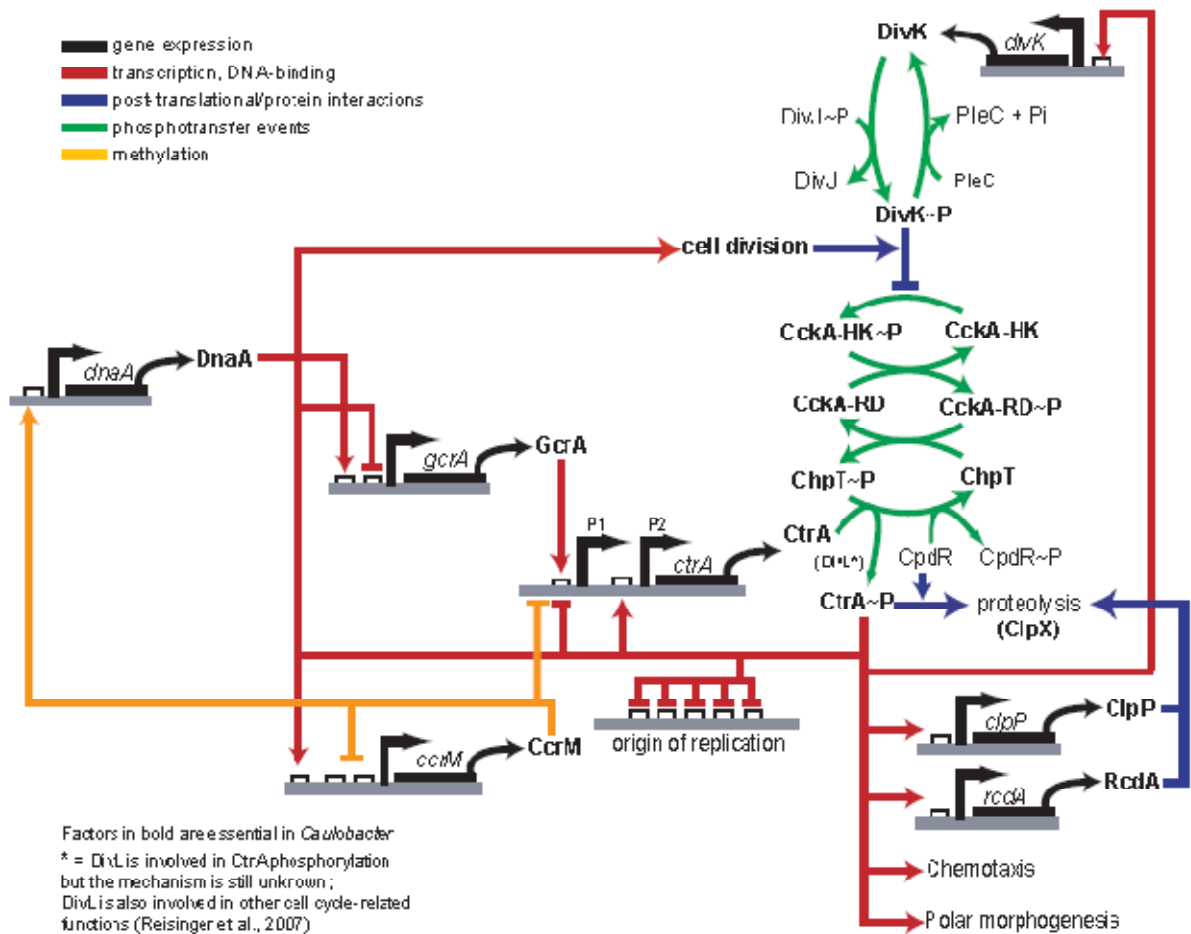
Figure 3. The cyclical genetic circuit driving cell cycle progression in *C. crescentus*. DnaA activates *gcrA*, GcrA activates *ctrA*, CtrA activates *ccrM*, and then CcrM closes the loop by activating *dnaA* by an epigenetic mechanism. GcrA is involved in activation of CtrA, and there may be another unidentified factor involved (Holtzendorff *et al.*, 2004). The CcrM methyltransferase enables *dnaA* expression by an epigenetic mechanism involving the methylation of the *dnaA* promoter region to restart the process. A second, interlocking cyclical circuit appears to control the phosphorylation of CckA, which in turn controls phosphorylation and stability of CtrA through the CckA→ChpT→(CtrA, CpdR) phospho-cascade (Biondi *et al.*, 2006a; Biondi *et al.*, 2006b). A model for control of the phosphorylation state of CckA involving DivK, as shown, along with differentially localized DivJ and PleC controlling DivK activity (not shown) has been proposed (Biondi *et al.*, 2006b) (see text). The positive autoregulation of CtrA through the P2 promoter serves to hold the concentration of CtrA~P high until the feedback loop is broken by interruption of the CckA→ChpT→(CtrA, CpdR) phospho-cascade with consequent dephosphorylation and degradation of CtrA.

A.2.7. The general architecture of C*aulobacter* cell cycle control

As recently reviewed in Laub e*t al.*, 2007, the core engine that controls cell cycle progression in *Caulobacter* is a genetic circuit involving four master regulatory proteins (CtrA, GcrA, DnaA, and CcrM) that together control expression of at least 200 genes (Collier *et al.*, 2006; Holtzendorff *et al.*, 2004; Hottes *et al.*, 2005; Laub *et al.*, 2002) (Figure 3). The four proteins are synthesized in succession to drive the series of modular functions that execute the cell cycle program (e.g., build the flagellum, replicate, separate, and methylate the chromosome, divide the cell, etc.). The overall design of the cell cycle control system includes epigenetic regulatory mechanisms, sensors, and signal transduction systems that provide feedback signals to synchronize the advance of the core engine with progression of chromosome replication and cytokinesis. The stabilities of CtrA, GcrA, and DnaA are actively controlled over the cell cycle. The molecular mechanisms of the pathway controlling CtrA proteolysis have been characterized (Biondi *et al.*, 2006b; Iniesta *et al.*, 2006; McGrath *et al.*, 2006), but those controlling GcrA and DnaA are largely unknown. CtrA, is the essential master DNA-binding response regulator member of the two-component signal transduction family. DnaA and GcrA, and the DNA methyltransferase, CcrM, are involved in controlling *ctrA* transcription (Collier et al. 2006; Collier et al. 2007). DnaA is a key element in the cell-cycle regulation because it is required for the initiation of DNA replication; it also controls the transcription of 40 genes involved in nucleotide biogenesis, cell division, and polar morphogenesis (Gorbatyuk and Marczynski 2005; Hottes et al. 2005), and it activates the transcription of *gcrA* gene (Holtzendorff et al. 2004). GcrA controls the transcription of *ctrA* and genes involved in DNA metabolism and chromosome segregation, including those encoding for DNA gyrase, DNA helicase, DNA primase, and DNA polymerase III (Holtzendorff et al. 2004). As a consequence of this genetic circuit, CtrA accumulates out-of-phase with GcrA (Holtzendorff et al. 2004). The transcriptional loop of *ctrA* is closed by CcrM. CtrA activated transcription of *ccrM*, which encodes for a DNA methyltransferase whose activity is cell cycle dependent. CcrM methylates and consequently activates *dnaA* promoter region, closing the positive feedback composed by CtrA, DnaA and GcrA. Thus, the protein concentrations of the master regulators, CtrA, GcrA and DnaA drive creation and operation of many modular subsystems that implement cell cycle progression.

A.3. CELL CYCLE REGULATION IN THE ALPHA-PROTEOBACTERIA GROUP

Living cells continuously receive and process signals coming from their environment, and, integrating this information with their own internal state, are able to react with appropriate responses. Ultimately cell cycle, comprising DNA replication, cell division and cell growth, together with coordination of biogenesis of cellular structures, must be controlled by environmental conditions. Regulation of cell cycle progression needs to be a robust but versatile process that integrates different exogenous and endogenous signals and that guarantees fidelity and controlled progression throughout the different phases. Different bacteria have evolved different regulation systems for cell cycle coordination, due probably to different ecological constrains and evolution (Fawcett *et al.*, 2000; Haeusser and Levin 2008). Alpha proteobacteria group is a very heterogeneous group of bacteria and includes symbionts of plants (Rhizobia), pathogens for animals (*Brucella*, *Rickettsia*), pathogens for plants (*Agrobacterium*), photosynthetic bacteria (*Rhodobacter*) and also several genera metabolizing C1-compounds (*Methylobacterium*). Moreover the precursors of the mitochondria of eukaryotic cells are thought to have originated in this bacterial group.

As explained above (A.2.6. and A.2.7.) and schematized in figure 3, regulation of cell cycle as in *Caulobacter* is composed by circuits that work at different regulatory levels: transcription, protein-protein interaction, phosphorylation and epigenetic mechanisms (DNA methylation). Basically two main oscillators are working during cell cycle progression: i) the transcriptional and epigenetic circuit (CtrA-DnaA-GcrA-CcrM); ii) the phosphorylation/proteolysis and transcription circuit (CckA-CtrA-DivK). The last one involves also coordination of CtrA proteolysis and cell division by the regulation of the DivK activity. So far, it has been demonstrated that only phosphorylation is not dispensable during cell cycle progression; in fact, cell cycle regulated transcription of *ctrA* can be substituted by constitutive transcription (Biondi *et al.*, 2006b) and also proteolysis can be removed, being probably compensated by a not yet identified dephosphorylation mechanism. *Caulobacter* regulation of cell cycle progression has evolved in order to respond to a life style in nutrient-poor environments but other alpha proteobacteria occupy different ecological niches suggesting that regulation of cell cycle must respond to different requirements although several features can be conserved.

Several preliminary studies have been carried out on regulation of cell cycle progression in other alphas such as *Brucella*, *Sinorhizobium*, *Silicibacter*, *Agrobacterium*, *Rickettsia* and *Rhodobacter*. It has been recently demonstrated that asymmetric division takes place in *Agrobacterium tumefaciens*, *Sinorhizobium meliloti* and *Brucella abortus* (Hallez *et al.*, 2004), indicating that at least some of the features governing cell cycle progression in *Caulobacter* might also be present in other species. Indeed, sporadic studies have been carried out in those organisms revealing a fairly consistent conservation of the properties of several factors involved in cell cycle regulation in *Caulobacter* but also remarkable differences. For example, in *R. capsulatus*, CtrA and CckA are not essential and are required for the expression of the GTA, a system for genetic exchanges (Bellefontaine *et al.*, 2002). CtrA in *Brucella* controls cellular events similar to those controlled by CtrA in *Caulobacter*, but through a direct effect on different targets (Bellefontaine *et al.*, 2002). Moreover CtrA from *Caulobacter* is able to bind the *B. abortus ccrM* promoter in vitro (Robertson *et al.*, 2000). CtrA of *Brucella abortus* binds to *ccrM, pleC, rpoD, ftsE* and *minC* but not to *divK, ftsZ* or the origin of replication (known targets in *Caulobacter*) (Bellefontaine *et al.*, 2002).

In *Silicibacter pomeroy* three known mutants affect the motility: *cckA, ctrA* and one concerns a gene with negligible homology to protein sequences from non-roseobacters (FlaA, ORF1857) (Miller and Belas 2006). In *A. tumefaciens ccrM* is essential and cell-cycle regulated (Kahng and Shapiro 2001). Also in *Brucella ccrM* is essential and its overexpression impairs proper intracellular replication in murine macrophages (Robertson *et al.*, 2000). A yeast two hybrid in *Brucella* with DivK as a bait returned DivL, DivJ, PleC and PdhS (Hallez *et al.*, 2007). In *S. meliloti ctrA* is essential (Barnett *et al.*, 2001) and the closest PdhS homolog, called CbrA is not essential and it appears to be involved in succinoglycan production (Gibson *et al.*, 2006). Although several features appeared conserved in alphas other features revealed by those studies showed unique features suggesting variability as well and the missing of a systematic comparison of factors that are involved in cell cycle regulation.

A.4. THE ALPHA-PROTEOBACTERIUM *SINORHIZOBIUM MELILOTI*

The organism object of this work is the Gram-, α-proteobacterium *S. meliloti*. *S. meliloti* is an important organism in research, representing the model system for the study of both plant-bacteria interaction and nitrogen fixation. *Sinorhizobium meliloti* is one of the most studied members of alpha-proteobacteria belonging to the family of *Rhizobiaceae* (Young *et al.*,

2001), which lives either free in soil or in symbiosis with leguminous (*Fabacae*) plants. During symbiosis *S. meliloti* invades root hairs of the plant and forms root nodules. Inside them the bacteria can perform nitrogen fixation.

For an overview of the nitrogen fixation process and the general features of *S. meliloti*, excluding information related to root nodules and cell cycle, see the Introduction of the Chapter II, section A.3.

A.4.1. Determinate and indeterminate nodules

The symbiosis between Rhizobia and their host plants results in the formation in the roots of specialized symbiotic organs called nodules. Nodules are formed by the plant tissue and occupied by the nitrogen-fixing bacteria. Mature nodules can be of two types, determinate or indeterminate.

Determinate nodules

Determinate nodules are formed on tropical and subtropical legumes (*Glycine max*, *Phaseolus vulgaris*, *Lotus japonicus*). These kind of nodules are characterized by disappearance of meristematic activity after nodulation. Thus, determinate nodules stop to grow after formation and have a globose shape (Brewin, 1991). Differentiation of infected cells occurs synchronously and the mature nodule contains symbiotic bacterial cells with a homogenous population of nitrogen-fixing bacteroids (Franssen *et al.*, 1992).

Indeterminate nodules

Indeterminate nodules are usually formed on temperate legumes (e.g., *Medicago sativa*, *Pisum sativum*, *Vicia hirsuta*) and are characterized by persistent meristematic activity, that causes elongated shape of nodules. The central tissue of such nodules consists of a number of distinct zones containing invaded plant cells at different stages of differentiation, in which bacteria also show a progressive differentiation (Figure 4) (Patriarca *et al.*, 2002; Pawlowski and Bisseling, 1996).

The bacteria object of this work, *Sinorhizobium meliloti*, forms indeterminate nodules, thus the following description will be focused on this kind of nodules.
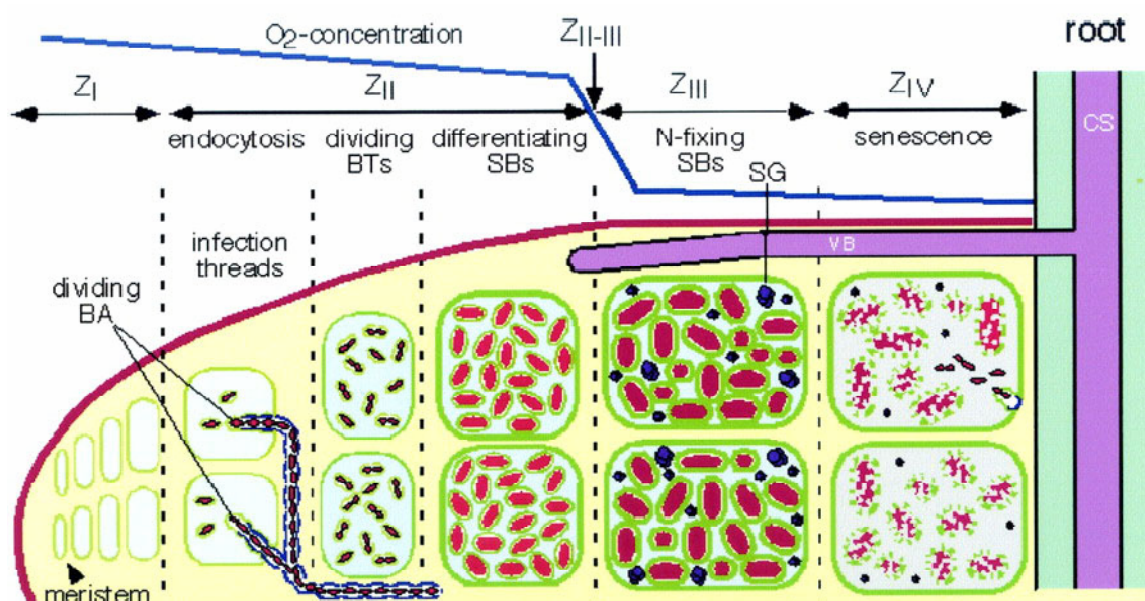
Figure 4. Scheme of the indeterminate elongated nodule. BA, bacteria; BTs, bacteroids; SBs, symbiosomes; SG, starch grain; CS, central stele; VB, vascular bundle. The nodule zones (Z) are indicated (Patriarca *et al.*, 2002).

A.4.2. Formation of indeterminate nodules

Formation of nodule is a complicated and selective process, characterized by the exchange of molecular signals between the symbionts. At the first step of the interaction, bacteria sense the specific compounds of plant root exudates. Amino acids, dicarboxylic acids and flavonoids present in the root exudates induce positive chemotaxis of rhizobia (van Rhijn and Vanderleyden, 1995). Furthermore, bacteria respond to the flavonoids by the production of the lipochitooligosaccharide signalling molecules known as Nod factors.

At the second step of nodule formation, the rhizobia attach to the plant root surface. At the same time, the root hair deforms by reinitiating tip growth with a changed growth direction in response to the Nod factors. Root hairs curl in the way that the bacterial cells become entrapped in a pocket of host cell wall. After the entrapment, a local lesion of the root hair cell wall is formed by hydrolysis of the cell wall, so that infection can be initiated. The plasma membrane invaginates, and a tube-like structure, called an infection thread, is formed. The infection thread grows down, inside of the root hair and into the body of the epidermal cell. Rhizobia inside the infection thread grow and divide, thereby keeping the tubule filled with bacterial cells (Gage, 2004). Even before the infection thread has crossed the epidermis, cells

of cortex and pericycle respond in a local manner to the presence of rhizobia. Cells in the inner cortex dedifferentiate by entering the cell cycle; later these cells will form the nodule tissue. The group of dividing cortical cells is called the nodule primordium (Geurts and Bisseling, 2002; Timmers *et al.*, 1999). In the following step, the infection thread branches in the nodule primordium, thereby increasing the number of sites from which bacteria can exit the thread and enter nodule cells. At this point, cells at the base of primordium establish a radial pattern consisting of a central tissue surrounded by peripheral tissues, and the cells at the apex of primordium form the meristem tissue, thus creating a young nodule (Pawlowski and Bisseling, 1996). Eventually, rhizobia are released from infection threads in the cytoplasm of postmitotic nondividing plant cells by endocytosis. At this step bacteria are called bacteroid. The term ''bacteroid'' refers to these intracellular membrane encapsulated bacteria. Bacteroid form the, so called "symbiosomes": the compartments containing nitrogen-fixing rhizobia surrounded by the plant-derived peribacteroid membrane (Becker *et al.*, 2005).

A.4.3. Structure of mature indeterminate nodules

Once inside nodule cells, the bacteria continue to differentiate and synthesize proteins required for nitrogen fixation. Ultrastructural studies of wild type nodules distinguish 5 steps in bacteroid differentiation (types 1 to 5), each of them being restricted to a defined histological region of the nodule (Zones I to IV) (Figure 4) (Luyten and Vanderleyden, 2000; Vasse *et al.*, 1990).

Zone I contains meristematic tissue, situated at the apex of the nodule. This is a region of actively dividing plant cells devoid of bacteria.

Zone II is called the infection zone. Here the bacteria enter the root cells via infection threads. Bacteria, released from the infection threads, are called type 1 bacteroids. These bacteroids divide and resemble free-living bacteria by size and cytoplasm content. They have a large periplasmic space, and the peribacteroid membrane (membrane of the plant origin that surrounds invading bacteria) appears irregular in shape due to local fusions with plant cytoplasmic vesicles. In the proximal part of Zone II, type 2 bacteroids are the most abundant. These bacteroids are elongated; their periplasmic and peribacteroid spaces are reduced, and the peribacteroid membrane is more regular in shape. The cell division stops once the type 2 bacteroid stage is reached.

Interzone II-III is a very restricted zone that contains only 3-4 layers of cells, separating the prefixation zone II and nitrogen-fixing Zone III. The Interzone II-III contains bacteroids of type 3 which have stopped elongating and are about seven times longer than the free-living bacteria. The membranes surrounding each bacteroid, including the peribacteroid membrane, are smooth, often in contact with each other, with small periplasmic and peribacteroid spaces.

Zone III is filled with the fully differentiated, nitrogen-fixing bacteroids of type 4. In this zone, the leghaemoglobin is produced giving the typical pink or red color of the nitrogen-fixing nodules. Leghaemoglobin is essential because of it binds oxygen molecules, protecting oxygen-sensitive nitrogenase, the crucial bacterial enzyme catalyzing nitrogen fixation. Thus, in Zone III, the bacteroids fix nitrogen and show a dispersion of the ribosome-enriched areas, thus becoming the bacteroids of type 5.

Zone IV is the senescence zone, located proximal to the point of attachment to the plant root. Here, both symbiotics partners degrade and the number of bacteroids gradually decreases. Ghost membranes of plant and bacteroid origin are the ultimate result of the senescing process.

In both nodule types, indeterminate and determinate, growth and differentiation of infected plant cells involve extreme cell enlargement. This cell enlargement is predominantly responsible for the growth of the nodule organ and is mediated by repeated endoreduplication cycles resulting in 64C or 128C polyploid nodule cells (C being the haploid DNA content) (Kondorosi *et al.*, 2000; Gonzalez-Sama *et al.*, 2006). In symbiotic nodule cells, high ploidy levels allow extreme cell growth, hosting thousands of bacteroids and sustaining the energy-demanding nitrogen fixation.

Cytological studies showed that, similarly to the hosting plant cells, the bacterial symbionts in the nodules of the galegoid legumes *Medicago sativa* (alfalfa) or *Vicia sativa* (vetch) undergo a profound differentiation process including important cell enlargement (Vasse *et al.*, 1990).

A.4.4. Free living bacteria versus bacteroids

A recent a study Mergaert *et al.* (2006) has shown that differentiation of bacteroids in galegoid legumes involves indeed genome amplification that is generated by endoreduplication cycles and correlates with elongation of bacteria.

The differentiation includes an important elongation of the cells, free-living cells were 1–2 μm long, whereas the bacteroids were 5–10 μm (Mergaert *et al.*, 2006). Moreover, the bacteroids exhibited higher fluorescence corresponding to higher DNA content and were polynucleoid. The multiple nucleoids appear randomly organized, with large cell-to-cell variations and differences in nucleoid sizes (Mergaert *et al.*, 2006). Moreover the DNA content and size of cultured rhizobia and bacteroids is 1C-2C DNA (C being the haploid DNA content) content of free-living *S. meliloti*, while the DNA content of bacteroids is 24C, when measured by flow cytometry (Mergaert *et al.*, 2006). Thus positive correlation exists between the DNA content and the size of the bacteroids. Comparison of the genomes of *S. meliloti* bacteroids and cultured *S. meliloti* cells by comparative genomic hybridization (CGH) shows that the hybridization ratio of DNA from bacteroids and cultured bacteria of strain Sm1021 is close to 1 for all genes as well as for the control comparing two samples of cultured Sm1021 bacteria (Mergaert *et al.*, 2006). This indicates neither amplification nor deletion of specific regions in the bacteroid genome. Thus the 24C DNA content in *S. meliloti* bacteroids arises from endoreduplication of the whole genome suggesting a deregulation of the DNA replication normal program.

Figure 5. Size, shape, and DNA content of free-living, cultured *S. meliloti* bacteria and *S. meliloti* bacteroids isolated from nitrogen-fixing *M. truncatula* nodules. Nomarski (*Upper*) and fluorescence (*Lower*) microscopy of DAPI stained bacteria and
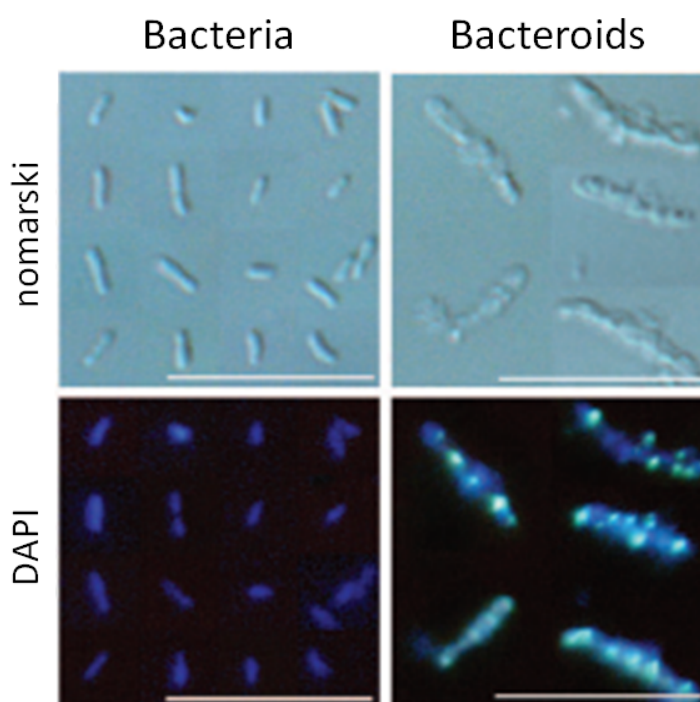
bacteroids (image from Mergaert *et al.*, 2006).

The viability of bacteroids (ability to resume growth and to produce descendants) is a long controversy in the literature (Oke and Long, 1999). But from bacteroid preparations only 0.8% of the cells, likely arisen from undifferentiated rhizobia, form colonies on agar plates, demonstrating that differentiated *S. meliloti* bacteroids are non-dividing (Mergaert *et al.*, 2006).

The reason of the loss of bacteroid viability in the galegoid legumes could be related to the endoreduplication and multiple nucleoids in bacteroid cells, which may preclude the ability to perform again cell division correctly (Mergaert *et al.*, 2006). This is also impossible in endoreduplicated, highly polyploid eukaryotic cells. Moreover, the membranes of the bacteroids of galegoid legumes became permeable for diffusion (Mergaert *et al.*, 2006), which could also compromise the capacity of bacteroids to reproduce.

The meaning of differentiation process from free-living form to bacteroid is under discussion. It could be either, the differentiated bacteroids have a better symbiotic performance, for example higher nitrogen fixation or better exchange of nutrients and fixed nitrogen, or the terminal bacteroid differentiation is a means by the plant to control proliferation of the bacterial endosymbiont.

Those observations reported above strongly suggest that the differentiation from free living rhizobia to bacteroids requires a different regulation of the normal cell cycle progression. Up to date, both the cell cycle regulation in rhizobia or a connection between the developmental process of nodulation and the cell cycle regulation have not been explored even if it represents one of the most interesting directions in the plant-rhizobia symbiosis research.

B. AIM OF THE WORK

As illustrated in the introduction, regulation of bacterial cell cycle is still poorly understood. A single complete model has been proposed, explaining the regulation of cell cycle progression only in the model organism *C. crescentus*, a member of the alpha proteobacteria group. Preliminary studies suggested that the regulation found in *Caulobacter* might be conserved also in alpha proteobacteria. One of the most studied members of alpha-proteobacteria group is *S. meliloti*, because of it is the symbiont of agriculturally important leguminous plants such as *Medicago* sp. To establish symbiosis *S. meliloti* forms root nodules where it can perform the fundamental process of nitrogen fixation. Inside the nodule, bacterial cells differentiate in a peculiar form called bacteroid, that is the one able to fix nitrogen. Bacteroids are both morphologically and metabolically different from free living bacteria. A recent study from Mergaert *et al.* (2006) has shown that mature bacteroids deeply increase both the cell size and the intracellular DNA content, accumulating multiple genome copies. Moreover, bacteroids lose the ability to divide, representing an irreversible form of differentiation. Those observations suggest that the differentiation process from free-living cell to bacteroid involves the mechanisms regulating the cell cycle progression. Unfortunately, up to date, both the cell cycle regulation and a connection between the differentiation process and the cell cycle regulation have not been explored in rhizobia, even if this field represents one interesting direction to comprise plant-rhizobia symbiosis.

In *Caulobacter*, cell cycle regulation is mostly controlled by two component system proteins (Skerker *et al.*, 2005; Biondi *et al.*, 2006b) and at the core of cell cycle regulation there is an essential response regulator named CtrA (Quon *et al.*, 1996). CtrA phosphorylated (CtrA~P) is able to bind and silence the origin of replication and in predivisional cells drives the expression of more than 50 genes, many of which are required for completing the cell cycle (Skerker and Laub, 2004).

The indication that bacteroids are bigger in size, lose ability to divide and accumulate multiple chromosomes (Mergaert *et al.* 2006) suggests that CtrA activity may be compromised in bacteroids. In *C. crescentus*, in fact, a typical CtrA-loss of function strain shows many features that resemble those found in bacteroids. A preliminary study from Barnett *et al.* (2001) individuated the CtrA orthologue in *S. meliloti* and demonstrated, such as for other alpha-proteobacteria, that it is essential.

Considering all those observation, the aim of this work is to explore, for the first time, the cell cycle progression mechanism in the nitrogen fixing bacterium *S. meliloti*. Thus, the experimental work will be addressed to the production and investigation of a conditional CtrA-loss of function strain of *S. meliloti*. To observe the phenotype of this strain in restrictive conditions it will be necessary to construct a *S. meliloti* mutant stain where the expression of *ctrA* gene can be opportunely induced or stopped by environmental controllable input. This particular mutant will allow verifying the hypothesis of a connection between cell cycle progression machinery and development of bacteroid state. Confirmation of this hypothesis will require to explore the mechanisms that regulate CtrA function and cell cycle progression in *S. meliloti*, to find the possible link between plant and bacteroid differentiation. Moreover, using bioinformatic tools, the analysis of genes in *S. meliloti*, that are orthologous of those genes involved in the *C. crescentus* cell cycle regulation, will be presented.

C. RESULTS

Results presented in this section were aimed at the investigation of a conditional CtrA-loss of function strain of *S. meliloti*. Preliminary work was focused on the construction of a system to obtain chromosomal *ctrA* deletion in *S. meliloti*, confirming it essentiality. Thus, two different strategies were explored to complement *ctrA* deletion in *S. meliloti* with a conditional allele. Finally, a depletion *ctrA* mutant of *S. meliloti* was described together with preliminary observations of CtrA-loss of function phenotype.

C.1. STRATEGY TO OBTAIN A *ctrA* DEPLETION MUTANT IN *S. MELILOTI*

In 2001 Barnett *et al.* demonstrated that an homologue of *ctrA* is present in *S. meliloti* and, as in *C. crescentus, ctrA* is essential for viability. The primary sequence of *S. meliloti* CtrA is very similar to *C. crescentus* CtrA (Barnett *et al.*, 2001). In fact, *C. crescentus ctrA* gene complements the deletion of chromosomal *ctrA* gene in *S. meliloti*. In 1996, Quon *et al.* isolated a *C. crescentus* temperature-sensitive (ts) mutant that grew at 28°C and had died at 37°C. This strain allowed many fundamental advances to explain *C. crescentus* cell cycle regulation (Laub *et al.*, 2000).

Our first strategy to obtain a constrictive-*ctrA* mutant of *S. meliloti* was based on the construction of a *S. meliloti* strain carrying deletion of the chromosomal copy of *ctrA*, where viability was restored by complementation with the *ctrA*-ts allele of *C. crescentus* (for details C.3.1.).

The second strategy, instead, was based on the fusion of *ctrA* coding region with an inducible promoter, to obtain conditional expression of CtrA (for details C.3.2.).

C.2. CONSTRUCTION OF A PLASMID TO DELETE *ctrA* GENE IN *S. MELILOTI*

Strategies for deleting a genomic region in bacteria are principally based on the use of suicidal vectors and selection of double crossing-over events. Suicidal vectors are narrow host range plasmids carrying, at least, a marker for antibiotic resistance, but are not replicative in the host organism. Thus, they need recombination to be integrated in the genome of the host. The sequence of the plasmid is not complementary to the sequence of the host, so recombination (crossing-over) can be driven in a specific site engineering the plasmid by adding a copy of the targeted gene disrupted with the insertion of an antibiotic-

resistance cassette. After the first integration the wild type copy of the gene and the disrupted one are separated by the plasmid. A second event of crossing-over, opportunely selected, will excise the wild-type copy of the targeted gene, leaving only the disrupted copy. This event can be selected because the vector carries a particular gene that is lethal in certain media conditions (in our case *sacB* gene from *B. subtilis*). The product of *sacB* gene provokes death of the cell when grown in presence of sucrose, consequently only cells that lose the vector (and *sacB*) in the second crossing-over event, can survive in medium supplemented with sucrose. The resulting strain will carry an irreversible deletion of the gene. Figure 6 shows the strategy utilized using the suicidal vector pNPTS138 (Spratt *et al.*, 1986).
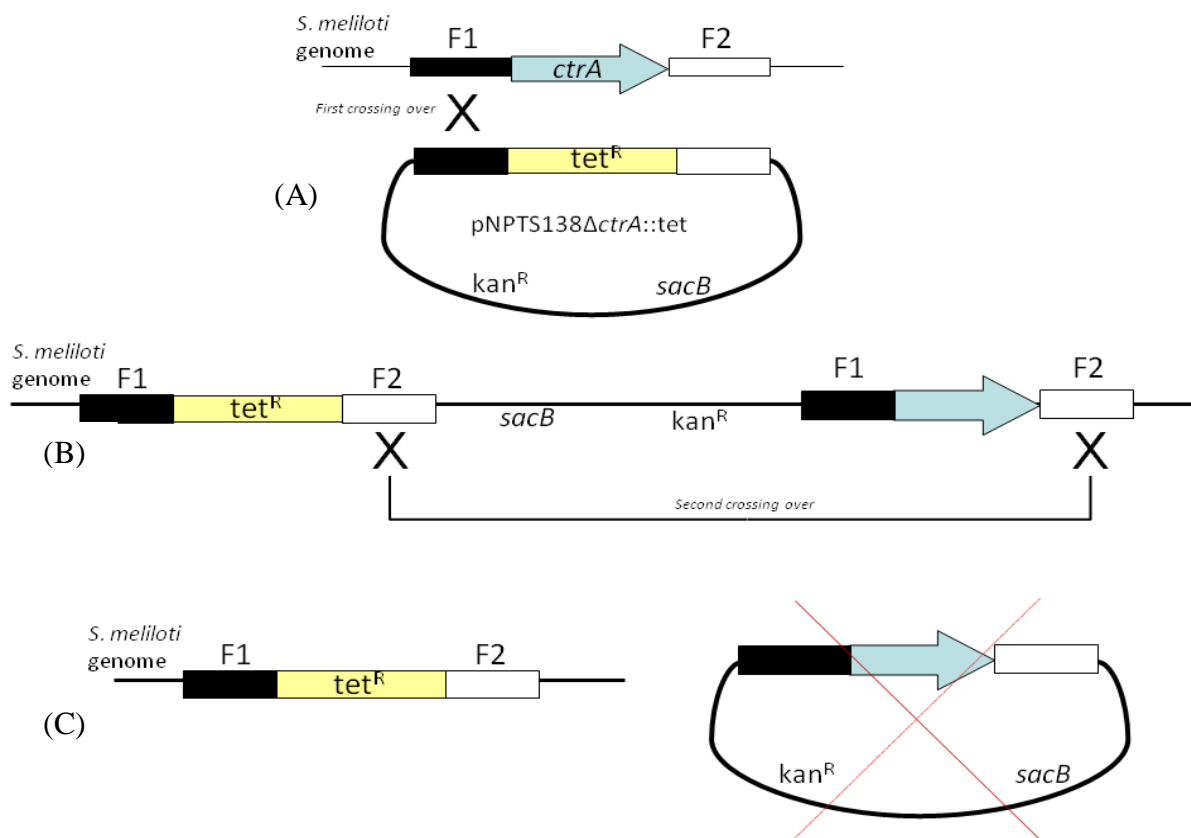
Figure 6. Scheme of targeted-gene deletion. (A) The suicidal plasmid, derivative of the vector pNPTS138, contains the tetracycline gene flanked by the regions upstream (F1) and downstream (F2) of the targeted gene (*ctrA*). Plasmid is moved in *S. meliloti* by conjugation and a first single crossing over event carries integration of the plasmid. (B) strain with plasmid integrated can be selected for this phenotype: resistance to kanamycin (kan^R), resistance to tetracycline (tet^R) and sensibility to sucrose (*sacB*). (C) ON growth with only tetracycline will produce cells where a second crossing-over event recircularize the plasmid, but deleting the gene. The plasmid will be lost because it is suicidal and these colonies will have this phenotype: resistance to tetracycline, resistance to sucrose and sensitivity to kanamycin.

The construction of the deletion cassette was carried out as follows (figure 7): upstream and downstream 1 Kb regions flanking the *ctrA* gene were amplified from *S. meliloti* type-strain Rm1021, using primers *HindIII*-P1 + *EcoRI*-P2 and *EcoRI*-P3 + *SphI*-P4. Tetracycline cassette was obtained by excision with *EcoRI* from the plasmid pKOC3. Tet-cassette, PCR fragments flanking *ctrA* and the vector pNPTS138 opportunely treated with restriction enzymes (see figure 7) where ligated and transformed in *E. coli* JM109 competent cells.
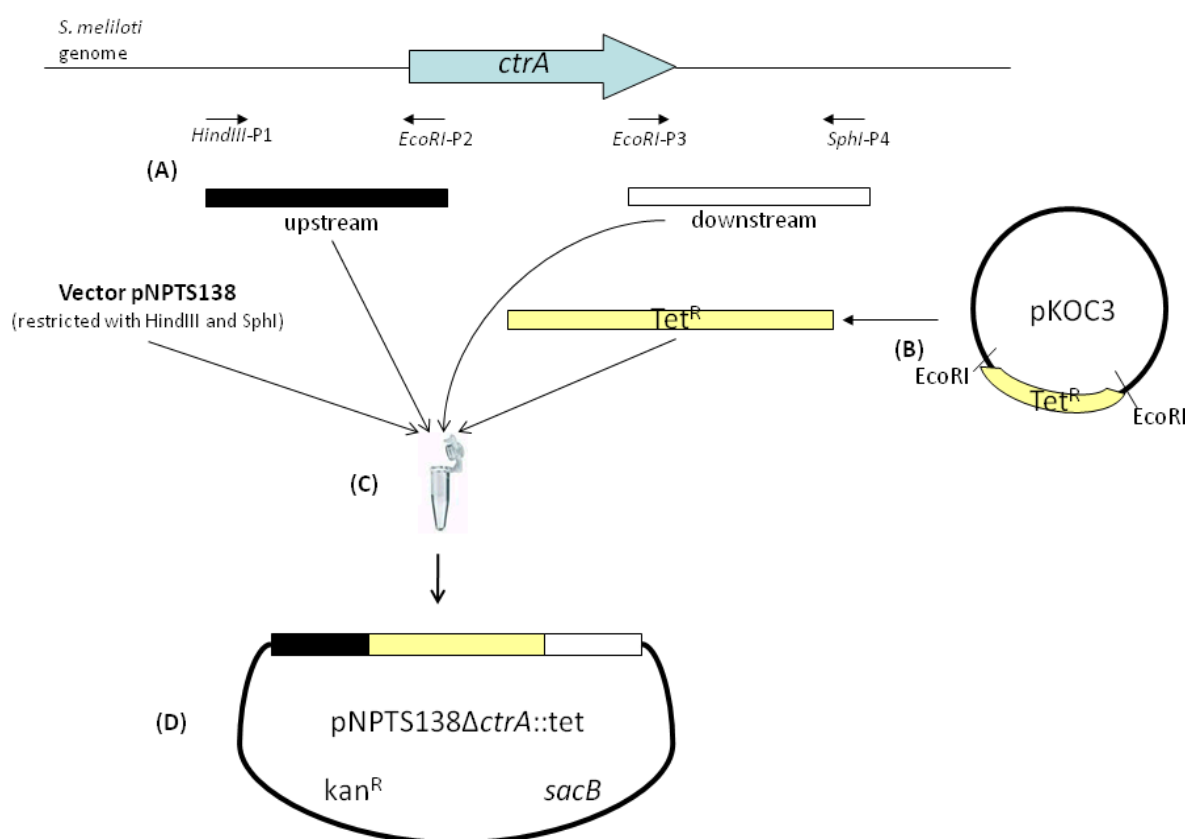
Figure 7. Construction of the deletion cassette for *S. meliloti ctrA*. (A) PCR amplification of the regions upstream and downstream of the target gene and subsequent restriction with appropriate enzymes. (B) Excision of the tetracycline cassette from the plasmid pKOC3 by restriction with *EcoR*I. (C) Ligase of all fragment produced with the vector pNPTS138 previously cut with both *Hind*III and *Sph*I. All restriction enzymes used generate sticky ends, so, using different enzymes, the ligation is forced to assemble the desired final product (D), that can be selected for tetracycline and kanamycin resistances and sucrose sensitivity.

The plasmids obtained from different tet^R, km^R and sacB^S (sucrose sensitivity) *E. coli* colonies were checked for the correct restriction profile. Different combinations of the same restriction enzymes used to construct the plasmid were assayed. Plasmids showing the expected profile were also confirmed by direct sequencing of the insert. Thus, a correct plasmid was selected and called pNPTSΔctrA::tet.

C.3. CONSTRUCTION OF A SET OF PLASMIDS TO COMPLEMENT THE DELETION OF *S. MELILOTI* CHROMOSOMAL COPY OF *ctrA*

C.3.1. First strategy: the *ctrA* ts allele of *C. crescentus*.

ctrA gene, including its promoter, was amplified by both strains of *C. crescentus,* the wild type CB15N and the *ctrA401^{ts}* strain, that possesses the allele of *ctrA* coding for a protein sensitive to temperature. This version of CtrA is functional at 30°C and loses its function at 37°C. Moreover, the wild type copy of *S. meliloti ctrA* complete gene was also amplified from strain Rm1021. Amplifications were carried out as reported in material and methods (E.2.13.). For *C. crescentus* primers pPctrA-ctrA-fw and pPctrA-ctrA-rev were used that amplify a fragment of about 1 Kbp composed by the coding sequence and 200 bp upstream. For *S. meliloti* two primers P1 and P4 (used for the construction of the deletion cassette) were used amplifying a fragment of about 2,7 Kbp. Amplifications were performed with Accuprime pfx (Invitrogen) that possesses proof-reading activity and gives blunt-ended PCR fragments. Gel-purified PCR fragments were ligated in the EcoRV site of the multicloning site (MCS) of the vector pJS14 (high copy number in *Caulobacter,* 15-20 copies per cell) that confers resistance to chloramphenicol, and ligation products were transformed in *E. coli* JM109 cells. Plasmids extracted from clones obtained after transformation were first checked by restriction. Nucleotide sequence of molecules with correct restriction pattern were finally confirmed by DNA sequencing. Plasmid obtained with the wild type *C. crescentus ctrA* was called pJS14-ctrA(C.cre), with the *ts* allele of *C. crescentus ctrA*, pJS14-ctrAts and plasmid with the wild type allele of *S. meliloti* Rm1021, was called pJS14-ctrA(S.mel), (strains were called BM20, BM21 and BM43 respectively).

The same inserts previously cloned in pJS14, were cloned also into the low copy plasmid pMR10: restriction of pJS14 derivative plasmids with both *Kpn*I and *EcoR*I allowed excision of the inserts which were gel-purified and ligated into the plasmid pMR10 previously treated with the same enzymes. Plasmid pMR10 carries a kanamaycin resistance cassette and,

differently from pJS14, is a low copy plasmid (2-5 copies per cell). Transformation was performed in *E. coli* JM109 cells and plasmids obtained from transformants were checked for the correct size by agarose gel electrophoresis. Correct plasmids were confirmed by restriction with *Kpn*I and *EcoR*I and plasmids obtained were called pMR10-ctrA(C.cre), pMR10-ctrA(ts) and pMR10-ctrA(S.mel) respectively (*E. coli* JM109 derivatives were designated BM118, BM120 and BM121, respectively).

C.3.2.Second strategy: conditional expression of CtrA

Recently, Khan *et al.* (2008) described a new family of vectors called pSRK, constituted by three members: pSRK-Km, kanamycin resistence, pSRK-Gm, gentamycin and pSRK-Tc, tetracycline, that are identical except for the resistance marker. All these vectors contain the wild type complete promoter of the *lac*-operon of *E. coli* engineered to be fused with a coding sequence of interest (figure 8). Although there are a number of broad-host-range vectors based on the *lac*-promoter system (Graupner and Wackernagel 2000; Luo and Farrand 1999; Chen and Winans, 1991; Bagdasarian *et al.*, 1983), in *S. meliloti* genes cloned in these vectors are known to be expressed also in the absence of induction (Hallez et al., 2007a). The *lac*-promoter of pSRK vectors, instead, allows complete suppression of gene expression in absence of the inducer also in *S. meliloti* (Khan *et al.*, 2008). Cloning of the appropriate insert must be performed between the *Nde*I and another restriction site of the MCS. Fusion in the *Nde*I of the first codon (ATG) of the cloned gene assures translation in the correct frame (figure 8).

Primers *Nde*I-Smc00654-pctrA-fw and *Kpn*I-Smc00659-pctrA-rev where designed and used to amplify the coding region of *ctrA* from *S. meliloti* Rm1021, including the start codon in the 5′-*Nde*I site and the stop codon before the 3′-end. PCR product obtained with Accuprime pfx (Invitrogen), was gel purified and treated with a common *Taq* polymerase to add A-tails at both ends (E.2.14). This fragment was cloned in the pGEM-T-Easy vector system (Promega) and transformed in *E. coli* JM109 cells. Plasmids obtained was checked by sizing on agarose gel, by *Nde*I-*Kpn*I restriction for the presence of the insert, and confirmed by sequencing. The correct plasmid selected, called pGEM-ctrA(S.mel), was restricted with both *Nde*I and *Kpn*I. Then the insert excised from pGEM-ctrA(S.mel) was gel purified and ligated in pSRK-Km, previously treated with both enzymes. By this way the final plasmid was obtained and called pSRK-Km-ctrA(S.mel) (The *E. coli* strain maintaining this plasmid was called BM221).

Following the same approach two other plasmids were constructed, pSRK-Km-ctrA(C.cre) and pSRK-Km-ctrAts, with respectively the *ctrA* coding region of *C. crescentus* wild type and the *ctrA401^{ts}* mutant allele (*E. coli* strains were respectivly BM204 and BM207. Primers used for amplification of the insert in both cases were *Nde*I-CC3035-pctrA-fw and *Kpn*I-CC3035-pctrA-rev.
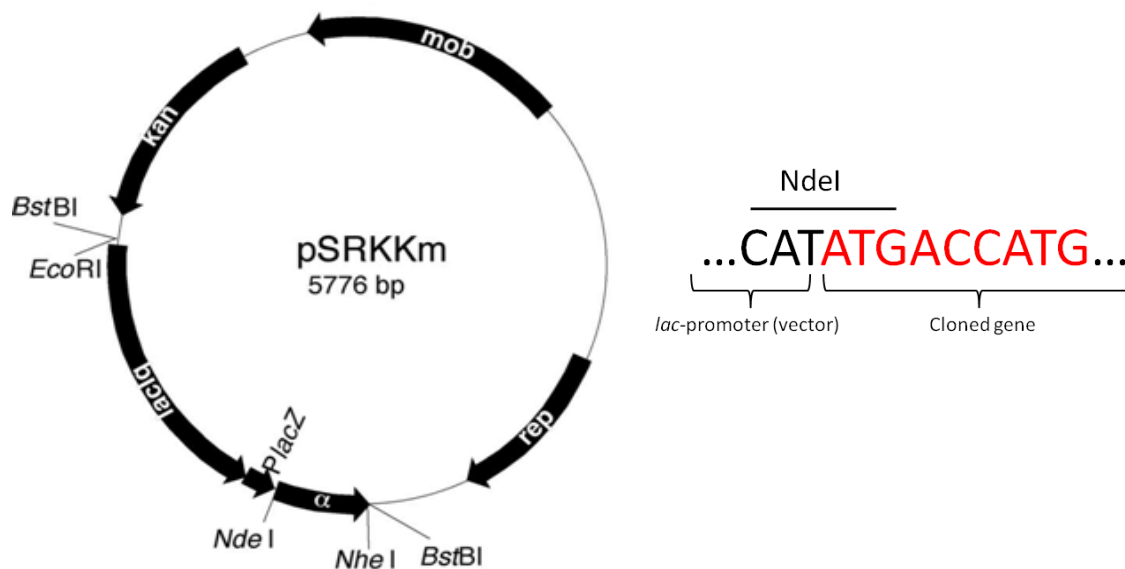
Figure 8. Structures of the pSRK-Km broad-host-range expression vector. The physical map shows the locations of the key genetic determinants, including replication (rep), antibiotic resistance (kanamycin (kan, Km)), mobilization (mob) and the expression cassette, composed of *lacI*q (coding for the repressor of transcription), the *lac* promoter (P*lacZ*) and the *lacZ*α (α) coding sequence. The sequence of the Nde I cloning site is shown.

C.4. CONSTRUCTION OF *S. MELILOTI* Rm1021 DERIVATIVE STRAINS CONTAINING PLASMIDS FOR THE COMPLEMENTATION OF *ctrA* DELETION

pJS14 derivatives, pMR10 derivatives and pSRK-Km derivatives produced in this work were moved in *S. meliloti* wild type strain Rm1021 by triparental mating, involving pRK2013 as helper plasmid. Also empty vectors pJS14 and pMR10 were moved in Rm1021 by the same way to create control strains. Transconjugants obtained were checked by plasmid extraction (see E.2.11.). In four cases the plasmid extraction failed from all transconjugants colonies tested: pJS14-ctrA(C.cre), pJS14-ctrAts, pSRK-Km-ctrA(C.cre) and pSRK-Km-ctrAts. In all these

cases however the presence of the plasmid was confirmed by specific PCR amplification of the insert with the same primers used to amplify it.

Both pJS14 and pSRK plasmids share the same group of incompatibility that is different from pMR10. These feature could be at the origin of the failure of the extraction such as the exogenous origin of the insert, because of the presence of the self *ctrA* gene doesn't compromise visualization of the plasmid extracted. Moreover, the protocol for plasmid extraction derive from the standard protocol for *E. coli* and it could not be sufficiently adapted to *S. meliloti*. Thus, strains that failed extraction but were positive to the specific PCR amplification were though considered corrected and were used for other experiments.

C.5. *ctrA* IS ESSENTIAL IN *S. MELILOTI*

C.5.1. Low-copy complementation of *ctrA* deletion

As shown in section C.2. the first step to produce *ctrA* deletion was the integration of pNPTS138ΔctrA::tet in the genome of *S. meliloti*. In order to transfer the plasmid in *S. meliloti*, triparental mating and electroporation (see Methods) were attempted, but no transfer of pNPTS138ΔctrA::tet was obtained probably because the efficiency of transfer of a suicidal plasmid is lower than that of a self replicative plasmid, because it needs recombination. Higher efficiency of conjugal transfer can be obtained by biparental mating, based on an *E. coli* strain S17-1 (Simon *et al.*, 1983), that carries genes for transfer in the chromosome and doesn't need the contribution of the helper plasmid from a third strain.

Thus, S17-1 was electroporated with pNPTS138ΔctrA::tet DNA, and transformants were confirmed by plasmid extraction. This *E. coli* strain, called BM61, was then mated with *S. meliloti* Rm1021 and transconjugants were obtained (with an efficiency of about 10^{-4} transconjugants/donor). All transconjugants tested were kanamycin and tetracycline resistant and sensitive to sucrose as expected, so one of these strains, BM65, was obtained which carries the *ctrA* deletion cassette within the chromosome.

In order to obtain the *ctrA* deletion mutant, the insertion cassette should be transferred to the strains with *ctrA* complementation plasmids; this was achieved by transduction with M12 phage (Finan *et al.*, 1984). A bacteriophage M12 lysate was produced from the *S. meliloti* strain BM65. BM65 phage-lysate was used to transduce the integration of pNPTS138ΔctrA::tet in strains BM113, BM130, BM131 and BM132 (strains Rm1021 containing pMR10 derivative plasmids). Transduction was successful in all cases giving

strains BM178, BM180, BM181 and BM182. All these strains were resistant for both kanamycin and tetracycline and were sensitive to sucrose.

Three colonies from each transduction were used for the selection of sucrose resistant colonies, whereby corresponding to the final deletion mutants (E.2.17.). Results of the selection are shown in Table 1.

Table 1. *ctrA* deletion: selection for sucrose resistant colonies.

Strain	Clones tested	Sucrose resistants (cells/ml)	Average of sucrose resistants
BM182	Clone 1A	$2,8 \times 10^3$	$2,83 \times 10^3$
	Clone 2A	$2,7 \times 10^3$	
	Clone 3A	$3,0 \times 10^3$	
BM178	Clone 2A	$1,99 \times 10^5$	$1,24 \times 10^5$
	Clone 2B	$1,02 \times 10^5$	
	Clone 2C	$7,0 \times 10^4$	
BM180	Clone 3B	$3,9 \times 10^3$	$4,8 \times 10^3$
	Clone 3C	$6,5 \times 10^3$	
	Clone 3D	$4,0 \times 10^3$	
BM181	Clone 4A	$> 1,0 \times 10^5$	$> 1,0 \times 10^5$
	Clone 4C	$> 1,0 \times 10^5$	
	Clone 4D	$> 1,0 \times 10^5$	

*All the strains were grown for selection at the same optical density in order to plate the same number of cells.

As previously shown by Barnett *et al.* (2001), *ctrA* gene is essential for viability, and therefore it is not surprising the low number of sucrose resistant colonies obtained from the strain BM182 (it has the pMR10 vector without any complementing insert). These colonies didn't lose the pNPTS138 plasmid, but represent the amount of spontaneous inactivation of *sacB* gene. Higher number of colonies were obtained by BM178 and BM181, with complementing plasmids. In the case of BM180, the strain carrying the *ctrAts* allele, the number of colonies was similar to the background, suggesting failure of complementation.

Sucrose resistant colonies obtained transducing BM178 and BM181 were analyzed by PCR to check for the deletion of the chromosomal copy of *ctrA* (Data not shown). All the colonies screened were confirmed to be effectively ΔctrA mutants, and strains BM146 (Rm1021ΔctrA + pMR10-ctrA(C.cre)) and strain BM196 (Rm1021ΔctrA + pMR10-ctrA(S.mel)) were produced.

Using BM146 or BM196, where *ctrA* was in fact deleted, it was possible to transfer the mutation in other genetic backgrounds by transduction and selection for tetracycline resistance. Thus, a M12 phage-lysate of BM146 was produced and used to infect recipient strains BM113, BM130, BM131 and BM132. All strains were successfully tested for sensitivity to phage infection (E.2.10) and then *ctrA* deletion was transduced. For each sample a non-infected control was performed permitting to evaluate the spontaneous tetracycline-resistantsthat were absent in each strain tested. Results obtained from transductions of *ctrA* deletion are shown in table 2.

Table 2. Transduction of *ctrA* deletion in low-copy complemented strains.

Recipient strain	Viable title (cfu/ml)	Number of transduced (cfu/ml)
BM113	$5,6 \times 10^{10}$	0
BM130	$4,5 \times 10^{10}$	$3,2 \times 10^{2}$
BM131	$2,3 \times 10^{10}$	0
BM132	$2,6 \times 10^{10}$	$3,1 \times 10^{2}$

Results confirmed unambiguously that *ctrA* is essential for viability as previously reported (Barnett *et al.*, 2001) and that viability can be restored by wild-type *ctrA* of *C. crescentus* as well as by the *S. meliloti ctrA*. As previously reported (Table 1), the temperature-sensitive allele of *C. crescentus ctrA* failed to complement chromosomal deletion of *S. meliloti ctrA* even at permissive temperature. Since the correct function of *ctrA* requires coordinated expression of the gene, phosphorylation, and proteolysis of the gene product, the heterologous product of the *ts*-allele may have failed at any one of these levels. The *ts*-mutation could reduce the functionality of the protein, becoming non functional when expressed in a low copy system. By the other hand an increment of the copy number of the gene, by using a high copy number vector, could increase the intracellular concentration of CtrA protein, improving its ability to complement deletion of the chromosomal copy. For this reason plasmid derivates of pJS14 were tested as carriers of the complementing *ts*-allele.

C.5.2. High-copy complementation of *ctrA* deletion

Phage-M12 lysate of mutant BM146 was used to transduce *S. meliloti* Rm1021 derivatives strains BM23, BM39, BM41 and BM42. These strains carry the same extra-copy of *ctrA* tested in low-copy plasmid, but in a high copy-plasmid (the plasmid pJS14). Since *C. crescentus ctrAts* allele failed complementation of chromosomal *ctrA* deletion of *S. meliloti* in a low-copy vector, this new experiment allows to verify if the increment of copy number of *ctrAts* gene can restore viability in *S. meliloti.*

All strains were first successfully tested for sensitivity to phage infection (E.2.10.) and then *ctrA* deletion was transduced. For each sample a non-infected negative control was performed permitting to evaluate the spontaneous tetracycline-resistants. Spontaneous tetracycline-resistants were absent for strains BM23, BM41 and BM42; instead strain BM39 gave spontaneous tetracycline resistant mutants with a frequency of about 10^{-7} resistants/ml. After transduction of *ctrA* deletion, no transduced colonies were obtained for strains BM23, BM41, BM42. For BM39 tetracycline resistant colonies were obtained and their number was comparable to the background of spontaneous resistants. These data indicate that high-copy complementing plasmids are unable to restore viability in a *S. meliloti* chromosomal *ctrA* deletion mutant. To confirm these results another approach was followed. Plasmid carrying the cassette for the deletion of *ctrA* gene pNPTS138ΔctrA::tet, was moved in the four recipients BM23, BM41, BM42 and BM39, using *E. coli* strain BM61 as donor. Transconjugants obtained were then selected for sucrose resistant colonies. The number of sucrose resistant colonies obtained from all the stains containing the *ctrA* extra-copy (BM41, BM42, BM39) was comparable to the number of spontaneous sucrose resistant colonies obtained from the control BM23 (without complementation) confirming indeed that the high copy number vector system used here was not able to complement the *ctrA* chromosomal deletion in *S. meliloti.* Considering that pJS14 derivatives have the same inserts as pMR10 derivatives and successful complementation was obtained from both pMR10-ctrA(C.cre) and pMR10-ctrA(S. mel) (C.5.1.), we conclude that some unknown aspect of the pJS14 vector is responsible for the non-correct expression of the inserted gene and thus for the failed complementation of deletion of *ctrA.*

C.6. DELETION OF CHROMOSOMAL *ctrA* CAN BE RESTORED BY CONSTITUTIVE EXPRESSION OF *S. MELILOTI* CtrA.

Plasmid pSRK-Km derivatives have chimerical genes in which the coding region of *ctrA* is cloned under the control of the *lac*-promoter and *lac* repressor (Khan *et al.*, 2008), so the expression of the gene needs the presence of an inducer (IPTG). Transcription of *ctrA* from pSRK-Km derivatives, differently from pMR10 and pJS14 derivatives, is constitutive under induction, while the native promoter is differently regulated during cell cycle progression (Barnett *et al.*, 2001). On the contrary, when IPTG is absent, the transcription is stopped. *S. meliloti* strains used were: BM210, carrying pSRK-Km-ctrA(C.cre), BM212, carrying pSRK-Km-ctrAts and BM240, carrying pSRK-Km-ctrA (S. mel). Two clones for each strain were infected with phage-M12 lysate of mutant BM146. All strains were successfully tested for sensitivity to phage infection (E.2.10.) and then *ctrA* deletion was transduced. For each sample a non-infected negative control was performed permitting to evaluate the percentage of spontaneous tetracycline-resistants. Spontaneous tetracycline-resistants were absent for each strain tested. Results of transductions of *ctrA* deletion are shown in table 4.

Table 4. Transduction of *ctrA* deletion in strain constitutively expressing *ctrA*.

Strain	Clones tested	Viable title (cfu/ml)	Number of transductants (cfu/ml) selected without IPTG	Number of transductants (cfu/ml) selected with IPTG 500 µg/ml
BM210	Clone A	$3,9 \times 10^9$	0	0
	Clone D	$4,3 \times 10^9$	0	0
BM212	Clone A	$4,8 \times 10^9$	0	0
	Clone B	$4,5 \times 10^9$	0	0
BM240	Clone B	$8,0 \times 10^8$	0	$5,8 \times 10^1$
	Clone C	$8,0 \times 10^8$	0	$7,4 \times 10^1$

Data obtained showed that, in pRSK-system, only the *S. meliloti ctrA* coding region is able to complement the deletion of chromosomal *ctrA* gene (the mutant strain obtained was called BM249). Interestingly, this data shows that regulation of CtrA transcription during cell cycle progression is not essential for viability in *S. meliloti* as for *C. crescentus* (Domian *et al.*, 1997), indicating that CtrA activity can be sufficiently regulated only at post-translational level by phosphorylation/dephosphorylation and proteolysis. Moreover strain BM240 without inducer failed to grow indicating the lack of complementation of *ctrA*.

C.7. MORPHOLOGICAL ANALYSIS OF THE *ctrA*-LOSS OF FUNCTION PHENOTYPE IN *S. MELILOTI*

To study the phenotype of CtrA-loss of function, strain BM249 was grown as described in E.2.18.

Optical densities (OD_{600}) of the cultures are reported in table 5:

Table 5. Growth of mutant strain BM249 in different conditions.

Time (hours)	no IPTG	250 mg/ml IPTG	500 mg/ml IPTG
0	0,103	0,103	0,104
8	0,188	0,186	0,180
24	0,275	0,852	0,868
32	0,295	2,260	2,390

Data reported demonstrated that when IPTG is absent the cell cycle progression is stopped and that IPTG is essential for growth of mutant strain BM249. The small increment of OD_{600} on the culture without IPTG can be interpreted as the increase of cell size (as shown in figure 9) but presumably don't involve cell division. Also in *C. crescentus* deletion of *ctrA* stops cell division inducing abnormal growth of the cells (Biondi *et al.*, 2006b).

Concerning the effect of IPTG concentration, it is known that increasing IPTG concentration rises the level of gene-expression (Khan *et al.*, 2008). Since both the concentration tested were equivalent for growth stimulation, we can conclude that 250 mg/ml of IPTG may ensure the highest level of induction of *ctrA* expression. Moreover this observation shows again that regulation of *ctrA* transcription during cell cycle progression is not essential for viability in *S. meliloti*.

Aliquots of cells from the above described cultures were taken at each time point and were observed under microscope. Figure 9 shows the morphology of *S. meliloti* cells determined by CtrA loss of function.
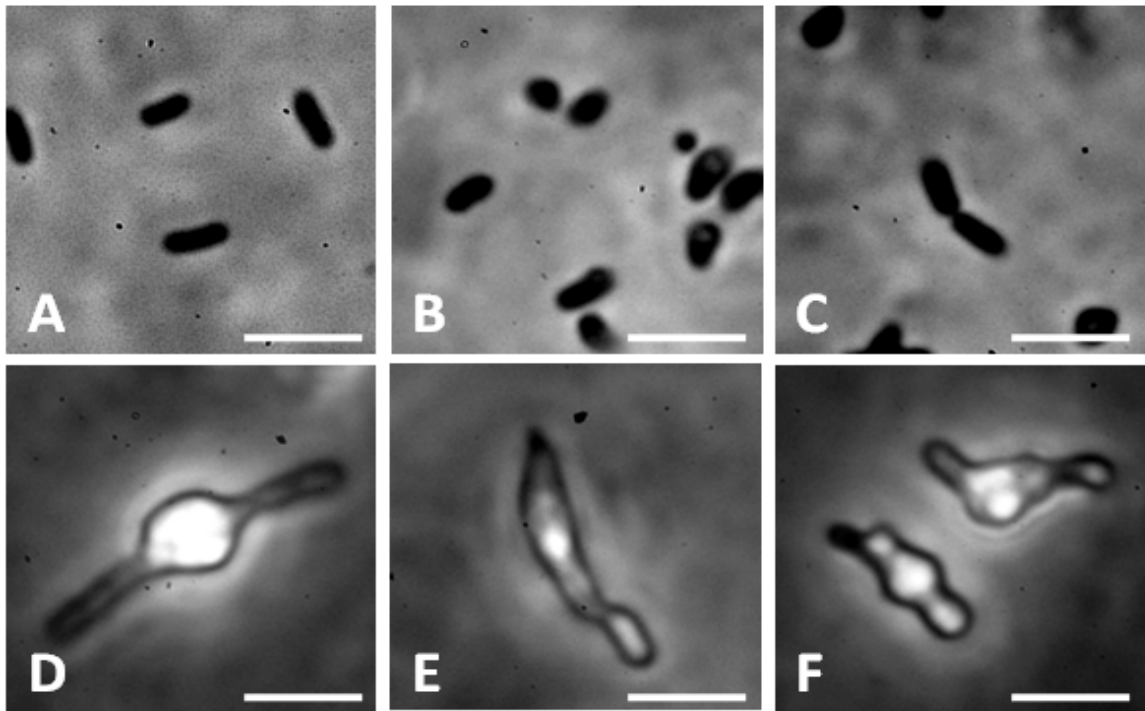
Figure 9. Changes of morphology determined by CtrA-loss of function in *S. meliloti* Rm1021. A) wild type Rm1021; B-C) conditional-mutant BM249 grown with IPTG 500 mg/ml; D-E-F) BM249 after 32 h of incubation without IPTG. Pictures were taken with a FinePixS1pro (Fujifilm) camera connected to a LEICA DM-L phase contrast microscope.

Figure 9 clearly shows that loss of CtrA function determines extreme morphological changes of *S. meliloti* cells. When *ctrA* is constitutively expressed by IPTG induction (figures 9B and 9C), cell's morphology doesn't show changes with respect to the morphology of the wild type strain *S. meliloti* Rm1021 (figure 9A). This demonstrates that the modifications determined by the absence of *ctrA* expression (figures 9D-E) are due to the deletion of CtrA activity. Pictures 9D-E shows three of the more frequent cell's shapes observed after 32 hours of incubation without induction which indicates that CtrA-function deletion resulted in a strong increase of the cell's volume. Length was increased between 3-10 times and width of about 3 times. Thus it seems that CtrA-loss of function changed the morphology of *S. meliloti* in a different way respect to *C. crescentus* (Biondi *et al.*, 2006b), that in fact developed highly elongated cells, maintaining the same width of the normal growing cells.

C.8.   CtrA REGULON IN *S. MELILOTI*

Laub and collaborators (2000, 2002) combining different sources of information were able to identify a set of genes possibly representing the CtrA regulon in *Caulobacter* (that is the whole set of genes whose expression is controlled by CtrA): 116 genes were identified through chromatin immunoprecipitation using phosphorylated CtrA; 88 of them were moreover identified as CtrA-dependent for normal expression levels, and 69 as cell cycle-regulated. The 54 genes within the overlap of all three data sets were identified as members of the CtrA cell cycle regulon, and were used in our computational analysis of CtrA regulons. Upstream sequences of these *C. crescentus* 54 genes were retrieved and used to find enriched sequence motifs using AlignAce (Roth *et al.*, 1998). The position weight matrix obtained (Appendix A) was found to correspond to a 16-mer containing the known CtrA binding motif and was used in a sliding window approach on the sequenced genome of *S. meliloti* Rm1021. An output was obtained where all genes have a score based on the presence of CtrA binding motif in the region comprised from 100 nucleotides within the coding sequence to 400 nucleotides upstream the start codon.

The complete list of genes obtained from this analysis is reported in Appendix B at the end of chapter I. Among cell cycle regulator factors, this analysis indicated that *S. meliloti* CtrA controls the DivJ-DivK-PleC system, the CpdR-RcdA-ClpPX system and the GcrA-DnaA-CcrM. Moreover, CtrA can bind its own promoter, as in *C. crescentus.*

As a second step the *Caulobacter* CtrA regulon, composed by the 54 genes previously described, was analyzed for its conservation in *S. meliloti.* For each gene the first blast hit in the genome of *S. meliloti* was selected; furthermore positive genes were sought in the promoter region for the presence of CtrA boxes. Results obtained showed that *S. meliloti* share with *C. crescentus* several cell cycle genes controlled by CtrA, such as *ccrM*, RcdA (responsible for proteolysis of CtrA) and the orthologs of two *C. crescentus* hybrid histidine kinases, CC2324 (59%) and CC3219 (62%). Other genes controlled by CtrA were the ortholog of CC2165 putatively involved in cell division and chromosome partitioning; the orthologs of CC1872 and CC0233 involved in cell envelope and outer membrane biogenesis; and genes involved in cell motility and secretion. Finally, processes of post-translational modification, protein turnover and chaperones can also be controlled by CtrA in *S. meliloti*, since it can putatively regulate the ATP dependent Clp-protease (CC1963).

All these bioinformatic predictions on the CtrA regulon in *S. meliloti* can now be confirmed using the conditional *ctrA* mutant strain with the inducible CtrA complementation. Microarray experiments in fact will be performed in the near future to analyze variations of global expression levels of strain BM249 revealing the real extent of CtrA control in *S. meliloti.*

D. DISCUSSION

Sinorhizobium meliloti is one of the most studied members of alpha-proteobacteria, belonging to the family of *Rhizobiaceae* (Young *et al.*, 2001), which lives either free in soil or in symbiosis with leguminous (*Fabacae*) plants. During symbiosis *S. meliloti* invades root hairs of the plant and forms root nodules. Inside them the bacteria differentiate in bacteroids that can perform nitrogen fixation. A recent study from Mergaert *et al.* (2006) has shown that differentiation from free-living cells to bacteroids involves significant changes in morphology, as increment of the cell size and genome amplification. Moreover after differentiation *S. meliloti* bacteroids stop to divide, and lose viability, suggesting an important involvement of the regulation of cell cycle progression in the process of bacteroid differentiation. The master regulator of cell cycle progression in alpha-proteobacteria is CtrA, deeply studied in the model organism *C. crescentus*, whose gene is present also in *S. meliloti,* with consistent similarities to the *C. crescentus* gene (Barnett *et al.*, 2001).

The work carried out in the present study was aimed at elucidating the role of CtrA in *S. meliloti* by the use of a mutant of *ctrA* gene. First, *ctrA* was showed to be essential for viability in *S. meliloti*, according with a previous report (Barnett *et al.*, 2001). Then, two strategies were developed to construct a *S. meliloti* strain with CtrA-loss of function phenotype, based on a *ctrA* deletion mutant with an extra-copy of *ctrA* for conditional complementation. The *ctrA* allele *ctrA401^{ts}* of *C. crescentus* was tested for complementation with both low-copy and high-copy plasmid, but none of them were able to complement the deletion of *ctrA* in *S. meliloti* chromosome. As known in *C. crescentus*, the correct functioning of *ctrA* requires coordinated expression of the gene, phosphorylation, and proteolysis of the gene product. The heterologous product of the *ts*-allele may have failed at any one of these levels. The *ctrA401ts* mutation, in fact, causes the substitution of a residue of tyrosine (Thr-170) with a residue of isoleucine. This modification, even if, at permissive temperature, it can restore viability in *C. crescentus*, in *S. meliloti* the function of the protein might be compromised by preventing the correct interaction with one of its substrates, regulatory proteins or DNA binding regions.

A second strategy for complementation of the *ctrA* deletion was developed by fusing the *ctrA* coding region with an IPTG-inducible promoter (Khan *et al.*, 2008). Three alleles were tested, *ctrA* of *S. meliloti*, *ctrA* of *C. crescientus* and *ctrA401^{ts}*. The *ctrA* coding region of *S.*

meliloti was found to restore viability of the chromosomal *ctrA* deletion in *S. meliloti*. Because the complementing gene is constitutively transcribed in the experimental conditions used, by the uninterrupted presence of IPTG, the result obtained demonstrates that the regulation of CtrA at the transcriptional level is not essential for cell cycle progression. This suggests that CtrA activity in *S. meliloti* can be sufficiently regulated only at the post-translational level by both phosphorylation/dephosphorylation and proteolysis, as demonstrated for *C. crescentus* (Domian *et al.*, 1997).

Conditional mutant is not viable when induction is interrupted, allowing the observation of the CtrA-loss of function phenotype in *S. meliloti*. Blocking CtrA expression provokes loss of motility and a strong modifications of the cell morphology. This result was expected as it was predicted by the bioinformatic study of CtrA regulon that cell motility and cell envelope should be regulated by CtrA in *S. meliloti*. Microscope observations revealed that cells that lost CtrA appeared clearly non-motile compared to the cells where CtrA expression is induced by IPTG and to the wild type Rm1021, confirming the bioinformatic prediction. Considering the overall morphology of the *ctrA* deletion mutant, all cells showed abnormal growth with larger cell volume in both dimensions. Moreover cells apparently develop an enlargement of the envelop located at the center or at one pole, it appears bright under phase contrast microscopy, suggesting a different three-dimensional structure. Cell elongation ranged between 3 and 10 times the size of the cells induced with IPTG and of the wild type Rm1021. Since a similar elongation is typical of bacteroids, a relation between the stop of cell cycle progression by inactivation of CtrA and the differentiation from free-living bacteria to bacteroids within the nodule can be hypothesized. These observations shed new light in elucidating cell cycle progression in *S. meliloti* and understanding basic mechanisms regulating bacteroids formation.

E. MATERIALS AND METHODS

E.1. MATERIALS

E.1.1. Strains and plasmids

Strain or plasmid	Relevant properties*	Source or Reference
S. meliloti		
Rm1021	SU47 *str-21*, sm^R	Meade *et al.*, 1982
BM23	Rm1021 + pJS14, sm^R, cm^R	This work
BM39	Rm1021 + pJS14-ctrA(S.mel), sm^R, cm^R	This work
BM41	Rm1021 + pJS14-ctrA(C.cre), sm^R, cm^R	This work
BM42	Rm1021 + pJS14-ctrAts, sm^R, cm^R	This work
BM65	Rm1021::pNPTS138ΔctrA::tet, sm^R, km^R, tc^R, sacB^S	This work
BM113	Rm1021 + pMR10, sm^R, km^R	This work
BM130	Rm1021 + pMR10-ctrA(C.cre), sm^R, km^R	This work
BM131	Rm1021 + pMR10-ctrAts, sm^R, km^R	This work
BM132	Rm1021 + pMR10-ctrA(S.mel), sm^R, km^R	This work
BM146	Rm1021ΔctrA + pMR10-ctrA(C.cre), sm^R, km^R, tc^R	This work
BM178	BM130:: pNPTS138ΔctrA::tet, sm^R, km^R, tc^R, sacB^S	This work
BM180	BM131:: pNPTS138ΔctrA::tet, sm^R, km^R, tc^R, sacB^S	This work
BM181	BM132:: pNPTS138ΔctrA::tet, sm^R, km^R, tc^R, sacB^S	This work
BM182	BM113:: pNPTS138ΔctrA::tet, sm^R, km^R, tc^R, sacB^S	This work
BM196	Rm1021ΔctrA + pMR10-ctrA(S.mel), sm^R, km^R, tc^R	This work
BM210	Rm1021 + pSRK-Km-ctrA(C.cre), sm^R, km^R	This work
BM212	Rm1021 + pSRK-Km-ctrAts, sm^R, km^R	This work
BM240	Rm1021 + pSRK-Km-ctrA(S.mel), sm^R, km^R	This work
BM249	Rm1021ΔctrA + pSRK-Km-ctrA(S.mel), sm^R, km^R, tc^R	This work
C. crescentus		
CB15N	Wild type strain	Evinger and Agabian, 1977
LS2195	*C. crescentus* derivative of CB15N carrying *ctrA401ts* allele (functional at 30°C, dies at 37°C)	Quon *et al.*, 1996
E. coli		
DH5α	F_ *supE44 _lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (_80*lacZ*_M15)	Hanahan, 1983
JM109	e14⁻(McrA⁻) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI^qZΔM15*]	Promega S.r.l.
S17-1	RP4-2, Tc::Mu,Km-Tn7, for plasmid mobilization	Simon *et al.*, 1983

BM18	DH5α + pNPTS138ΔctrA::tet, km^R, tc^R, sacB^S	This work
BM20	JM109 + pJS14-ctrA(C.cre), cm^R	This work
BM21	JM109 + pJS14-ctrAts, cm^R	This work
BM43	JM109 + pJS14-ctrA(S.mel), cm^R	This work
BM61	S17-1 + pNPTS138ΔctrA::tet, km^R, tc^R, sacB^S	This work
BM118	JM109 + pMR10-ctrA(C.cre), km^R	This work
BM120	JM109 + pMR10-ctrAts, km^R	This work
BM121	JM109 + pMR10-ctrA(S.mel), km^R	This work
BM214	DH5α + pGEMT-Easy-ctrA(S.mel), am^R	This work
BM204	DH5α + pSRK-Km-ctrA(C.cre), km^R	This work
BM207	DH5α + pSRK-Km-ctrAts, km^R	This work
BM221	DH5α + pSRK-Km-ctrA(S.mel), km^R	This work

Bacteriophage

φM12	Phage specific for *S. meliloti* derivates of SU47 and useful to carry out general transduction	Finan *et al.*, 1984

Plasmid

pNPTS138	Kan^R; *sacB*-containing integration vector	Alley, 2001
pNPTS138ΔctrA::tet	4 Kb *S. meliloti ctrA* deletion cassette cloned in HindIII-SphI sites of pNPTS138. km^R, tc^R, sacB^S	This work
pRK2013	Helper plasmid carrying the conjugal-transfer genes of RK2	Ditta *et al.*, 1980
pKOC3	Plasmid containing *EcoRI* sites-flanked Tet^R-cassette	West *et al.*, 2002
pJS14	pBBR1-derived medium copy number broad host range vector. cm^R	J. Skerker, unpublished
pJS14-ctrA(C.cre)	1 Kb PCR fragment containing complete *ctrA* gene of *C. crescentus* CB15N, cloned in the EcoRV site. cm^R	This work
pJS14-ctrAts	1 Kb PCR fragment containing complete *ctrA* gene of *C. crescentus* LS2195, cloned in the EcoRV site. cm^R	This work
pJS14-ctrA(S.mel)	2,7 Kb PCR fragment containing complete *ctrA* gene of *S. meliloti* Rm1021, coned in the EcoRV site. cm^R	This work
pMR10	RK2-derived low copy number broad host range vector. km^R	Mohr C and Roberts R, unpublished
pMR10-ctrA(C.cre)	KpnI-EcoRI fragment from pJS14-ctrA(C.cre) containing complete *ctrA* gene of *C. crescentus* CB15N, cloned in KpnI-EcoRI sites of pMR10. km^R	This work
pMR10-ctrAts	KpnI-EcoRI fragment from pJS14-ctrAts containing complete *ctrA* gene of *C. crescentus* LS2195, cloned in KpnI-EcoRI sites of pMR10. km^R	This work
pMR10-ctrA(S.mel)	KpnI-EcoRI fragment from pJS14-ctrA(S.mel) containing complete *ctrA* gene of *S. meliloti* Rm1021, cloned in KpnI-EcoRI sites of pMR10. km^R	This work
pGEM-T-Easy	cloning vector, am^R	Promega
pGEM-ctrA(S.mel)	PCR fragment containing *S. meliloti ctrA* coding region (T/A)-cloned in pGEM-T-Easy, am^R	

pSRK-Km	pBBR1MCS-2 derived broad host range expression vector containing *lac* promoter and *lacI*^q, *lacZα*⁺, km^R	Khan *et al.*, 2008
pSRK-Km-ctrA(C.cre)	*ctrA* coding region of *C. crescentus* CB15N cloned in NdeI-KpnI sites of pSRK-Km. km^R	This work
pSRK-Km-ctrAts	*ctrA* coding region of *C. crescentus* LS2195 cloned in NdeI-KpnI sites of pSRK-Km. km^R	This work
pSRK-Km-ctrA(S.mel)	NdeI-KpnI fragment from pGEM-ctrA(S.mel), consisting of *ctrA* coding region of *S. meliloti* Rm1021, cloned in NdeI-KpnI sites of pSRK-Km. km^R	This work

*abbreviations: am^R, ampicillin; cm^R, chloramphenicol; km^R, kanamycin; sm^R, streptomycin; tc^R, tetracycline; sacB^S, sensitivity to sucrose.

E.1.2. Primers

Primer	Sequence (5′-3′)
Construction of ctrA deletion-cassette	
pSMc00654-*HindIII*-P1	GGAAGCTTCACAATGCGCCGATTCAACA
pSMc00654-*EcoRI*-P2	GGGAATTCGATCAGTAGAACCCGCAT
pSMc00654-*EcoRI*-P3	GGGAATTCGAGCCGGAAGGCAGCGAC
pSMc00654-*SphI*-P4	GGGCATGCTTGCCGAGGCTGCGGAATAG
Amplification of ctrA alleles from C. crescentus	
pCC3035-ctrA-fw	GGCTGCAGTTCTCGATTTCTTGCGGC
pCC3035-ctrA-rev	GGAAGCTTAGTTCCAACGACTCAGGC
Amplification of ctrA for pSRK-Km-derivates	
pCC3035-*NdeI*-ctrA-fw	GGCATATGCGCGTACTGTTGATCGA
pCC3035-*KpnI*-ctrA-rev	GGGGTACCAGTTCCAACGACTCAGGC
pSMc00654-*NdeI*-ctrA-fw	GGCATATGCGGGTTCTACTGATCGAAG
pSMc00654-*KpnI*-ctrA-rev	GGGGTACCATGTCGCCTACGGGAATGCC
Confirmation of ctrA deletion	
pSMc00654-(-900)-ctrA-seq	TTGGCCCTTCACGCGATCGA
pSMc00654-ctrA-ctrA-rev	ATGGCGATCGACGTCGTATC
Sequencing	
M13-fw	GTAAAAGGACGGCCAG
M13-rev	CAGGAAACAGCTATGAC
pSMc00654-PctrA-PctrA-fw	CACCTCGACCACTAGAGAAGCCGGTT

E.1.3. Growth media

All the media were dissolved in distilled water and autoclaved.

LB medium (Luria-Bertrani Broth, (Sambrook *et al.*, 1989))

> 10 g/l Tryptone
> 5 g/l Yeast Extract
> 5 g/l NaCl

LB/MC medium

LB medium supplemented with 2,5mM of $CaCl_2$ and 2,5mM of $MgSO_4$.

Stock solutions for salts were prepared 2,5M and sterilized by 0,2µm-pores filtration.

TY medium (Beringer, 1974)

> 5 g/l Tryptone
> 3 g/l Yeast extract
> 0.4 g/l CaCl2

PYE medium (Ely, 1991)

> 2 g/l Tryptone
> 1 g/l Yeast extract
> 0.3 g/l $MgSO_4$

SOB medium (Sambrook *et al.*, 1989)

> Stock A: 20 g Tryptone
>   5 g Yeast extract
>   0.5 g NaCl
>   dissolved in 980 ml H2O
> Stock B: 18.65 g/l KCl
> Stock C: 406.6 g/l MgCl2 × 6 H2O
> Stock D: 493 g/l MgSO4 × 7 H2O

10ml of the stock solution B were added to the stock solution A prior to autoclaving. Solutions C and D were autoclaved separately, and 5 ml of each were added to the autoclaved and cooled mixture of A and B solutions.

SOC medium

SOB medium supplemented with 20 mM of a 2 M solution of glucose.

E.1.4. Supplements for growth media

Agar

For the solid bacterial media, 16 g/l were added.
For LB/MC top agar the concentration of 0,3% was used.

Sucrose

To select sucrose-resistant *S. meliloti* Rm1021 colonies (for loss of *sacB* gene) 10 g/l of sucrose were added directly to TY medium before autoclaving. To test *E. coli* sucrose-sensitivity 5 g/l were added to LB medium before autoclaving.

IPTG

IPTG stock solution was prepared 50 mg/ml, sterilized by 0,2 nm-filtering and stored at -20°C in aliquots of 1 ml.

Antibiotics

Ampicillin (Am)

For the selection of Am-resistant *E. coli* clones 100 µg/ml of antibiotic were added to both solid and liquid media.

Chloramphenicol (Cm)

For the selection of Cm-resistant *E. coli* clones, 30 µg/ml of antibiotic were added to solid media; 20 µg/ml were added to liquid media to prevent the loss of plasmids.
For the selection of Cm-resistant *S. meliloti* clones 50 µg/ml of antibiotic were added to both solid and liquid media.

Kanamycin (km)

For the selection of kanamycin-resistant *E. coli* clones 50 µg/ml of antibiotic were added to solid media; 30 µg/ml were added to liquid media to prevent the loss of plasmids. For the selection of kanamycin-resistant *S. meliloti* clones 200 µg/ml of antibiotic were added to both solid and liquid media.

Streptomycin (Sm)

For the selection of *S. meliloti* strains Rm2011, 600 µg/ml of antibiotic were added to both solid and liquid media.

Tetracycline (tc)

For the selection of tetracycline-resistant *E. coli* clones 10 µg/ml of antibiotic were added to both solid and liquid media. For the selection of tetracycline-resistant *S. meliloti* Rm1021 2 µg/ml of antibiotic were added to solid media; 1 µg/ml were added to liquid media.

E.1.5. Buffers and solutions

TEA 50X

242 g TRIS
57,1 g Acetic Acid
100ml EDTA pH.8 (0,5M)
H_2O up to a final volume of 1 liter

Glycerol 10%

100ml of Glycerol 99,9% (Sigma)
900ml distilled water

Physiological solution

0,85 % (W/V) of NaCl.

E.2. METHODS

E.2.1. Cultivation of bacteria

Bacteria were grown using solid media, as well as liquid media. In special cases, the media were supplemented with additives listed above (E.1.3.). *E. coli* cells were cultivated at 37°C in LB. *S. meliloti* cells were cultivated at 30°C in TY. Liquid cultures were also shaken at 180 RPM.

E.2.2. Storage of bacterial strains

Bacteria were grown in the liquid medium overnight until the optical density (OD_{600}) was more than 1 OD and than 500 µl of bacterial culture were mixed with 500 µl of glycerol 50% and stored at -80°C.

E.2.3. Determination of viable titre

Viable titres were determined preparing serial 10-fold dilutions. 100 microliter of the appropriate dilutions were then plated on non selective plates using sterile handles. Plates were then incubated at the appropriate temperature until colonies were grown.

E.2.4. Preparation of *E. coli* electrocompetent cells.

- From an over-night culture of *E. coli* DH5α grown in LB medium at 37°C, inoculate 500 ml of LB medium to an optical density of 0,2 OD.

- Incubate the culture at 37°C to an optical density of 0.6-0.7 OD.

- Chill the culture in ice for 15 min.

- Pellet the cells centrifuging 5 min at 8000 RPM at 4°C and discard the supernatant.

- Resuspend the pellet with 500 ml of sterile distilled H_2O by vortexing and centrifuge again.

- Resuspend the pellet with 250 ml of sterile distilled H_2O by vortexing and centrifuge again.

- Resuspend the pellet with 20 ml of sterile glycerol 10 % solution by vortexing and centrifuge again.

- Resuspend the pellet in a final volume of 2 ml of glycerol 10 % and aliquot (50 µl) the suspension in eppendorfs. Store at -80°C.

  NB. Title of cells of each 50 µl aliquot will be ranged between 10^9 and 10^{10}.

E.2.5. Electroporation of *E. coli* DH5α

All electroporations were performed using sterile electroporation cuvette (inter-electrode distance of 0.1 cm) supplied from Molecular BioProducts and a Gene Pulser® Apparatus connected to the Pulse controller, version 2-89 supplied from Biorad.

- Thaw in ice electrocompetent cells, about 10 min.

- Add DNA in a volume of no more than 4-5 µl and mix well by vortexing few seconds. Keep in ice.

  All the following sequence were performed quickly.

- Transfer the suspension in a new electroporation cuvette.

- Place immediately in the Gene Pulser apparatus and apply the following impulse: 12,5 KV/cm; 5 msec (200 Ω, 25 µF).

- Immediately after pulse application resuspend the cells with 1 ml of SOC medium and transfer in a 13 ml tube.

- Incubated at 37°C, with shaking at 200 RPM, for 1h and 30 secs without any antibiotic.

- Proceed by plating aliquots from serial dilutions on non-selective and selective medium.

- Incubate plates at 37°C until colonies are grown.

E.2.6. Preparation of *E. coli* DH5α chemically competent cells

- Grow DH5α overnight in 5 mL of SOB at 37°C.

- Inoculate 100 mL of SOB to and OD_{600} = 0.1.

- Grow at 37°C, with shaking, until OD_{600} = 0.5 -0.7.

- Place the culture in ice for 15 minutes.

- Divide the culture into 2 tubes with ~40 mL each (ok Falcon).

- Centrifuge at 4000 rpm for 10 minutes at 4°C .

- Gently re-suspend each pellet with 15 mL of cold Mg^{2+} /Ca^{2+} solution (see below).

- Incubate in an ice bath for 30 minutes.

- Centrifuge at 4000 rpm for 10 minutes at 4°C.

- Resuspend each pellet with 1.6 mL of cold 0,1M $CaCl_2$ solution.

- Incubate in an ice bath for 20 minutes.

- Combine cells to one tube.

- Add 0.5 mL of cold 80% glycerol and swirl to mix.

- Flash-freeze in liquid nitrogen as 100-µL aliquots.

    Note: when liquid nitrogen is not available prepare the aliquots and rapidly transfer in the -80°C freezer.

- Store in the -80°C freezer.

    Note: efficiency of cells prepared by this way should range about 10^7 transformants/µg of DNA.

Mg^{2+} /Ca^{2+} solution:
3,25g $MgCl_2$ x $6H_2O$
0,6g $CaCl_2$ x $2H_2O$ = 0,45g $CaCl_2$ anhydrous

200mL H_2O

Sterilize by filtration with filter of 0,2μm-pores

0,1M Ca^{2+} solution:

0,56g $CaCl_2$ anhydrous

50mL H_2O

Sterilize by filtration with filter of 0,2μm-pores

E.2.7. Transformation of *E. coli* DH5α chemically competent cells

- Add 5-7 μL of ligation mixture to one aliquot of chemo-competent DH5α cells.

- Incubate on ice for 30 minutes.

- Heat shock at 42°C for 45 seconds.

- Incubate on ice for 2 minutes.

- Add 900 μL SOC.

- Incubate 37°C for 1 hour and 30 minutes.

- Plate onto the appropriate selective medium.

E.2.8. Bacterial cells lysate

From a fresh ON plate cells were resuspended in 20 μl of dH_2O (alternatively 20 μl of fresh ON liquid culture), heated for 10 min at 99°C and then placed in ice for at least 3 min. 80 μl of dH_2O were added. Bacterial lysates were stored at – 20°C until use.

E.2.9. Conjugation

Biparental mating

- Grow *E. coli* S17-1 overnight in liquid LB medium supplemented with the opportune antibiotics, and recipient *S. meliloti* (Rm1021) in liquid TY supplemented with opportune antibiotics.

- Dilute the cultures to an OD_{600} of 1.0 OD and take 4 ml of *S. meliloti* cells suspension and 2 ml of *E. coli* cells suspension.

- Pellet separately the cells centrifuging 8000 RPM for 2 min.

- Wash twice with 1 ml of physiological solution.

- Resuspend, mixing together with 100 µl of physiological solution and transfer the suspension, as a unique drop without spreading, in a TY plate without antibiotics.

- Incubate overnight at 30 °C.

- Resuspend cells in 1ml of physiological solution using a sterile handle.

- Proceed by plating onto TY plates containing streptomycin to select recipient Rm1021 and the appropriate antibiotic to select the plasmid.

Triparental conjugation

- Grow *E. coli* donor and helper (pRK2013) strains overnight in liquid LB medium supplemented with the opportune antibiotics, and recipient *S. meliloti* (Rm1021) in liquid TY supplemented with opportune antibiotics.

- Dilute the cultures to an OD_{600} of 1.0 OD and take 4 ml of *S. meliloti* and 2 ml of each *E. coli*.

- Pellet separately the cells centrifuging 8000 RPM for 2 min.

- Wash twice with 1 ml of physiological solution.

- Resuspend, mixing together with 100 µl of physiological solution and transfer the suspension, as a unique drop without spreading, in a TY plate without antibiotics.

- Incubate overnight at 30 °C.

- Resuspend cells in 1ml of physiological solution using a sterile handle.

- Proceed by plating onto TY plates containing streptomycin to select recipient Rm1021 and the appropriate antibiotic to select the plasmid.

E.2.10. Transduction

Preparation of lysates of bacteriophage φM12

- Grow over-night *S. meliloti* donor strain at 30°C in LB/MC medium supplemented with opportune antibiotics.

- Estimate the number of cells by direct count with the Burker's chamber. It should be about 10^9 cells/ml.

- Inoculate 5 ml with φM12 phage with a ratio cells:phages of 1 : 1.

- Incubate at 30°C by shaking over-night or until lysis occurs (no more than 24h).

- Add several drops of chloroform (at least 100µl) and mix by vortexing.

- Centrifuge 10,000 RPM for 10 min.

- Recover the supernatant and aliquot in sterile eppendorfs over a drop of chloroform.

- Store at 4°C.

Titering φM12 lysates (sensibility to phage)

- Make appropriate dilutions of the phage stock in LB/MC (expect more than 10^{10} pfu/ml).

- Mix 100µl of *S. meliloti* fresh overnight colture grown in LB/MC and 100µl of diluted phage stock. Mix gently by pipetting few times an incubate at room temperature for 30 minutes.

- Add 5 ml of molten (50°C) LB/MC top agar, mix and pour evenly onto an LB/MC agar plate.

- Incubate at 30°C over-night.

Transduction with φM12 lysates

- Grow over-night *S. meliloti* recipient strain at 30°C in LB/MC medium supplemented with opportune antibiotics.

- Estimate the number of cells by direct count with the Burker's chamber. It should be more than 10^9 cells/ml.

- Inoculate at least 10^9 *S. meliloti* cells with φM12 phage with a ratio cells to phages of 2 : 1 (total volume about 1-1,5ml).

- Incubate 30 min at room temperature.

- Centrifuge 8,000 RPM for 2 min.

- Wash once the pellet with 1 ml of LB and resuspend in LB.
  Note: do not use LB/MC to avoid further phage infection.

- Plate cells on LB containing the appropriate antibiotics and supplements.

- Incubate at 30°C until colonies are grown (usually 3-4 days).
  Note: all transductions should include the control of "recipient only" on the selective medium. Lysate can also checked for contamination by plating it on LB plate.

E.2.11. Isolation and purification of plasmid DNA
Isolation

Plasmid DNA was isolated by using NucleoSpin® Plasmid kit supplied from Macherey-Nagel. Supplier's instruction were followed for *E. coli*, while for *Sinorhizobium* the following changes were applied:

1) Each pellet of cells was obtained from 5ml of a dilution to 1.0 OD_{600} of an overnight culture.

2) Time for Lysis (after added buffer A2 and before to add buffer A3) was extended to 10 min.

3) Time of centrifuging to pellet cell debris (after added buffer A3) was of 10 min.

4) Elution of DNA was performed with 55 µl of nuclease free dH_2O (Ambion) incubating 1 min at room temperature and then centrifuging as indicated. After centrifugation 50 µl of the eluate was recovered and loaded again in the same column incubating 1 min at room temperature and then centrifuging again.

E.2.12. Visualization and quantification of DNA

Agarose gel electrophoresis

This method is used for visualization, as well as quantification of double-stranded DNA. The agarose gels were prepared of 0.6 - 1 % (W/V).

- Mix the agarose with TEA 1X buffer and boil till diluted.
- Chill the agarose-TEA to 50 °C, add Ethidium Bromide solution to a final concentration of 1mg/L and pour in a horizontal chamber with a comb.
- After the gel becomes solid, cover it with TEA buffer and remove the comb.
- Mix the DNA with BBF loading buffer 6X and load into the pockets of the gel.
- Run the gel using the voltage of 100 V.
- Image of gel was collected making a photograph under UV light on the transilluminator.

Quantification of DNA

The concentration and purity of DNA was determined calculating the UV_{260} absorbance using spectrophotometer (BioPhotometer, Eppendorf). Analysis was performed as recommended by the manufacturer.

E.2.13. PCR amplification

PCR amplification were performed as following. For PCR mixture 20 pmol each primer, 0,5 U of Accuprime pfx (Invitrogen) and 1X Accuprimer buffer (it comprises Mg^{2+} and dNTPs) were mixed with 2 μl of bacterial cells lysate. Sterile distilled H_2O was added to a final volume of 25 μl. Amplification was carried out as follow, 95°C 2min, then 35 cycles of denaturation at 95°C 15sec, annealing (for temperature see table below) 30sec, extension 68°C for a time period related to the length of the product (1min for 1Kb). A final step kept reactions at 8°C, before to be moved and stored at -20°C.

E.2.14. T/A cloning

T/A cloning was performed with pGEM-T-Easy vector System I (Promega), according with supplier's instruction. An insert:vector molar ratio 3:1 was applied.

To add A-tail, blunt-end PCR product were mixed with 0,5 U of GoTaq (Promega), 1X of appropriate buffer, 1,5mM of $MgCl_2$ and 100mM of dATP. H_2O was added to a final volume of 50μl. Reaction was incubated 20min at 72°C then stopped in ice. Purification was carried out by using QIAquik gel extraction kit (Qiagen) following appropriate supplier's instruction for DNA purification from enzymatic reactions.

E.2.15. Restriction reactions

Restriction reactions of PCR products and plasmids were performed by mixing purified DNA 10 U of each enzyme and opportune buffer. Buffer for each single and double digestion was chosen as suggested by Double Digest Finder (New Englan Biolab, http://www.neb.com/nebecomm/DoubleDigestCalculator.asp). Reactions were incubated over-night at room temperature.

E.2.16. Ligation

Ligations were performed by mixing plasmid and insert (molar ratio 1 : 3) with 1 U of T4 ligase (Invitrogen or Promega) and its appropriate buffer. Mixtures were performed in ice and then incubated at 4°C over night, for blunt-ends, or room temperature, for sticky-ends. For the construction of pNPTS138ΔctrA::tet, ligation of four fragments was performed with the molar ratio of 1 : 3 : 3 : 3, respectively vector, tet-cassette and PCR products.

E.2.17. Selection of sucrose resistant colonies

Following procedure allows the selection of deletion mutants from the strain with plasmid pNPTS138-derivative integrated in the genome. *S. meliloti* strain was grown in TY medium with tetracycline, to select the deletion cassette, but, without kanamycin, that select the plasmid. After over-night grown, 100μl of colture were spread-plated in TY medium with tetracycline and sucrose 10%. Plates were incubated at 30°C until colonies were grown.

E.2.18. Growth of the *ctrA* deletion mutant

Strain BM249 was grown in 20 ml of TY medium supplemented with IPTG (500mg/ml) and with opportune antibiotics. The following morning optical density (OD$_{600}$) was measured. Cells were centrifuged at 8000 RPM for 5 min at room temperature and washed tree times with an equal volume of TY medium without any supplement. Finally cells were resuspended in a final volume of 1 ml and OD$_{600}$ was measured. Tree different, 10 ml each, TY coltures (with appropriates antibiotic) were prepared, one without IPTG, one with IPTG 250 mg/ml, and one with IPTG 500 mg/ml. All coltures were inoculated with 170 μl of the suspension, OD$_{600}$ was measured, and then were incubated at 30°C by shaking 180 RPM.

E.2.19. *S. meliloti* CtrA regulon characterization

Genes directly regulated by CtrA in *S. meliloti* genome were identified using the following multi-level approach:

1) A Position Weight Matrix (PWM) describing CtrA binding sites in *C. crescentus* was obtained by using the program AlignAce (Roth *et al.*, 1998) on upstream sequences of 55 genes previously identified as being part of the CtrA regulon (Laub *et al.*, 2002). We used such matrix to scan *S. meliloti* Rm1021 genome with a sliding window approach and a scoring function from Schneider *et al.* (1986): $S_i=(1/16) \sum_j [2 + \log_2(F_{ij})]$,, where F_{ij} is the frequency of base i at position j of the 16-mer. This score, whose maximum for the best match using CtrA position weight matrix is ≈1.22, is a measure of the information content of a potential binding site.

2) We retained only motifs having a score greater than or equal to 30% of the maximum score attainable with the given matrix and moreover located in the range -100 to 400 nucleotides from the start codon of a gene.

3) Then we applied a Z-score transformation to highlight significant occurrences and take into account the background DNA implicitly: $Z_i=(S_i - <S_i>)/\sigma_i$, where S_i is

calculated using the above formula, and $<S_i>$) is the average score in an organism and σ_i is the corresponding standard deviation.

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Appendix A

CtrA motif found using AlignAce (Roth et al., 1998) used to characterize the CtrA regulon in the alphas. The known consensus sequence is TTAANNNNNNNTTAA(C). We have obtained a matrix that contains the known consensus and moreover is characterized by the presence of a conserved G (in bold) that has not been recognized in previous works.

		A	C	G	T
1	T	0.1064	0.0213	0.1915	0.6809
2	T	0.1064	0.1915	0.01	0.7021
3	A	0.8298	0.1277	0.01	0.0426
4	A	0.5957	0.1064	0.2979	0.01
5	N	0.2128	0.2553	0.2766	0.2553
6	N	0.383	0.234	0.234	0.1489
7	N	0.1277	0.234	0.383	0.2553
8	N	0.2766	0.2128	0.2553	0.2553
9	N	0.234	0.2128	0.2128	0.3404
10	N	0.2128	0.4681	0.2128	0.1064
11	**G**	**0.2553**	**0.01**	**0.7447**	**0.01**
12	T	0.01	0.01	0.0426	0.9574
13	T	0.1277	0.0426	0.01	0.8298
14	A	0.8723	0.0426	0.0213	0.0638
15	A	0.97	0.01	0.01	0.01
16	C	0.01	0.8723	0.1277	0.01

APPENDIX B

CtrA regulon of *S. meliloti*

Gene Name	Function	Strand	COG cat.*	N occurrences*	Max Z-score**
SMb21524	septum formation inhibitor	-	-	4	4,56661912
SMc00776	PUTATIVE SENSOR HISTIDINE KINASE PROTEIN	-	COG0642T	2	4,43747328
SMc00777	hypothetical protein	+	-	2	4,43747328
SMc03835	PUTATIVE 2'-5' RNA LIGASE PROTEIN	-	COG1514J	3	4,01501827
SMc00639	PUTATIVE HEAT RESISTANT AGGLUTININ 1 SIGNAL PEPTIDE PROTEIN	-	COG3637M	5	3,93936706
SMc03241	hypothetical protein	-	-	5	3,91775327
SMc03040	FLAGELLIN PROTEIN	+	COG1344N	6	3,78367284
SMc02051	hypothetical protein	-	COG5457S	4	3,75992168
SMc03143	hypothetical protein	-	-	6	3,71678641
SMc03808	PUTATIVE CELL DIVISION TRANSMEMBRANE PROTEIN	+	COG1674D	2	3,69106496
SMc02463	PROBABLE SUCCINATE DEHYDROGENASE CYTOCHROME B-556 SUBUNIT TRANSMEMBRANE PROTEIN	-	COG2009C	2	3,65951879
SMc03225	HYPOTHETICAL/UNKNOWN PROTEIN	+	-	3	3,65951879
SMb20343	putative aldehyde dehydrogenase subunit protein	-	COG2080C	2	3,6109561
SMc00718	hypothetical protein	-	-	5	3,57065126
SMc00717	PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN	+	COG2884D	5	3,57065126
SMc03046	PUTATIVE TRANSCRIPTION REGULATOR PROTEIN	+	COG0745TK	5	3,53737303
SMc01335	PROBABLE N-ACETYLMURAMOYL-L-ALANINE AMIDASE AMIC PRECURSOR TRANSMEMBRANE PROTEIN	+	COG0860M	1	3,47637249
SMc01163	PUTATIVE OXIDOREDUCTASE PROTEIN	-	COG0673R	2	3,43319255
SMc01162	hypothetical protein	+	COG0121R	2	3,43319255
SMc00638	PUTATIVE HEAT RESISTANT AGGLUTININ 1 PROTEIN	-	COG3637M	3	3,43026976
SMb20652	putative asparagine synthetase protein	-	COG0367E	3	3,40065887
SMb20654	hypothetical protein	+	-	3	3,40065887
SMc00120	hypothetical protein	-	-	2	3,38741119
SMc00062	hypothetical protein	+	COG4991S	2	3,38741119

SMc02396	PROBABLE OUTER MEMBRANE PROTEIN	+	-	3	3,28394945
SMc00100	hypothetical protein	-	COG0433R	2	3,269338
SMc00041	hypothetical protein	+	-	2	3,269338
SMc01871	D-alanine--D-alanine ligase	-	COG1181M	3	3,26840646
SMc02229	PUTATIVE ACYL-COA DEHYDROGENASE PROTEIN	-	COG1960I	4	3,26654853
SMc02230	hypothetical protein	+	-	4	3,26654853
SMc01563	RNA polymerase sigma factor RpoD	-	COG0568K	2	3,25759412
SMb20909	hypothetical protein	-	-	1	3,22246774
SMc02855	hypothetical protein	-	COG0697GER	2	3,21416359
SMc02856	PUTATIVE PENICILLIN-BINDING PROTEIN	+	COG0744M	3	3,21416359
SMa1016	hypothetical protein	-	COG1835I	1	3,1588988
SMa1018	hypothetical protein	+	-	1	3,1588988
SMc00469	PUTATIVE DNAK SUPPRESOR PROTEIN	-	COG1734T	3	3,14727716
SMc00468	hypothetical protein	+	COG2062T	3	3,14727716
SMb20039	putative transcriptional regulator protein	-	COG1167KE	1	3,09353098
SMb20040	hypothetical protein TRANSMEMBRANE	+	-	1	3,09353098
SMc02139	hypothetical protein	-	-	1	3,06239912
SMc02138	bifunctional N-succinyldiaminopimelate-aminotransferase/acetylornithine transaminase protein	+	COG4992E	1	3,06239912
SMc00360	hypothetical protein	-	COG3672S	3	3,06237575
SMc00743	hypothetical protein	+	COG2244R	3	3,0578412
SMc04112	PUTATIVE PILUS ASSEMBLY SIGNAL PEPTIDE PROTEIN	+	-	1	3,03244941
SMc02060	LIPOPROTEIN PRECURSOR	+	COG0739M	3	3,02977047
SMa0794	hypothetical protein	+	COG2141C	1	3,01931142
SMc01000	hypothetical protein	-	-	1	3,01333324
SMc01001	hypothetical protein	+	COG0705R	1	3,01333324
SMc00289	PUTATIVE COLD SHOCK TRANSCRIPTION REGULATOR PROTEIN	-	-	1	3,00773889
SMc00290	PROBABLE LACTOYLGLUTATHIONE LYASE METHYLGLYOXALASE PROTEIN	+	COG0346E	1	3,00773889
SMb20303	hypothetical protein	+	-	1	3,00151887
SMb20302	hypothetical protein	-	-	2	3,00151887
SMa1097	hypothetical protein	-	-	1	2,99571371
SMa1099	Putative CycB1 cytochrome c-552 precursor	+	-	1	2,99571371
SMb20724	hypothetical protein	+	COG3181S	2	2,99205881

SMc03037	FLAGELLIN A PROTEIN	+	COG1344N	2	2,98191083
SMc00456	hypothetical protein	-	-	6	2,961406
SMc00059	PUTATIVE SENSOR HISTIDINE KINASE PROTEIN	+	COG0642T	6	2,961406
SMa2219	probable decarboxylase	+	COG0163H	4	2,94670394
SMb20808	putative membrane-anchored protein	-	-	1	2,94320344
SMb20809	putative protein involved in assembly, operation or regulation of an export machinery for a cell surface saccharide	+	COG0794M,COG0517R	1	2,94320344
SMb21244	putative membrane protein, similar to putative polysaccharide transporter	-	COG2244R	3	2,91345601
SMb21245	putative OMA family outer membrane protein precursor, similar to ExoF	+	-	3	2,91345601
SMc04113	PUTATIVE PILUS ASSEMBLY TRANSMEMBRANE PROTEIN	+	COG4960OU	3	2,91111496
SMc04115	hypothetical protein	-	COG4964U	4	2,91111496
SMb20128	putative deaminase protein	-	COG0402FR	3	2,9074106
SMb20129	putative transcriptional regulator protein	+	COG1802K	3	2,9074106
SMc00584	hypothetical protein	-	-	3	2,90458254
SMc00585	leucyl aminopeptidase	+	-	3	2,90458254
SMc02278	HYPOTHETICAL UNKNOWN TRANSMEMBRANE PROTEIN	-	-	1	2,90108708
SMc02279	HYPOTHETICAL SIGNAL PEPTIDE PROTEIN	+	-	1	2,90108708
SMc01358	hypothetical protein	-	COG0679R	4	2,89719129
SMc01356	hypothetical protein	+	-	4	2,89719129
SMc01320	50S ribosomal protein L1	+	-	1	2,89380683
SMc03983	PROBABLE FRUCTOSE-BISPHOSPHATE ALDOLASE CLASS I PROTEIN	+	COG3588G	1	2,87422394
SMb21511	putative plasmid stability protein	+	COG1487R	2	2,86848851
SMc02369	PUTATIVE SENSOR HISTIDINE KINASE TRANSMEMBRANE PROTEIN	-	COG0642T	2	2,85852348
SMb21597	putative exported oxidoreductase protein	-	COG0673R	2	2,85606652
SMb21598	putative transcriptional regulator protein	+	COG1609K	2	2,85606652
SMc00191	hypothetical protein	-	-	4	2,84217825
SMc00190	hypothetical protein	+	-	4	2,84217825
SMb21513	putative cell-surface polysaccharide exporter protein, PST family	-	COG2244R	2	2,83692492
SMb21514	putative modification methylase protein	+	COG2890J	2	2,83692492
SMb20483	putative catabolite repressor protein	-	COG1609K	1	2,83070779
SMb20484	putative ABC transporter periplasmic sugar-binding protein	+	COG1879G	1	2,83070779
SMb21080	putative response regulator protein	+	COG2197TK	2	2,82523915
SMc02678	hypothetical protein	-	COG5465S	1	2,8203079

SMb21599	hypothetical protein	+	-	3	2,8162699
SMc01579	hypothetical protein	-	COG1192D	5	2,81248038
SMc00137	hypothetical protein	+	-	1	2,79646594
SMb20372	hypothetical protein	-	COG1593G	1	2,7704092
SMc00655	hypothetical protein	-	-	6	2,75956534
SMc00654	RESPONSE REGULATOR,CONTROLS CHROMOSOMAL REPLICATION INITIATION PROTEIN	+	COG0745TK	8	2,75956534
SMb21008	putative transcriptional regulator, arsR family protein	-	COG0640K	2	2,75527754
SMc03989	hypothetical protein	+	-	4	2,74134294
SMc02376	PUTATIVE HEAT SHOCK PROTEIN	-	COG0443O	6	2,73999143
SMc02377	PROBABLE ELECTRON TRANSFER FLAVOPROTEIN-UBIQUINONE OXIDOREDUCTASE	+	COG0644C	6	2,73999143
SMc03889	PUTATIVE TRANSPORT PROTEIN	-	-	1	2,7388663
SMb20048	putative transcriptional regulator protein	+	COG2188K	3	2,73048559
SMb20893	probable sugar uptake ABC transporter permease protein	-	COG4214G	2	2,7129156
SMb21334	hypothetical exported glutamine-rich protein	+	-	1	2,71216685
SMc00729	PUTATIVE ELECTRON TRANSFER FLAVOPROTEIN BETA-SUBUNIT BETA-ETF FLAVOPROTEIN SMALL SUBUNIT	+	COG2086C	2	2,70938165
SMa0473	hypothetical protein	+	COG3668R	1	2,70548082
SMc02729	hypothetical protein	-	COG5345S	1	2,6980267
SMc00651	hypothetical protein	+	-	3	2,68713613
SMc00652	hypothetical protein	-	-	4	2,68713613
SMb20403	putative oxidoreductase subunit protein	+	COG2080C	2	2,68136484
SMc00003	PUTATIVE CHAPERONE PROTEIN	+	COG2214O	2	2,67892396
SMc01794	PUTATIVE POLYSACCHARIDE EXPORT SYSTEM PERIPLASMIC TRANSMEMBRANE PROTEIN	-	-	6	2,67547566
SMc01795	PUTATIVE POLYSACCHARIDE SYNTHESIS/TRANSPORT TRANSMEMBRANE PROTEIN	+	COG0489D,COG3206M	6	2,67547566
SMa0063	putative GntR-family transcriptional regulator	+	COG2186K	4	2,67547126
SMc02823	PUTATIVE TRANSPOSASE PROTEIN	+	COG3385L	1	2,6674161
SMc00084	HYPOTHETICAL/UNKNOWN PROTEIN	-	-	1	2,66116459
SMc00026	hypothetical protein	+	-	1	2,66116459
SMa2000	Putative ABC transporter, periplasmic solute-binding protein	-	COG1879G	3	2,64433659

SMa2002	hypothetical protein	+	COG2755E	3	2,64433659
SMb20245	putative NDP-glucose dehydrataseepimerase protein	-	COG0451MG	1	2,6341749
SMb20246	putative oxidoreductase protein	+	COG1063ER	1	2,6341749
SMc02660	PUTATIVE TOXIN-ACTIVATING LYSINE-ACYLTRANSFERASE PROTEIN	+	COG2994O	1	2,63334309
SMc02447	hypothetical protein	-	-	2	2,63289764
SMc01014	hypothetical protein	-	COG2301G	4	2,63148948
SMc01015	hypothetical protein	+	-	4	2,63148948
SMb20560	hypothetical protein	-	COG2608P	1	2,62947474
SMc02556	hypothetical protein	-	-	3	2,62643811
SMc02848	hypothetical protein	-	COG3807S	2	2,62590809
SMc02849	2-hydroxyacid dehydrogenase	+	COG1052CHR	2	2,62590809
SMa1362	Putative inner-membrane permease	-	COG0395G	2	2,62343957
SMa1724	hypothetical protein	-	COG1335Q	1	2,62284354
SMa1725	putative AraC-family transcriptional regulator	+	COG4977K	1	2,62284354
SMa0774	NoeB host specific nodulation protein	+	-	1	2,59956795
SMc04018	PROBABLE 5'-NUCLEOTIDASE PRECURSOR (SIGNAL PEPTIDE) PROTEIN	+	COG1652S,COG0737F	2	2,59943951
SMb21523	putative cell division inhibitor protein	-	COG2894D	1	2,59736474
SMc01148	hypothetical protein	+	COG2764S,COG4319S	1	2,59398618
SMc00548	CONSERVED HYPOTHETICAL SIGNAL PEPTIDE PROTEIN	+	-	1	2,59377766
SMc01237	ribonucleotide-diphosphate reductase subunit alpha	-	COG0209F	7	2,58694456
SMc00731	hypothetical protein	+	COG2096S	1	2,58518298
SMb21506	putative auxiliar protein involved in export of cell surface polysaccharides, MPA1 family without cytosolic domain, slightly exoP-like	-	COG3206M	2	2,58243519
SMb21507	putative amino acid transporter, exporter protein	+	COG1280E	2	2,58243519
SMc00336	hypothetical protein	-	-	2	2,5797238
SMc00337	HYPOTHETICAL/UNKNOWN PROTEIN	+	-	2	2,5797238
SMc00932	DNA mismatch repair protein	-	COG0323L	3	2,57213917
SMc00888	PUTATIVE CONTAINS A 2-COMPONENT RECEIVER DOMAIN PROTEIN	+	-	3	2,57213917
SMc02094	PUTATIVE OUTER MEMBRANE TRANSMEMBRANE PROTEIN	+	COG4775M	3	2,57119489
SMa0252	hypothetical protein	+	COG1638G	2	2,55600132
SMb20596	hypothetical protein	-	-	2	2,55213013

SMb20597	hypothetical protein	+	COG2259S	2	2,55213013
SMc03039	PROBABLE FLAGELLIN D PROTEIN	+	COG1344N	4	2,54774297
SMb20856	putative sugar uptake ABC transporter periplasmic solute-binding protein precursor	-	COG1879G	2	2,53779988
SMb20857	glucose-6-phosphate isomerase	+	COG2140GR	2	2,53779988
SMb20881	hypothetical protein	-	COG3019R	1	2,53768696
SMc03832	CONSERVED HYPOTHETICAL SIGNAL PEPTIDE PROTEIN	-	-	2	2,53031109
SMc01029	hypothetical protein	+	COG2919D	2	2,53003428
SMc03942	hypothetical protein	-	-	1	2,52836503
SMc03943	hypothetical protein	+	COG2071R	1	2,52836503
SMa1746	putative iron uptake protein	-	COG0614P	2	2,52688964
SMa1747	putative ferrichrome-iron receptor	+	COG1629P	2	2,52688964
SMc02150	hypothetical protein	-	COG2070R	2	2,51913877
SMc02149	hypothetical protein	+	-	2	2,51913877
SMc00989	HYPOTHETICAL SIGNAL PEPTIDE PROTEIN	+	-	2	2,51832493
SMc01494	PUTATIVE PHOSPHOSERINE PHOSPHATASE PROTEIN	+	COG0560E	1	2,51829655
SMc02203	hypothetical protein	+	-	1	2,50871262
SMb20905	putative transposase protein	-	COG2801L	2	2,50581768
SMb20906	hypothetical protein	+	-	2	2,50581768
SMa2367	putative ABC transporter, permease	+	COG4603R	2	2,5028666
SMb20907	hypothetical protein	+	-	3	2,50249839

* COG categorists refer to Tatusov *et al.* (2000); ** Number of CtrA binding sites; ***See Materials and Methods (E.2.19).

CHAPTER II

Horizontal gene transfer

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A. INTRODUCTION

A.1. HORIZONTAL GENE TRANSFER (HGT)

Horizontal gene transfer (HGT) represents an important source of genetic variability in bacteria. The first evidence that HGT occurred was the recognition that virulence determinants could be transferred between pneumococci in infected mice, a phenomenon that was later shown to be mediated by the uptake of the genetic material DNA (Griffith, 1928). In laboratory, horizontal transfer of genetic material between different bacteria has been detected in a wide variety of several bacterial species and genera. There are also data demonstrating HGT between phylogenetically very distant organisms such as Bacteria and Eucaria. In fact, the transfer of part of the Ti plasmid DNA from *Agrobacterium tumifaciens* to plants (reviewed in Zupan and Zambryski, 1995) and to yeast (Bundock and Hooykaas, 1996), demonstrated the horizontal transfer of genes between different phylogenetic kingdoms.

Each HGT event takes place in, at least, three consecutive fundamental steps:

- release of DNA from an organism called Donor.
- intracellular acquisition of DNA from the receiving organism, called Recipient.
- integration of the exogenous DNA in the genome of the Recipient.

Three different mechanisms of HGT have been identified in bacteria: conjugation, transformation and transduction. These DNA transfer methods enhanced our understanding of bacterial molecular genetics and provided elegant tools for the development of genetic engineering technology.

A.1.1. Conjugation

Conjugation is a cell contact-dependent DNA transfer mechanism found in most bacterial genera. Conjugative transfer is mediated by cell-to-cell junctions through which DNA can pass (Figure 1). Conjugative transfer systems are frequently associated with plasmids, probably because, to evolve, they would normally need the genetic element of which they are a part to be completely transferred to the recipient before the genes for the conjugative system can be reconstituted in an active form (Thomas and Nielsen, 1995). A plasmid is an autonomously replicating genetic element that, if it enters in a host cells as complete double-stranded, can remove the need for a foreign gene to integrate into the recipient

chromosome to become established. Conjugation may be of several types: a) Transfer of a self-transmissible conjugative plasmid. The classic examples are the F-plasmid and plasmid RP4 of *E. coli.* b) Mobilization, whereby a non-self-transmissible plasmid, which nonetheless contains an origin of conjugal transfer (*oriT*), can be transferred by the action of a conjugative plasmid. An example is the mobilization of the IncQ plasmid RSF1010 by conjugative IncP1 plasmids (Derbyshire *et al.*, 1987). (c) Cointegration, whereby two different circular plasmids fuse to become one. Thus, a non self-transmissible non-mobilizable plasmid may nonetheless be sexually transferred due to the action of its cointegrated self-transmissible partner. Conjugation may also be effected by conjugative transposons (Clewell *et al.*, 1995). Many plasmids and conjugative transposons are of very wide host range. For example, the non-conjugative, mobilizable IncQ plasmids (e.g., RSF1010) have an extremely broad host spectrum and RP4, and conjugative transposons, such as Tn916, are also of very wide host range (Clewell *et al.,*1995; Salyers and Shoemaker, 1996).
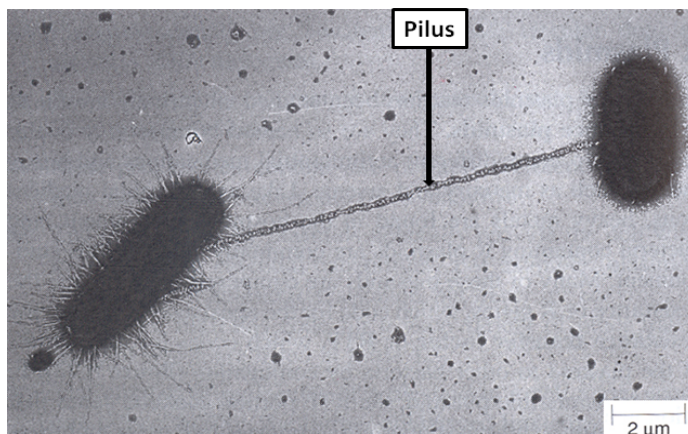
Figure 1. Bacterial conjugation. The donor cell is on the left side and the recipient cell is on the right side. It is evident the conjugative pilus connecting donor with recipient.

A.1.2. Transformation

Transformation is the process involving the uptake and incorporation of naked DNA (Figure 2). Many species of bacteria are naturally transformable as reported by Lorenz and Wackernagel (1994). Several species (e.g., *Streptococcus pneumoniae*) become competent in the natural course of their life cycle (Lunsford, 1998). Others (e.g., *Neisseria gonorrhoeae*) are always in a competent state (Lorenz and Wackernagel, 1994). Some species (e.g., *E. coli*) can be induced to take up DNA by a number of chemical or physical processes including

treatment with CaCl2, EDTA, temperature shifts, electro-shocks, and protoplast formation. Despite its sensitivity to nucleases, DNA is relatively common in almost all environments and may be excreted by living bacteria or be liberated during autolysis (Lorenz and Wackernagel, 1994). Environmental DNA can be stabilized by adsorption to sand and clay particles, thereby becoming 100- to 1000-fold more resistant to DNase. Such adsorbed DNA may retain its transforming ability for weeks or even months (Romanowski *et al.,* 1993; Khanna and Stotzky, 1992; Chamier *et al.,* 1993; Lorenz and Wackernagel, 1994). The potential dilution of DNA in aqueous environments may seem a barrier to interactions with recipient cells. However, Baur *et al.* (1996) have pointed out that many genetic interactions may take place in a biofilm, rather than between pelagic bacteria. Transformation has been demonstrated in different bacteria in a variety of natural ecosystems. Stewart and Sinigalliano (1991) demonstrated transformation of *Pseudomonas stutzeri* in sterile or non sterile marine sediments. Williams *et al.* (1996) demonstrated that transformation could take place in *Acinetobacter calcoaceticus* growing in biofilms attached to river stones and incubated in natural rivers. Sikorski *et al.,* 1988 demonstrated transformation of *P. stutzeri* in soil microcosm.

Figure 2. Bacterial transformation. 1) DNA donor fragment bind to protein on cell surface. 2) Donor DNA fragment is transported into host cell as single strand. 3) Single stranded donor DNA is integrated in the genome of the host. 4) Donor DNA is degraded and not integrated in the genome.
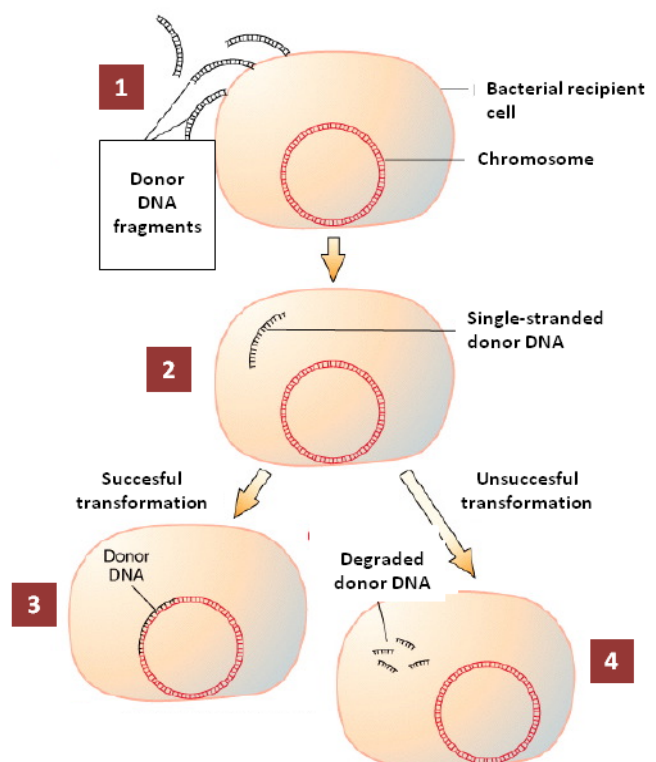
A.1.3. Transduction

In the process of transduction, bacterial genes are incorporated by bacteriophage particles and transferred to another bacterium. Transduction may be either "generalized" (e.g., by coli-phage P1), whereby any bacterial gene may be transferred, or "specialized" (e.g., by coli-phage lambda), where only genes located near the site of prophage integration are transferred. Bacteriophages have a restricted host range, sometimes being limited to a single bacterial species or even a strain. Furthermore, bacteria may mutate to become resistant (incapable of phage adsorption). For these reasons, transduction would seem an un-unlikely candidate for gene transfer in the environment. However, phages are very common in the environment (Jiang and Paul, 1998) and are relatively stable, being protected by a protein coat. Phages are also more compact and thus more diffusible than naked DNA. Finally, temperate phages may continue to coexist with the bacteria in the form of lysogens and be liberated in some distant future, in response to environmental factors.

A.1.4. HGT and speciation

The process of speciation can start only when genetic exchange is interrupted between two populations (Matic *et al.*, 1996). Thus, the study of genetic isolation between closely related bacterial species might indicate what triggered the process of speciation. One theory considers isolation to be an accidental by-product of genetic divergence. Another regards isolation as a product of natural selection. The two theories for the origin of genetic isolation are not mutually exclusive. The degree of genetic isolation in nature between bacterial species may depend on several factors: differences in their microhabitats, the host ranges of genetic exchange vectors, and restriction-modification systems, as well as DNA sequence divergences and functional incompatibilities. It is not easy to identify functions involved in the initial phases of speciation because other functions contributing to genetic isolation may become fixed after speciation is complete (Matic *et al.*, 1996). Barriers, such as different microhabitats and the host ranges of genetic exchange vectors, seem to slow down DNA transmission rather than to block it. The large overlap in host range, among conjugal plasmids, allows sequential DNA transmission among almost all bacterial species (Amabile-Cuevas and Chicurel, 1992). Transformation and transduction may provide routes for gene transfer between conjugationally incompatible groups. The promiscuous transfer of DNA among virtually all eubacteria, yeasts and even plant cells suggests that there is no strong

selection against it (Heinemann, 1991). The major limitation for interspecies gene transfer seems to consist of barriers that block the establishment of the acquired genetic information.

A.2. BARRIERS TO HGT

Horizontal gene transfer is an important mechanism of bacterial genome evolution (Gogarten *et al.*, 2002). Within this context, barriers limiting gene transfer induce bacterial genetic isolation (Matic *et al.*, 1996).

Depending on the bacterial species involved and the gene-transfer mechanisms that are active, a number of processes limit transfer, uptake and stabilization of foreign DNA molecules in bacteria. Whereas in transformation, it is the recipient that has the more active role that promotes HGT, in conjugative transfer it is the donor that seems to have the positive role and, by contrast, the recipient often has a negative role, limiting entry or establishment of the incoming DNA. Factors that can reduce horizontal gene transfer between bacteria, besides geographical separation, are surface exclusion barriers, cytoplasmic barriers as endonuclease restriction, DNA divergence and homologous recombination (Matic *et al.*, 1996; Berndt *et al.*, 2003; Thomas & Nielsen, 2005). More space will be spent to explain the biology of restriction-modification systems since they are crucial in this work.

A.2.1. Surface exclusion as a barrier

Surface exclusion creates an effective barrier against conjugative transfer into bacterial cells that already carry the genes for a closely related transfer apparatus (Frost *et al.*, 1984). Surface exclusion is widespread (but not universal) in conjugation systems of both Gram-negative (Haase *et al.*, 1996; Hochhut *et al.*, 2001; Pohlman *et al.*, 1994) and Gram-positive elements (Possoz *et al.*, 2003). It might be that surface exclusion has not evolved as a barrier to promote recombinational isolation of plasmids, but is more related to promoting the breakdown of mating pairs after gene transfer has taken place and release of the recipient to disperse the plasmid to new potential recipients (Thomas and Nielsen, 2005).

A.2.2. Barriers to plasmid replication and establishment in a heterologous host.

A key property that allows a plasmid to promote horizontal transfer of genes that have no orthologue in the recipient genome is its ability to replicate and therefore avoid the need for recombination into a replicon like the chromosome. Some plasmids have a broad host range, whereas others are more limited (Thomas and Nielsen, 2005). The broad host range of IncQ plasmids like RSF1010 could be due to the plasmid encoding three replication proteins — an origin activation protein, RepA; a HELICASE, RepB; and a primase, RepC (Scherzinger *et al.*, 1991). Moreover its segregational stability is largely due to its copy number (Becker and Meyer, 1997). The absence of lagging strand synthesis, and therefore accumulation of single-stranded DNA-replication intermediates, puts constraints on the amount of DNA that these plasmids can acquire and therefore transfer horizontally without becoming recombinationally unstable. An IncG (IncP-6) plasmid, called Rms149, the DNA sequence of which was determined (Haines *et al.*, 2005), contains the IncQ-family *mob* genes but has acquired a different, more typical low-copy-number θ replicon with linked active partitioning genes, explaining its ability to have expanded to 57 kb through insertion of multiple transposable elements. Studies on plasmids with narrower host ranges have revealed various limitations to successful replication. For plasmid F of *E. coli*, the barrier to replication in *Pseudomonas* species seems to be due to the inability of its replication protein, RepE, to effectively recruit DnaB to complete the activation of the replication origin after the initial RepE–DnaA–*ori* complex has been formed (Zhong *et al.*, 2005). For pPS10, originally from *Pseudomonas syringae*, the reason for the plasmid's temperature-sensitive replication in *E. coli* is the lack of productive interactions between the plasmid-encoded Rep and DnaA, as indicated by mutations that allow replication at 37°C in *E. coli* mapping in either *rep* or *dnaA* (Maestro *et al.*, 2002; Maestro *et al.*, 2003).

A.2.3. DNA divergence and homologous recombination

To become replicable and stably inherited, chromosomal DNA transferred by HGT must become integrated into the recipient chromosome. The efficiency of integration by homologous recombination depends on the genomic sequence divergence between species. The increase in DNA sequence divergence has been shown to reduce the rate of transformation between *Bacillus* species (Zawadzki *et al.*, 1995), and also to reduce the efficiency of recombination in *E. coli* (Shen and Huang, 1986). Recombination enzymes

appear to be highly selective for sequence identity only at the initial stage of the strand-exchange process, which requires a minimal length of sequence identity below which recombination becomes inefficient (Shen and Huang, 1986). However, once initiated, strand exchange occurs despite large numbers of mismatches and even large insertions (DasGupta and Radding 1982; Bianchi and Radding, 1983). During this stage of recombination, fidelity is controlled by the mismatch-repair system. The mismatch repair system is a potent inhibitor of recombination between genomes of related bacterial species. The frequency of conjugational and transductional genetic exchange between *E. coli* and *S. typhimurium* is enhanced up to a thousand-fold when the MutHLSU mismatch-repair system is inactivated in recipients (Matic *et al.*, 1995; Rayssiguier *et al.*, 1989). Also in *P. stutzeri* up-regulation of MutS can enforce sexual isolation and down-regulation can increase foreign DNA acquisition (Meier and Wackernagel, 2005).

A.2.4. Restriction as a barrier

Bacterial restriction-modification (RM) function as systems that attack foreign DNA entering the cell. DNA that is recognized as foreign because it does not have the same sequence specific chemical signatures can be broken into pieces by restriction endonucleases (Jeltsh, 2003). The fact that DNA entering through conjugative transfer or natural transformation is single-stranded instead of double-stranded might provide some protection, and indeed, comparison with transformation frequencies of double-stranded DNA does confirm a greater ability to avoid destruction through the former mechanism (Lacks and Springhorn, 1984). Nevertheless, it is well established that the frequency of transconjugants can be reduced if the recipient has a restriction system to which the incoming plasmid is susceptible (Moser *et al.*, 1993; Pinedo and Smets, 2005). Plasmids carrying genes encoding products that are known to interfere with the action of type I restriction systems can enter restriction-positive hosts more efficiently than plasmids without such anti-restriction systems (Belogurov *et al.*, 1992; Belogurov *et al.*, 1993). Many plasmids carry restriction-modification systems (Kobayashi, 2001) and this can potentially affect transfer to new hosts. If the host DNA is not modified, it should therefore be susceptible to degradation by the newly introduced restriction endonuclease. In practice, expression of restriction-modification systems in a virgin host proceeds in such a way that the modification component of the system is active first (Nakayama and Kobayashi, 1998).

A.2.5. Restriction modification systems

Typically, RM systems have enzymes responsible for two opposing activities: a restriction endonuclease (REase) that recognizes a specific DNA sequence for cleavage and a cognate methyltransferases (MTase) that confers protection from cleavage by methylation of adenine or cytosine bases within the same recognition sequence (Figure 3). REases recognize 'non-self' DNA, such as that of phage and plasmids, by its lack of characteristic modification within specific recognition sites (Pingoud and Jeltsch, 2000). Foreign DNA is then inactivated by endonucleolytic cleavage. Generally, methylation of a specific cytosine or adenine within the recognition sequence confers protection from restriction. Host DNA is normally methylated by the MTase following replication, whereas invading non-self DNA is not. R-M systems are classified into four major groups according to their subunit composition, recognition site, cofactor requirement and cleavage position (Tock and Dryden. 2005).
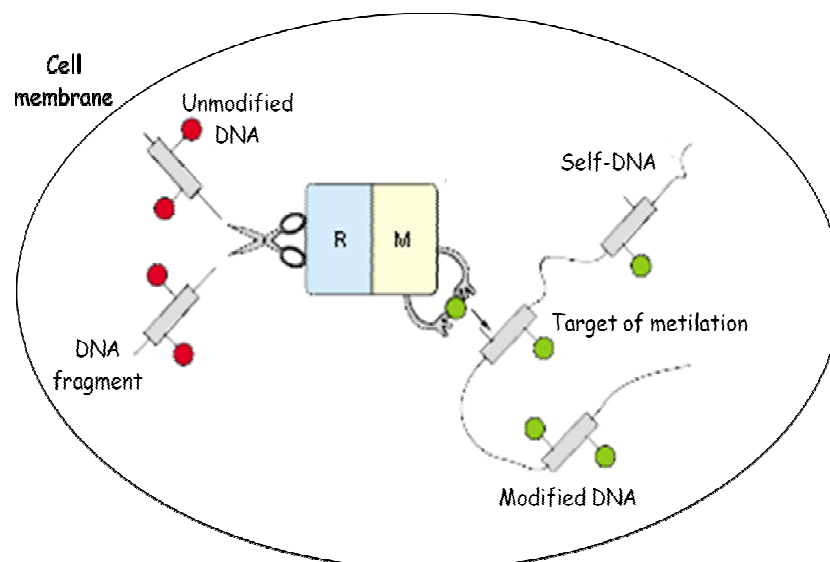
Figure 3. The function of R-M system. These enzymes recognize the methylation state of their specific target sequence. Fully methylated DNA (shown as two green circles on the target sequence on the host DNA) is recognized to be part of the bacterial genome. Hemimethylated DNA (a single green circle on host DNA target sequence) is recognized as newly replicated bacterial DNA, and the MTase (M) modifies the other strand by methylation. Invading DNA, for example a phage genome, generally lacks specific modification (red circles on the target sequence of phage DNA) and is recognized to be foreign by the REase (R) and cleaved into harmless fragments (from Tock and Dryen 2005).

Type I restriction-modification enzymes

Type I RM enzymes are hetero-oligomeric complexes that typically contain two REase subunits that are required for DNA cleavage, one Specificity subunit (S) that specifies the DNA sequence recognized, and two MTase subunits that catalyze the methylation reaction (Dryden *et al.*, 2001; Murray 2000). Depending upon the methylation status of DNA, this complex can function as either an REase or an MTase. Unmethylated DNA is targeted for restriction, hemi-methylated molecules are targeted for further methylation, and fully methylated DNA is immune to restriction (Vovis et al., 1974).

Type II restriction-modification enzymes

Most, but not all, type II R-M systems contain separate REase and MTase enzymes. The REase and MTase recognize the same DNA sequence, which is typically a 4–8 base pair (bp) palindrome. All Type II REases cleave within or adjacent to this specific DNA sequence to generate a defined restriction pattern of products (Pingoud and Jeltsch, 2000). Some Type II REases are active as homodimers, with each monomer cutting one strand in a coordinated fashion to generate double-strand breaks. Other Type II REases are able to act as monomers or tetramers, and there is evidence that many Type II REases must bind to two or more copies of their recognition site before the DNA is cleaved (Kruger *et al.*, 1988; Halford and Marko, 2004). Type II MTases generally act as monomers to modify a specific base of their recognition sequence on each strand of the duplex (Sistla and Rao, 2004).

Type III restriction-modification enzymes

Type III RM enzymes are less complex but share many similarities with Type I R-M enzymes (Dryden *et al.*, 2001). They are hetero-oligomers that consist of a modification (mod) subunit, which is required for substrate recognition and modification, and a restriction subunit (res), which is only active when associated in a res_2mod_2 complex (Janscak *et al.*, 2001). For cleavage to occur, a Type III RM enzyme must interact with two inversely oriented copies of its 5–6 bp asymmetric recognition sequence (Meisel *et al.*, 1992). As with Type I R-M enzymes, cleavage is preceded by DNA translocation during which two res_2mod_2 complexes maintain contact with their recognition sequence (Dryden *et al.*, 2001; Reich *et al.*, 2004). Stalled DNA translocation and/or collision of res_2mod_2 complexes initiates cleavage by each monomer at a point that is 25–27 bases from the recognition site; one DNA strand is cut by each complex. In all known cases, Type III mod subunits are able to act

independently of their cognate res subunits. Type III R-M systems have been identified almost exclusively in phage and in Gram-negative bacteria.

Type IV restriction-modification enzymes

Type IV R-M enzymes are REases that will only cleave DNA substrates that have been modified, for example bases that have been methylated, hydroxymethylated and glucosyl-hydroxymethylated. This enzyme detects two copies of a dinucleotide sequence, consisting of a purine followed by a cytosine methylated at either the N^4 or the C^5 position, which are separated by between 40 and 3000 nucleotides, and preferentially cuts 30 base pairs away from one of the sites (Sutherland *et al.*, 1992).

A.2.6. Evolutionary implications of RM systems

Traditionally, RM systems are regarded as defense mechanism against bacteriophage infection. The efficiency of RM systems against phages is also documented by the occurrence of various anti-restriction defense mechanisms in phages, like incorporation of modified nucleotides, phage encoded multi-specific methyltransferases, and the reduction of the number of sites for RM systems in the DNA of many phages (Bickle and Kruger, 1993). In addition, conjugating plasmids can encode anti-restriction proteins (Velkov, 1999).

On the other hand, RM systems never provide a full protection of a bacterial culture against bacteriophage infection (Arber and Dussoix, 1962). Population genetic experiments suggested that protection of bacteria is only a transient phenomenon and RM systems provide a significant selective value only under certain environmental conditions (Korona et al., 1993). Thus, although RM systems undoubtedly protect bacteria against bacteriophage infection to a certain degree, it remains questionable if this function can explain the enormous spreading of these biological systems in the prokaryotic world (Jeltsh, 2003). As reported above bacteria frequently exchange DNA among each other as well with organisms of different species, but maintaining the genetic uniqueness of species including the adaptations of each species to its special ecological niche requires genetic isolation to a certain degree. Furthermore, genetic isolation is a prerequisite to the evolution of new species, because only under conditions of genetic isolation a subpopulation is able to develop new biological properties. In bacteria, one way to achieve a genetic isolation is to control the uptake of DNA from the environment (Tortosa and Dubnau, 1999). As an alternative, the intracellular fate of DNA taken up can be regulated, which is the function of

RM systems. Moreover, bacteria evolved many different RM systems with different DNA recognition specificities. Thus, a function of RM systems in the maintenance of species identity explains why so many different RM systems are found in single bacterial species (Jeltsh, 2003). Such division of one species into different biotypes expressing mutually exclusive RM systems is an ideal starting point for a rapid adaptation to different ecological niches. It is interesting to note that under unfavorable environmental conditions, bacteria can shut down their RM systems (Velkov, 1999), which stimulates the uptake and integration of DNA.

A.3. *SINORHIZOBIUM MELILOTI* AND *SINORHIZOBIUM MEDICAE*

Sinorhizobium meliloti and *S. medicae* are two phylogenetically closely-related species (Rome *et al.*, 1996; Roumiantseva *et al.*, 1999; Young *et al.*, 2001) belonging to the family of *Rhizobiaceae* (alpha-proteobacteria) which live either free in soil or in symbiosis with leguminous plants, forming root nodules where they perform nitrogen fixation.

A.3.1. Nitrogen fixation

Nitrogen (N_2) is one of the most abundant elements on Earth and together with hydrogen, oxygen and carbon, one of the most important components of macromolecules fundamental for the living cells. All of the nutritional nitrogen is obtained by humans and animals directly or indirectly from plants. Plants, in turn, acquire nitrogen from two principal sources: the soil, through commercial fertilizer and/or mineralization of organic material, and the atmosphere, through nitrogen fixation by bacteria (Vance, 2001). The process of biological nitrogen fixation is limited to prokaryotes. The prokaryotes that can fix nitrogen due to an evolutionarily conserved nitrogenase protein complex is diverse and contains both eubacteria and archaea (Zehr *et al.*, 2003). Limiting factors in maintaining a high rate of nitrogen fixation are the large quantity of energy needed to break the N–N triple bond, and the high sensitivity of nitrogenase to oxygen. Different mechanisms have evolved to overcome this second limitation. For the anaerobic microorganisms (*Clostridium*) that predominate in waterlogged soils, where organic substrates are available but oxygen supply to the micro-environment of the bacteria is severely restricted, this is not problematic (Chen and Johnson, 1993). In cyanobacteria, nitrogen fixation occurs in special cells known as heterocysts which do not photosynthesize but are devoted solely to N_2 fixation (Golden and

Yoon, 2003). The obligate aerobe *Azotobacter* protects the nitrogenase in two ways: producing special auxiliary proteins which cause nitrogenase to aggregate when exposed to oxygen and by very high respiratory rate that creates a nearly anoxygenic environment in the cytoplasm of the cells (Poole and Hill, 1997). The most efficient way of nitrogen fixation, however, is the symbiosis between bacteria and plants, where the plant supplies the carbon source for the reduction of nitrogen and at the same time, creates micro-anaerobic environment to protect the nitrogenase. Bacteria, in turn, provide the plant with the nitrogen in form of ammonia. This process is mostly restricted to a limited number of bacterial groups, including the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* (*Ensifer*), and *Bradyrhizobium* (collectively referred to as rhizobia), and *Frankia*. All these genera except *Frankia* belong to the *Rhizobiacae* family in the alpha-proteobacteria, and establish symbiosis with plants from the family of *Fabacae* (the leguminous plants). *Frankia* is an actinomycete that enters symbiosis with plants from the families *Rosaceae*, *Casuarinaceae*, *Betulaceae*, *Myricaceae*, *Rhamnaceae*, *Datisticaceae*, *Eleganaceae* and *Coriariaceae* (Gage, 2004). *Sinorhizobium meliloti* and *S. medicae* are organism, from the genus *Sinorhizobium*, able to establish nitrogen fixing symbiosis with *Fabacae* plants of genera *Medicago*, *Melilotus* and *Trigonella* (Figure 4).
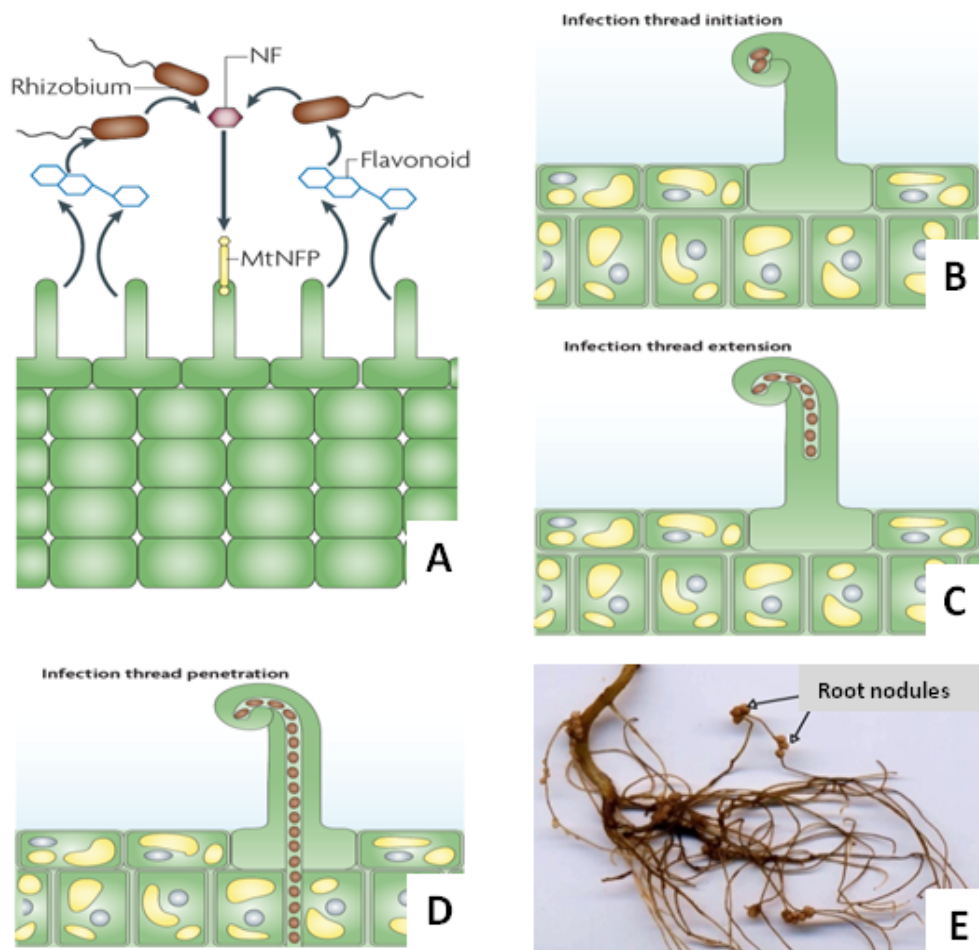
Figure 4. Schematic representation of the interaction between *Sinorhizobium* and the host plant. (A) Recognition: flavonoids produced by root cells are released in the soil and induce the production of nodulation factor (NF) by bacterial cells. NF interact with specific receptors present on the plant root cells. (B) Bacterial cells enter in the root hair and initiate the infection process. (C) Bacterial cells grow and divide themselves extending the infection thread. (D) The infection thread penetrates in the root where plant cells can pick up within themselves the bacterial cells. Within plant cells bacteria can differentiate in bacteroids initiating the nitrogen fixation process. (E) Root nodules (Jones *et al.*, 2007a).

A.3.2. *S. meliloti* and *S. medicae* as a model

Among the various symbiotic bacteria that are used to study the interaction between *Fabaceae* and Rhizobia, *Sinorhizobium* sp. interacting with *Medicago* sp. are particularly interesting. *Sinorhizobium meliloti* and *S. medicae* are, in fact, the sole symbionts of *Medicago* species with the exception of *Medicago ruthenica* (de Lajudie *et al.*, 1994; Rome *et al.*, 1996; van Berkum *et al.*, 1998). *Medicago* species can be clustered in four groups depending on their symbiotic partners: a) species that establish efficient symbiosis with both *S. meliloti* and *S. medicae*, such as *M. sativa* alfalfa, or *M. truncatula*; b) species that interact solely with *S. medicae*, such as *M. polymorpha*; c) species that interact with specific *S. meliloti* biovars, such as *M. laciniata* with *S. meliloti* bv. m*edicaginis* (Villegas *et al.*, 2006) or *M. rigiduloides* (Materon 1991); d) *M. ruthenica*, which is specifically associated to a biovar of *Rhizobium gallicum* (Silva *et al.*, 2005). Considering phylogeny, *S. meliloti* and *S. medicae* are closely related (Figure 5) and their genome show the same tripartite architecture (one chromosome, and two megaplasmids) (Roumiantseva *et al.*, 1999) and a relative DNA homology value of 60% (Rome *et al.*, 1996). The complete genome sequence of both species is available (Galibert *et al.*, 2001, http://genome.jgi-psf.org/finished_microbes/sinme/sinme.home.html). Their genome comprises a chromosome and two megaplasmids called pSymA and pSymB. The sizes of these different replication units are respectively 3.65 Mb, 1.35 Mb and 1.68 Mb in *S. meliloti* strain 1021 and 3,78 Mb, 1,57 Mb and 1,24 Mb in *S. medicae* strain WSM419, that has a one more smaller plasmid of 0,22 Mb. Furthermore, these replication units display distinct functional features. Indeed, whereas housekeeping genes are located on the chromosome, genes involved in secondary metabolic pathways are located on both megaplasmids, pSymA harbouring also nearly all symbiotic genes. Thus, the two nitrogen fixing bacteria *S. meliloti* and *S. medicae* appear to be an ideal biological model for such studies, including studies about HGT in sympatry.

A.3.3. HGT between *S. meliloti* and *S. medicae*

Host selective pressures and lateral gene transfers are key mechanisms that shape the genetic population structure of symbiotic microorganisms (Tibayrenc 1996). Several studies focused on the genetic structure of natural population have demonstrated that the genetic structure of *Sinorhizobium* is strongly influenced by HGT (Maynard Smith *et al.* 1993; Bailly *et*

al., 2006; Bailly *et al.*, 2007; Sun *et al.*, 2006; Van Berkum *et al.*, 2006). Studies focused on genetic structure of both *S. meliloti* and *S. medicae*, show that, considering HGT, *Sinorhizobium* strains are separated on the bases of the species (Bailly *et al.*, 2007; Bailly *et al.*, 2006; Biondi *et al.*, 2003). On the other hand, within each species, there are evidence of intra-specific gene flow but strains are clustered in separated subgroups, not related by gene flow (Bailly *et al.*, 2007; Bailly *et al.*, 2006; Biondi *et al.*, 2003). This observations reveal a complicate scenario with an elaborate web of gene exchange linkages between *Sinorhizobium* strains consisting with the presence of barrier to HGT.
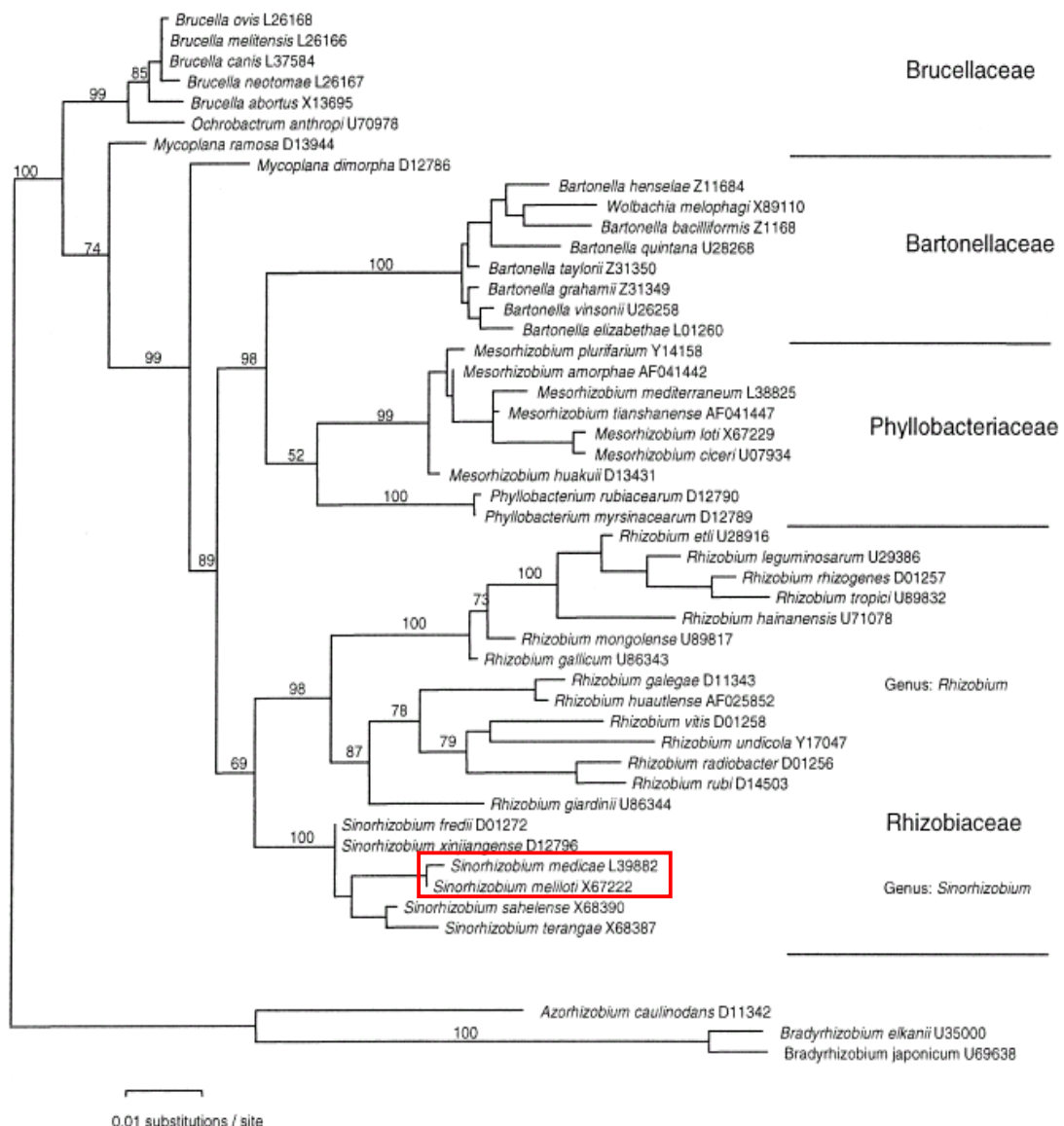

Figure 5. Phylogenetic tree of 16S rDNA of *Rhizobiaceae* and their relatives from Young *et al.* (2001). *Sinorhizobium meliloti* and *Sinorhizobium medicae*, indicated by the red line, are clearly closely related.

A.4.   HGT BETWEEN *S.MELILOTI* AND *S. MEDICAE* IN VITRO

Few and fragmentary information are available about in vitro experiments to investigate gene exchange between *Sinorhizobium* strains. Transduction is reported and described in lab conditions only for *S. meliloti* (Finan *et al.*, 1984) and the published isolated phages are strain specific for the *S. meliloti* stain SU47 and for all the strain derived from SU47 (Finan *et al.*, 1984), like the sequenced strain Rm1021 (Meade *et al.*, 1982). For those reasons transduction is not applicable to study gene flow between different *S. meliloti* and *S. medicae* strains, until it will be demonstrated the existence of phages able to infect strains of both species. Conjugation is the commonly used technique to transfer plasmid DNA into *Sinorhizobium* cells. It is performed in two way, biparental mating and triparental mating. In the first case the donor is an engineered *E. coli* strain, called S17-1 (Simon *et al.*, 1983), that carries itself, within its genome, the genes coding for conjugative functions. In the case of triparental mating there are two *E. coli* strains: the donor that is a non conjugative *E. coli* strain and the helper that has a plasmid carrying genes for conjugative functions, like pRK2013 (Figurski, D. & Helinski, 1879). Concerning conjugation between *Sinorhizobium* cells only one paper reports the mating between two *S. meliloti* strains (Jones *et al.*, 2007b) and no data are reported about *S. medicae* or inter-specific mating between them. In the paper of Jones *et al.* (2007b) the authors find a very low rate, approaching zero, of efficiency of conjugation between *S. meliloti* strain Rm1021 as donor and a natural *S. meliloti* strain as recipient. Thus, except for conjugation with engineered *E. coli* strains as donor, this mechanism is inefficient in lab conditions to study HGT between *S. meliloti* strains.

Concerning transformation, *Sinorhizobium* cells can be naturally competent (Balassa 1963; Courtois *et al.*, 1988), chemically competent (Kiss and Kalman, 1982) and competent for physical-transformation(Gage *et al.*, 1996; Hayashi *et al.*, 2000; Vincze & Bowra, 2006).

Development of natural competence described by Courtois *et al.* (1988), allows transformation of *S. meliloti* cells with an efficiency of about 10^{-4} or 10^{-5} cells per microgram of DNA if the transforming DNA is chromosomal or a self replicable plasmid respectively, but transformation requires quantity of donor DNA extremely great (6 microgram for sample). *S. meliloti* chemically competent cells can be also produced as described by Kiss and Kalman (1982). By this way the efficiency of transformation of strain 41 is reported to be greater than 10^{3} cells per microgram of DNA, if the plasmid is extracted from the same strain *S. meliloti* 41, but it decrease strongly to lower than 3×10^{1} cells per microgram of DNA if

transformed with DNA from *E. coli.* Physical methods described to transform *S. meliloti* are essentially two: the so called freeze-thaw method and electroporation that will be described separately. Freeze-thaw method was described by Vincze and Bowra (2006) and permits transformation of opportunely treated cells by thawing frozen samples with a "soft" heat shock at 37°C after addition of plasmid DNA. The efficiency reported for this method is 6 x 10^1 for the *S. meliloti* sequenced strain Rm1021, and 1,1 x 10^4 for *S. meliloti* 2011.

A.4.1. Electroporation

Electroporation is a transformation technique based on the application of high voltage electric field that opens transient pores in the plasma membrane of cells. Electro-pores permit the entrance of several kinds of molecules, including DNA, inside the cytoplasm (Dower *et al.*, 1988). Main advantages of electroporation are the extremely easy preparation of electro-competent cells, the high frequency of transformation and the high reproducibility. Main disadvantages are the high degree of salt purity required for transforming DNA suspension and, of course, the availability of the electroporator machine.

Electroporation of rhizobia is well described, with high efficiency, for *Rhizobium leguminosarum* (Garg *et al.*, 1999), *Bradyrhizobium japonicum* (Guerinot *et al.*, 1990; Hatterman and Stacey, 1990) and *Mesorhizobium huakuii* (Hayashi *et al.*, 2000).

Concerning *S. medicae*, electroporation, like transformation in general, have never been tested as far as we know, while *S. meliloti* can be electroporated (Gage *et al.*, 1996; Hayashi *et al.*, 2000), but no data are available about its optimization. A mutation that increased electroporation efficiency was previously reported for strain Rm1021, but the characterization of the specific mutation is still missing (Gage *et al.*, 1996).

In the study of barriers to foreign DNA acquisition from bacterial cells, electroporation overcomes the barriers to surface exclusion, allowing to focus the investigation on the intracellular barriers. Moreover if the DNA used is a self-replicating plasmid, this avoids also barriers against integration of exogenous DNA. Therefore, electroporation represents a convenient technique to test the specificity and selectivity of cytoplasmic barriers limiting gene transfer. Moreover, although electroporation is an artificial technique, its role in horizontal gene transfer in soil is under discussion, since it is possible to obtain bacterial electro-transformation in soil samples by lightning (Demanèche *et al.*, 2001; Cérémonie *et al.*, 2004).

B. AIM OF THE WORK

Sinorhizobium meliloti and *S. medicae* are two important nitrogen fixing bacteria sharing particular features that can constitute a model to study barriers to horizontal gene transfer. These bacteria, in fact, are genetically and ecologically closely related. They share a relative high degree of DNA homology, are phylogenetically closely related and can live in sympatry, sharing the same ecological niche. Several studies focused on the genetic structure of natural populations demonstrated that HGT plays a dominant role influencing the genetic structure of *Sinorhizobium* (Maynard Smith *et al.* 1993; Bailly *et al.*, 2006; Bailly *et al.*, 2007; Sun *et al.*, 2006; Van Berkum *et al.*, 2006). Considering inter-specific HGT, *S. meliloti* and *S. medicae* are separated on the bases of the species, while considering intra-specific HGT, there are evidence of intra-specific gene flow, but, within each species, strains are clustered in separated subgroups, not related by gene flow (Bailly *et al.*, 2007; Bailly *et al.*, 2006; Biondi *et al.*, 2003). Several barriers, such as DNA restriction, can reduce horizontal gene transfer between different bacteria and have not been studied so far in *Sinorhizobium*. Thus, barriers to the horizontal transfer were studied here by electroporation in strains of *S. meliloti* and *S. medicae*. Electroporation represents a physical method for bacterial transformation that, if combined with the use of plasmid DNA, allows the investigation of cytoplasmic barriers to foreign DNA acquisition in bacterial cells.

Thus, the aim of this work is the optimization of the transformation of *S. meliloti* and *S. medicae* by electroporation and the study of possible cytoplasmic barriers to horizontal plasmid DNA acquisition between different *Sinorhizobium* strains.

C. RESULTS

C.1. *SINORHIZOBIUM* STRAINS

Sinorhizobium strains analyzed in this work are listed in E.1.1. and chosen to be not geographically related. *S. meliloti* strains chosen comprise Rm1021, whose sequence is determined (Galibert *et al.*, 2001) and two natural strains AK58 and BL225C. The strain BM7 is a derivative of the strain Rm1021 and was produced in this work (E.2.6.). Strain AK58 (Giuntini *et al.*, 2005) was trapped from *M. sativa* (alfalfa), from soil samples collected in the northern Aral sea region by RIAM (St. Petersburg, Russia). Strain BL225C is an Italian strain, from Lodi, and was trapped on *M. sativa* (Carelli *et al.*, 2000).

S. medicae strains comprise the type strain LMG18864 (or LMG16580) (de Lajudie *et al.*, 1998), that was isolated from Syria by trapping with *M. sativa*, and two natural strains SS54 and SS55 that were isolated in the Centre de Biotechnologie de Borj-Cedria (Hammam-Lif, Tunisia) by alfalfa trapping from agricultural soil samples collected in Soliman, Tunisia.

C.2. THE PLASMID pMR20

For the experiment presented in this work we chose to use a plasmid called pMR20. This plasmid was constructed by Roberts *et al.* (1996) as a derivative of another plasmid, pRK290, which is known to easily establish in *S. meliloti* (Ditta *et al.*, 1980). A map showing of the plasmid pMR20 is shown in Figure 6.
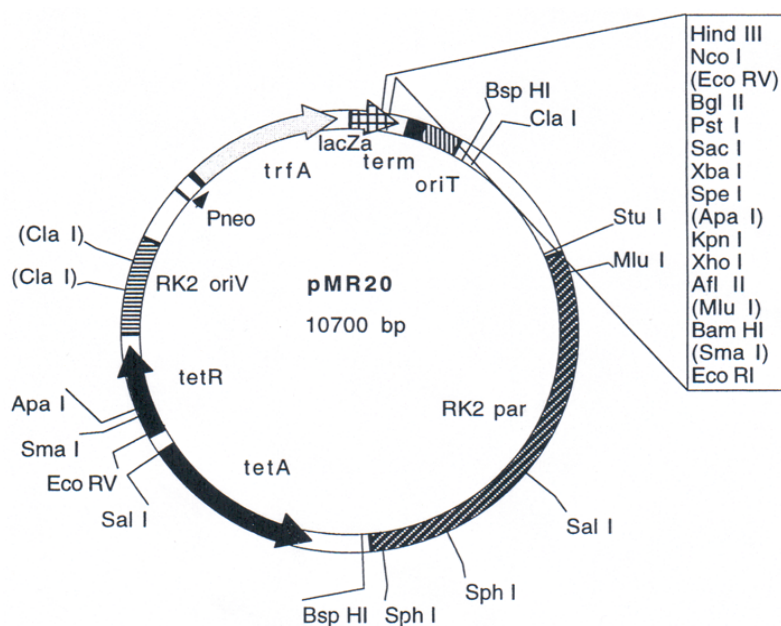
Figure 6. The plasmid pMR20. RK2 oriV is the broad host origin of replication of RK2. *tetR* and *tetA* constitute the tet-resistance cassette. Restriction enzymes indicated without parenthesis cut at unique site.

C.3. DETERMINATION OF THE MIC OF TETRACYCLINE

The Minimum Inhibitory Concentration (MIC) is defined as the minimum concentration of antibiotic which will inhibit the growth of the isolated microorganism. The antibiotic of interest is the tetracycline that allow selection of the plasmid pMR20 used in this work. The MIC was determined on agar plates: more than 10^9 bacterial cells from a fresh over-night (ON) colture were spread-plated on TY medium plates with increasing concentration of antibiotic. Data collected are reported in the following table (Table 1).

Table 1. Test of the MIC for tetracycline.

Strain name	Tetracycline concetration				
	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL	10 µg/mL
Rm1021	+	-	-	-	-
BM7	+	-	-	-	-
AK58	/	+	+	+	-
BL225C	/	+	+	+	-
LMG18864	+	+	-	-	-
SS54	/	+	+	+	-
SS55	/	+	+	+	-

+ = growth; **-** = bacterial growth totally inhibited; **/** = not tested

The minimum concentration determined were used in the following experiments for both to select plasmid acquisition by the strains and to prevent plasmid loss.

C.4. OPTIMAL CONDITIONS OF ELECTROPORATION OF *S. MELILOTI*

Optimal conditions of electroporations are that combinations of variable parameters that yields the highest value of efficiency of transformation.

The physical parameter fundamental to have electroporation is the Electric Field. In fact, Electric field is responsible to produce the poration of the membrane and the entry of the DNA in the cytoplasm of the cell.

Electric field (**E**) is defined by the following formula:

E = KV/cm

where **KV** is the difference of voltage expressed in kilovolts and **cm** is the distance between electrodes expressed in centimeters.

Electroporation cuvettes are supplied in three formats: 0,4 cm, useful for eukaryotic cells; 0,2 cm, useful for yeast and bacteria; 0,1 cm, useful for bacteria. In our experiment we chose the 0,1 cm cuvettes because they allow to obtain the highest electric field value.

Voltage can be varied in range from 0 to 2,5 KV by setting the electroporator, thus the Electric Field can vary **from 0 to 25 KV/cm**.

Another important variable parameter is the time constant (τ), that represents the time needed to the Electric Field to turn back to zero after the impulse is supplied. **Time constant** is expressed in milliseconds (msec) and is determined by the following formula:

τ = R x C

where R is the Resistance expressed in kiloohm (Ω) and C is the capacitance expressed in microfarad (μF). Electroporator machinery allows to change both Resistance and Capacitance. Usually capacitance can be hold fixed at 25 μF and resistance can be varied in a range from 100 up to 1000 Ω.

To find optimal conditions of electroporation of *S. meliloti* we chose the type strain Rm1021. Electrocompetent cells were prepared as reported in E.2.4. and electroporated as described in E.2.5. Each sample was transformed with 1 μg of plasmid pMR20 DNA extracted from *E. coli* DH5α.

As first step the time constant was fixed at 5 msec (200 Ω; 25μF) varying the voltage. The graphical representation of the average of the data is shown in Figure 8. Data obtained are reported in the Table 2. Negative controls were performed as follow, electrocompetent cells not treated with DNA and not electroporated were resuspended with 1 ml of TY medium and then treated as each other sample. Spontaneous mutants resistant to tetracycline were never found.
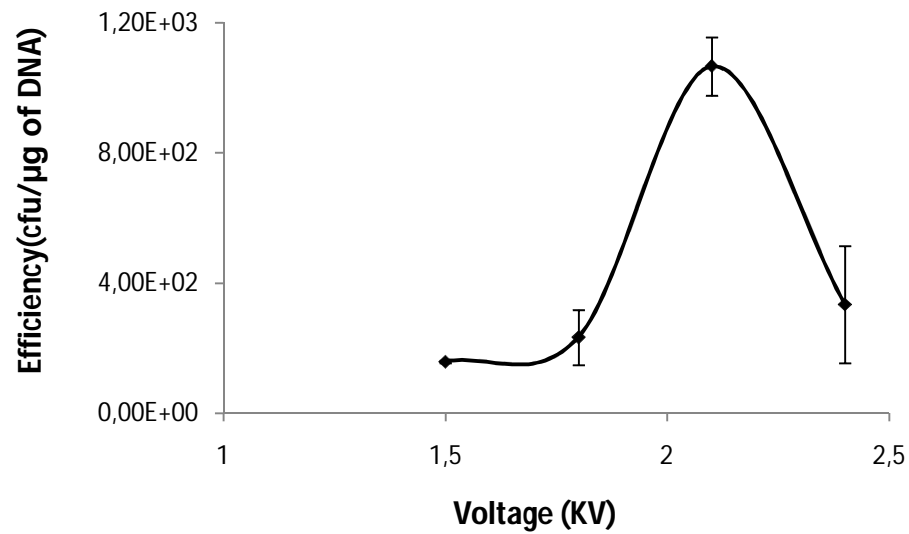
Figure 8. Transformation efficiencies of *S. meliloti* type strain Rm1021 varying the voltage from 1,5 to 2,4 KV. Each value is the average of three independent replicates and bars indicate the standard deviation.

Data obtained show clearly that the optimal value of voltage for *S. meliloti* is of 2,1 KV. The following step was the variation of the time constant to determine its optimal value. Thus the voltage was fixed at 2,1 KV and the resistance was changed to vary the time constant. Data obtained are showed in Table 3.

Table 2. Efficiency of electroporation of *S. meliloti* Rm1021 at different voltages.

Sample	Voltage applied (KV)	Effective time constant (msec)[a]	Viable title (cfu/ml)	Survival (%)[b]	Transformants (cfu/ml)	Efficiency (cfu/µg of DNA)
1	**1,5**	4,1	$1,19 \times 10^{10}$	$3,31 \times 10^{1}$	$1,60 \times 10^{2}$	$1,60 \times 10^{2}$
2	**1,5**	4,1	$1,31 \times 10^{10}$	$3,64 \times 10^{1}$	$1,55 \times 10^{2}$	$1,55 \times 10^{2}$
3	**1,5**	4,1	$1,08 \times 10^{10}$	$3,00 \times 10^{1}$	$1,62 \times 10^{2}$	$1,62 \times 10^{2}$
4	**1,8**	3,9	$7,32 \times 10^{9}$	$2,03 \times 10^{1}$	$1,87 \times 10^{2}$	$1,87 \times 10^{2}$
5	**1,8**	4,1	$7,12 \times 10^{9}$	$1,98 \times 10^{1}$	$3,32 \times 10^{2}$	$3,32 \times 10^{2}$
6	**1,8**	4	$7,40 \times 10^{9}$	$2,05 \times 10^{1}$	$1,83 \times 10^{2}$	$1,83 \times 10^{2}$
7	**2,1**	3,9	$2,32 \times 10^{9}$	6,44	$1,12 \times 10^{3}$	$1,12 \times 10^{3}$
8	**2,1**	3,9	$2,50 \times 10^{9}$	6,94	$9,63 \times 10^{2}$	$9,63 \times 10^{2}$
9	**2,1**	3,9	$2,25 \times 10^{9}$	6,25	$1,1 \times 10^{3}$	$1,1 \times 10^{3}$
10	**2,4**	3,8	$7,50 \times 10^{8}$	2,08	$5,42 \times 10^{2}$	$5,42 \times 10^{2}$
11	**2,4**	3,8	$7,98 \times 10^{8}$	2,22	$2,30 \times 10^{2}$	$2,30 \times 10^{2}$
12	**2,4**	3,8	$7,80 \times 10^{8}$	2,17	$2,33 \times 10^{2}$	$2,33 \times 10^{2}$

[a](200 Ω; 25 µF; 1µg DNA)

[b]Survival was determined as the percentage of the viable title divided by the viable title of the control that was $3,60 \times 10^{10}$ (the average of more than three separate measurements) .

Table 3. Electroporation of *S. meliloti* Rm1021 changing the time constant.

Sample	Resistance (Ω)	Attended time constant (msec)	Effective time constant (msec)	Efficiency (cfu/µg of DNA)
1	100	2,5	2,2	$1,95 \times 10^2$
2	100	2,5	2,2	$1,15 \times 10^2$
3	100	2,5	2,2	$1,42 \times 10^2$
4	400	10	0,1	/
5	400	10	0,1	/
6	400	10	0,1	/

/ = not reliable (see text below).

Data obtained showed that reduction of the time constant results in decrease of the efficiency of transformation, if compared with the average value obtained with 5 msec, $1,07 \times 10^3$ cfu/µg of DNA.

Increase of resistance to 400 Ω resulted in an effective time constant very low respect to the attended one of 10 msec. Thus, this condition were not reliable. In this condition current passes throw the cuvette so the strength of electric field is very low and its duration is very short. This determines strong reduction of both recovery of viable cells and transformants.

Finally the parameters of **2,1 KV and time constant of 5 msec (200 Ω; 25 µF) were considered as optimal for electroporation of *S. meliloti*** and were used in the following experiments.

C.5.   OPTIMAL CONDITIONS OF ELECTROPORATION OF *S. MEDICAE*

The same procedure followed to determine the optimal conditions of electroporation of *S. meliloti* was followed for *S. medicae* strain LMG18864. As first step the time constant was fixed at 5 msec (200 Ω; 25µF) varying the electric field. Negative controls were performed as follow, electrocompetent cells not treated with DNA or not electroporated were resuspended with 1 ml of TY medium and then treated as each other sample. Spontaneous mutants resistant to tetracycline were never recovered. The graphical representation of the average of the results is shown in Figure 9. Data obtained for each proof are reported in the Table 4.

Table 4. Efficiency of electroporation of *S. medicae* LMG18864 at different voltages.

Sample	Voltage applied (KV)	Effective time constant (msec)[a]	Viable title (cfu/ml)	Survival (%)[b]	Transformants (cfu/ml)	Efficiency (cfu/µg of DNA)
1	**1,5**	4,3	$4,20 \times 10^9$	$3,81 \times 10^1$	$8,20 \times 10^4$	$8,20 \times 10^4$
2	**1,5**	4,3	$6,25 \times 10^9$	$5,68 \times 10^1$	$1,18 \times 10^5$	$1,18 \times 10^5$
3	**1,5**	4,3	$5,70 \times 10^9$	$5,18 \times 10^1$	$9,89 \times 10^5$	$9,89 \times 10^4$
4	**1,7**	4,2	$4,20 \times 10^9$	$3,93 \times 10^1$	$1,60 \times 10^5$	$1,60 \times 10^5$
5	**1,7**	4,2	$3,94 \times 10^9$	$3,58 \times 10^1$	$2,13 \times 10^5$	$2,13 \times 10^5$
6	**1,7**	4,2	$4,01 \times 10^9$	$3,64 \times 10^1$	$1,90 \times 10^5$	$1,90 \times 10^5$
7	**1,9**	4,2	$3,70 \times 10^9$	$3,35 \times 10^1$	$3,31 \times 10^5$	$3,31 \times 10^5$
8	**1,9**	4,1	$3,98 \times 10^9$	$3,61 \times 10^1$	$5,30 \times 10^5$	$5,30 \times 10^5$
9	**1,9**	4,2	$3,60 \times 10^9$	$3,27 \times 10^1$	$4,23 \times 10^5$	$4,23 \times 10^5$
10	**2,1**	4,2	$2,79 \times 10^9$	$2,99 \times 10^1$	$6,00 \times 10^5$	$6,00 \times 10^5$
11	**2,1**	4,1	$3,63 \times 10^9$	$3,3 \times 10^1$	$1,10 \times 10^6$	$1,10 \times 10^6$
12	**2,1**	4,1	$3,20 \times 10^9$	$2,9 \times 10^1$	$7,32 \times 10^5$	$7,32 \times 10^5$
13	**2,3**	4,1	$2,57 \times 10^9$	$2,41 \times 10^1$	$1,14 \times 10^6$	$1,14 \times 10^6$
14	**2,3**	4,1	$2,89 \times 10^9$	$2,63 \times 10^1$	$8,61 \times 10^5$	$8,60 \times 10^5$
15	**2,3**	4,1	$2,33 \times 10^9$	$2,11 \times 10^1$	$9,78 \times 10^5$	$9,78 \times 10^5$

[a](200 Ω; 25 µF; 1µg DNA).

[b]Survival was determined as the percentage of the viable title divided by the viable title of the control that was $1,10 \times 10^{10}$ (the average of more than three separate measurements).
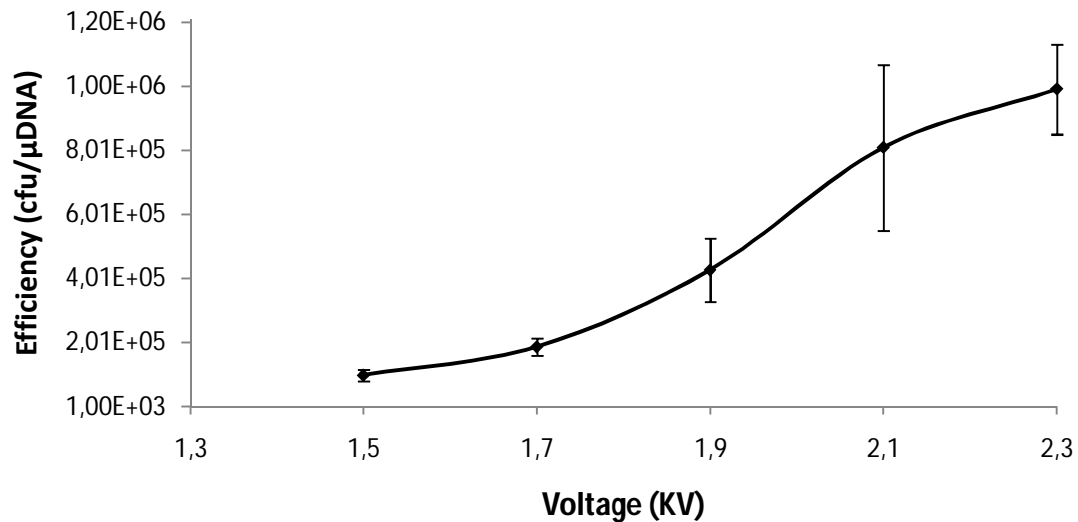
Figure 9. Transformation efficiencies of *S. medicae* type strain LMG18864 varying the voltage from 1,5 to 2,3 KV. Each value is the average of three independent replicates and bars indicate the standard deviation.

Data obtained show that the optimal value of voltage for *S. medicae* is of 2,3 KV. Increase of voltage up to 2,3 KV resulted in an effective time constant very low (0,1-0,5 msec) respect to the attended one of 5 msec. Thus, this condition was not reliable and so it is not included in Figure 9.

The following step was the variation of the time constant to determine its optimal value. Thus the voltage was fixed at 2,3 KV and the resistance was changed to vary the time constant. Data obtained are showed in Table 5.

Table 5. Electroporation of *S. medicae* LMG18864 changing the time constant.

Sample	Resistance (Ω)	Attended time constant (msec)	Effective time constant (msec)	Efficiency (cfu/μg of DNA)
1	100	2,5	1,7	$1,95 \times 10^5$
2	100	2,5	1,8	$2,00 \times 10^5$
3	100	2,5	1,7	$2,12 \times 10^5$
4	400	10	7,1	$8,00 \times 10^5$
5	400	10	7,0	$4,68 \times 10^5$
6	400	10	7,0	$3,60 \times 10^5$

Data obtained showed that reduction or increase of the time constant resulted in decrease of the efficiency of transformation, if compared with the average value obtained with 5 msec, $1,00 \times 10^6$ cfu/μg of DNA.

Finally the parameters of **2,3 KV and time constant of 5 msec (200 Ω; 25 μF) were considered as optimal for electroporation of *S. medicae*** and were used in the following experiments.

C.6. ELECTROPORATION OF *S. MELILOTI* WITH SELF DNA

For both strains *S. meliloti* Rm1021 and *S. medicae* LMG18864 four colonies of transformants were selected and analyzed for the presence of the plasmid. Plasmid was checked by direct plasmid extraction and subsequent visualization on agarose gel.

The plasmid extracted from each colony (E.2.7.) was run on agarose gel (E.2.8.) and the size of the molecules was found to be as attended for pMR20. This confirmed that the plasmid was not integrated in the genome and that was possible to extract it from *Sinorhizobium* cells.

A large amount of plasmid DNA from *S. meliloti* Rm1021, from *S. medicae* LMG18864 and from *E. coli* DH5α was obtained purifying the supercoiled form of the plasmid from gel (E.2.7.) Those plasmids were used to transform Rm1021 and LMG18864 and to compare the efficiency of transformation of self DNA to that of exogenous DNA from *E. coli*.

Each transformation was performed using 50 ng of DNA per sample and results obtained are reported in Figure 10. Data are listed in the following Table 6.
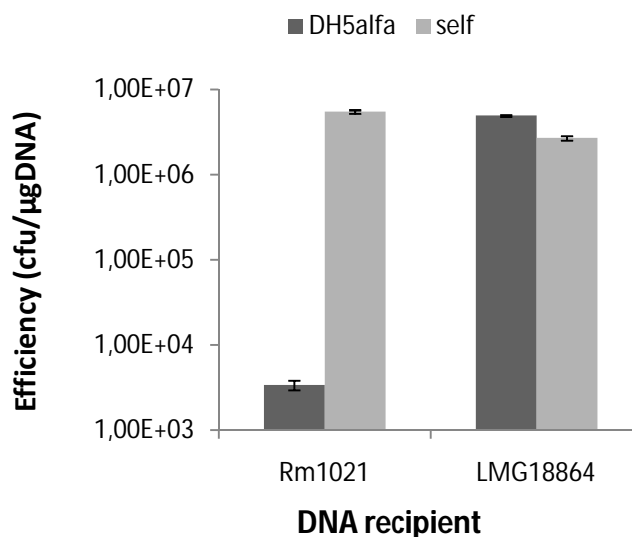
Figure 10. Transformation efficiencies of *S. meliloti* Rm1021 and *S. medicae* LMG18864 with plasmid DNA from *E. coli* DH5α and with self plasmid DNA. Each bar represent the average of three independent replicates with standard deviation.

Table 6. Efficiencies of electroporation (cfu/µg of DNA) of *S. meliloti* Rm1021 and *S. medicae* LMG18864 with DNA from DH5α and with self DNA.

Recipient	DNA from DH5α			Self DNA		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
Rm1021	$3,83 \times 10^3$	$2,94 \times 10^3$	$3,44 \times 10^3$	$5,70 \times 10^6$	$5,40 \times 10^6$	$5,20 \times 10^6$
LMG18864	$5,00 \times 10^6$	$4,80 \times 10^6$	$4,90 \times 10^6$	$2,76 \times 10^6$	$2,75 \times 10^6$	$2,50 \times 10^6$

Results obtained showed that transforming the two strains with 50 ng of plasmid DNA, instead 1 µg, increased the efficiency by more than 3 time (from $1,06 \times 10^3$ to $3,41 \times 10^3$) for Rm1021 and 5 times (from $9,78 \times 10^5$ to $4,90 \times 10^6$) for LMG18864. This demonstrates that transformations can be performed reducing the quantity of the DNA to 50 ng increasing the efficiency, thus each subsequent transformation was performed using 50 ng of DNA per sample.

Data obtained shows that, differently from LMG18864, the origin of the DNA strongly influenced the efficiency of transformation of the strain Rm1021, suggesting the presence of an efficient cytoplasmic barrier against non-self DNA in this strain.

C.7. PUTATIVE CYTOPLASMIC BARRIERS OF *S. MELILOTI* Rm1021

Cytoplasmic barriers to the acquisition of foreign DNA can be the barriers of restriction and, in case of plasmids, the barrier to the establishment in the genome (see A.2.). The plasmid used in this work is known to establish easily in *Sinorhizobium* cells, so we focused on restriction. In the genome of Rm1021 a putative gene coding for a type I restriction modification system is annotated. The system is composed by the tree genes *hsdM*, *hsdS* and *hsdR*, the first coding for the putative modification subunit, the second responsible for the sequence specificity and the third coding for the putative restriction system. Moreover these genes are clustered, suggesting to be organized as an operon (Figure 11).

Figure 11. The putative type I restriction system in the genome of *S. meliloti* Rm1021, composed by *hsdR* (Smc02292); *hsdS* (Smc02295) and *hsdM* (Smc02296), colored in green.
(Image from http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi).

C.8. CONSTRUCTION OF THE MUTANT FOR THE PUTATIVE *hsdR* GENE

To test the involvement of the gene Smc02292 (*hsdR*) in the barrier to the acquisition of foreign DNA, the mutant strain called BM7 was constructed. The mutation was produced disrupting the gene by plasmid integration, using the strategy of Luo *et al.* (2005), showed in Figure 12.

The plasmid, kindly provided by A. Becker (Molekulare Genetik, Institut für Biologie III, Albert-Ludwigs-Universität Freiburg), was derived from the suicidal plasmid pK19mob2ΩHMB (Luo *et al.*, 2005) and carries a PCR fragment of 341 nucleotides corresponding to the middle part of the Smc02292 gene (position 1041-1382 nucleotides downstream the start codon) inserted into the *Hind*III-*BsrG*I site. Mutant strain obtained by biparental mating (E.2.6.) was tested to evaluate its electroporation efficiency with plasmid pMR20 from *E. coli* DH5α as donor of DNA.

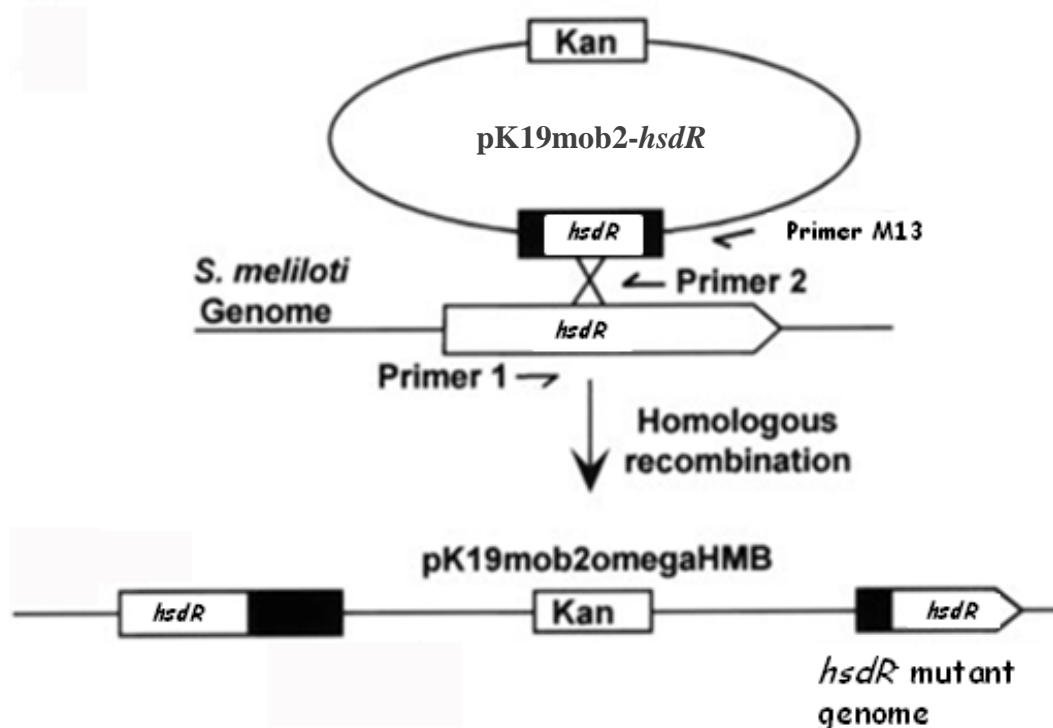**Figure 12.** The strategy of mutagenesis by plasmid integration of pK19mob2ΩHMB. Plasmid pK19mob2ΩHMB is suicidal in *S. meliloti* but can be established in its genome by single crossing-over on the bases of the homology between the *hsdR* gene region cloned in the plasmid and the *hsdR* gene sequence on the genome. Integration disrupt the gene (image obtained modifying the original one of Luo *et al.*, 2005).

C.9.   ELECTROPORATION OF THE MUTANT BM7

To test the electroporability of the mutant BM7, the strain was transformed with the plasmid pMR20 from *E. coli* DH5α. Results are shown in Table 7.

Table 7. Efficiencies of electroporation of BM7 with plasmid DNA from *E. coli.*

Sample	Viable title (cfu/ml)	Survival (%)[a]	Transformants (cfu/ml)[b]	Efficiency (cfu/µg of DNA)
Sample 1	$2,13 \times 10^9$	7,08	$1,00 \times 10^3$	$2,00 \times 10^4$
Sample 2	$1,87 \times 10^9$	6,21	$1,60 \times 10^3$	$3,20 \times 10^4$
Sample 3	$1,92 \times 10^9$	6,38	$3,00 \times 10^3$	$6,00 \times 10^4$

[a] Survival was determined as the percentage of the viable title divided by the viable title of the control that was $3,01 \times 10^{10}$ (the average of more than three separate measurements).
[b] 50ng of DNA were used.

The efficiency of transformation of the strain BM7 with plasmid DNA from *E. coli* was $3,73 \times 10^4$ cfu/µg of DNA (± SD $2,05 \times 10^4$), of 10,9 times higher than the efficiency of the wild type Rm1021 ($3,41 \times 10^3$).

C.10. ELECTROPORATION OF *S. MELILOTI* Rm1021 AND BM7 WITH PLASMID DNA FROM *SINORHIZOBIUM* DONOR

The experiments were carried out to evaluate the barrier of *S. meliloti* Rm1021 against DNA acquisition from other *Sinorhizobium* strains. To prepare donor DNA, all *Sinorhizobium* strains analyzed (E.1.1.) were electroporated with pMR20 plasmid DNA from *E. coli* DH5α, using the optimal conditions previously determined. Tetracycline resistance transformants were selected and pMR20 plasmid DNA was extracted from each strain. Those preparations were used in following experiments.

Rm1021 and its putative *hsdR* mutant BM7 were transformed with pMR20 plasmid DNA from all other *Sinorhizobium* strains analyzed in this work (E.1.1.). Efficiencies of transformation obtained are reported in Figure 13. Data from each single experiment are reported in Table 8 and Table 9.
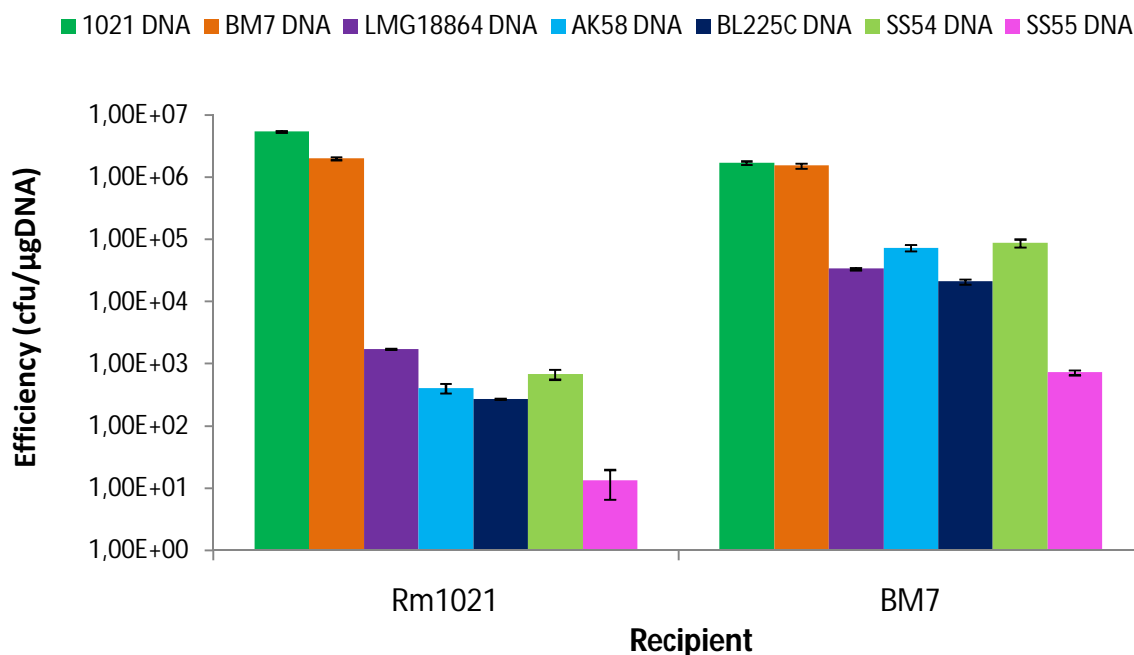
Figure 13. Transformation efficiencies of *S. meliloti* Rm1021 and mutant BM7 with plasmid DNA from different *Sinorhizobium* donors including self DNA. In abscissa there are the recipient strains and each differently colored bar represents a different donor. Data are the average of three independent replicates with standard error.

Data obtained show clearly that the barrier of Rm1021 is against all the non-self DNA tested, including DNA from conspecific strains as AK58 and BL225C. *S. meliloti* Rm1021 transformed with DNA from other strains showed an efficiency from 10^3 to 10^5 times lower than that with self DNA. The efficiency of transformation of mutant BM7, instead, is higher than that of the wild type for each foreign DNA tested. The increase ranged from nearly 20 times, for DNA from LMG18864, to 180 times, for DNA from AK58. Increase for other DNAs was 77 times for DNA from BL225C, 128 times for DNA from SS54 and 110 times for DNA from SS55.

Surprisingly mutant strain BM7, however, was transformed less efficiently by foreign DNA than self-DNA. This observation indicates that *hsdR* gene does not represent the sole barrier mechanism of strain Rm1021 toward foreign DNA.

Table 8. Electroporation of *S. meliloti* Rm1021 with pMR20 plasmid DNA from different *Sinorhizobium* donors.

DNA Donor	Species	Viable title (cfu/ml)	Transformants (cfu/ml)	Efficiency (cfu/μg of DNA)
BM7	*S. meliloti*	$1,92 \times 10^9$	$1,09 \times 10^5$	$2,18 \times 10^6$
BM7	*S. meliloti*	$1,59 \times 10^9$	$9,95 \times 10^4$	$1,99 \times 10^6$
BM7	*S. meliloti*	$1,34 \times 10^9$	$9,20 \times 10^4$	$1,84 \times 10^6$
AK58	*S. meliloti*	$2,96 \times 10^9$	$2,75 \times 10^1$	$5,50 \times 10^2$
AK58	*S. meliloti*	$1,14 \times 10^9$	$1,60 \times 10^1$	$3,60 \times 10^2$
AK58	*S. meliloti*	$3,46 \times 10^9$	$1,80 \times 10^1$	$3,20 \times 10^2$
BL225C	*S. meliloti*	$6,50 \times 10^8$	$1,30 \times 10^1$	$2,60 \times 10^2$
BL225C	*S. meliloti*	$5,00 \times 10^8$	$1,40 \times 10^1$	$2,80 \times 10^2$
BL225C	*S. meliloti*	$7,95 \times 10^8$	$1,40 \times 10^1$	$2,80 \times 10^2$
LMG18864	*S. medicae*	$1,64 \times 10^9$	$9,20 \times 10^1$	$1,84 \times 10^3$
LMG18864	*S. medicae*	$1,28 \times 10^9$	$8,60 \times 10^1$	$1,72 \times 10^3$
LMG18864	*S. medicae*	$1,95 \times 10^9$	$8,30 \times 10^1$	$1,66 \times 10^3$
SS54	*S. medicae*	$1,03 \times 10^9$	$2,40 \times 10^1$	$4,80 \times 10^2$
SS54	*S. medicae*	$1,54 \times 10^9$	$4,50 \times 10^1$	$9,00 \times 10^2$
SS54	*S. medicae*	$1,40 \times 10^9$	$3,40 \times 10^1$	$6,80 \times 10^2$
SS55	*S. medicae*	$5,55 \times 10^8$	$1,00 \times 10^0$	$2,00 \times 10^1$
SS55	*S. medicae*	$1,14 \times 10^9$	$1,00 \times 10^0$	$2,00 \times 10^1$
SS55	*S. medicae*	$1,20 \times 10^9$	0	0

Table 9. Electroporation of *S. meliloti* mutant BM7 with pMR20 plasmid DNA from different *Sinorhizobium* donors.

DNA Donor	Species	Viable title (cfu/ml)	Transformants (cfu/ml)	Efficiency (cfu/µg of DNA)
Rm1021	*S. meliloti*	$1,62 \times 10^9$	$7,90 \times 10^4$	$1,58 \times 10^6$
Rm1021	*S. meliloti*	$1,55 \times 10^9$	$9,65 \times 10^4$	$1,93 \times 10^6$
Rm1021	*S. meliloti*	$1,25 \times 10^9$	$8,05 \times 10^4$	$1,61 \times 10^6$
BM7	*S. meliloti*	$1,33 \times 10^9$	$8,55 \times 10^4$	$1,71 \times 10^6$
BM7	*S. meliloti*	$2,40 \times 10^9$	$6,25 \times 10^4$	$1,25 \times 10^6$
BM7	*S. meliloti*	$1,38 \times 10^9$	$8,25 \times 10^4$	$1,65 \times 10^6$
AK58	*S. meliloti*	$1,57 \times 10^9$	$2,96 \times 10^3$	$5,92 \times 10^4$
AK58	*S. meliloti*	$1,36 \times 10^9$	$3,60 \times 10^3$	$7,20 \times 10^4$
AK58	*S. meliloti*	$1,18 \times 10^9$	$4,45 \times 10^3$	$8,90 \times 10^4$
BL225C	*S. meliloti*	$5,10 \times 10^8$	$1,26 \times 10^3$	$2,52 \times 10^4$
BL225C	*S. meliloti*	$4,10 \times 10^8$	$9,18 \times 10^2$	$1,84 \times 10^4$
BL225C	*S. meliloti*	$3,65 \times 10^8$	$9,85 \times 10^2$	$1,97 \times 10^4$
LMG18864	*S. medicae*	$8,90 \times 10^8$	$1,85 \times 10^3$	$3,70 \times 10^4$
LMG18864	*S. medicae*	$1,26 \times 10^9$	$1,60 \times 10^3$	$3,20 \times 10^4$
LMG18864	*S. medicae*	$1,70 \times 10^9$	$1,65 \times 10^3$	$3,30 \times 10^4$
SS54	*S. medicae*	$9,40 \times 10^8$	$5,65 \times 10^3$	$1,13 \times 10^5$
SS54	*S. medicae*	$1,08 \times 10^9$	$3,40 \times 10^3$	$6,84 \times 10^4$
SS54	*S. medicae*	$1,45 \times 10^9$	$4,15 \times 10^3$	$8,30 \times 10^4$
SS55	*S. medicae*	$8,00 \times 10^8$	$4,00 \times 10^1$	$8,00 \times 10^2$
SS55	*S. medicae*	$1,08 \times 10^9$	$4,00 \times 10^1$	$8,00 \times 10^2$
SS55	*S. medicae*	$1,69 \times 10^9$	$3,00 \times 10^1$	$6,00 \times 10^2$

C.11. ELECTROPORATION OF *S. MELILOTI* AND *S. MEDICAE* STRAINS WITH PLASMID DNA FROM *SINORHIZOBIUM* DONORS

To evaluate the presence of a cytoplasmic barriers to foreign plasmid DNA acquisition in other *S. meliloti* and *S. medicae* strains, all the *Sinorhizobium* strains used in this work (E.1.1.), other than Rm1021 and BM7 (C.11.), were analyzed. Recipient strains were transformed with 50 ng of pMR20 plasmid DNA extracted from three different donors:

i)   Rm1021 as representative donor of *S. meliloti* species;

ii)   LMG18864 as representative donor of *S. medicae* species;

iii)   The same strain used as recipient, as self DNA.

Efficiencies of transformation obtained are reported in Figure 14. Data from each single experiment are reported in the following Table 10.
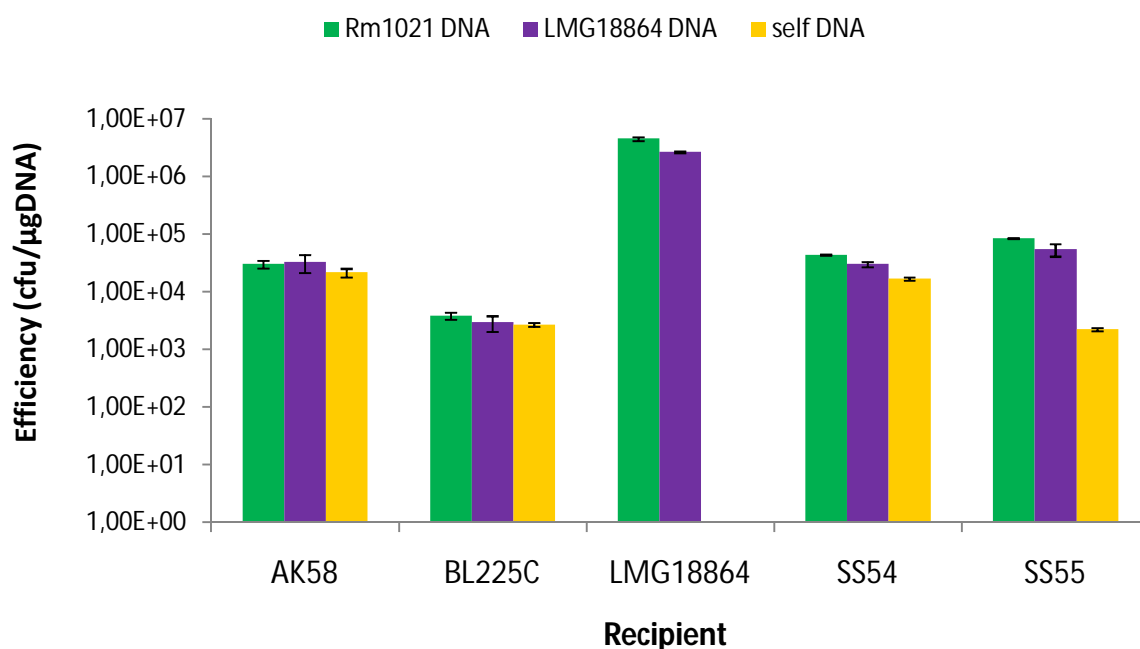
Figure 14. Transformation efficiencies of *S. meliloti* AK58, *S. meliloti* BL225C, *S. medicae* LMG18864, *S. medicae* SS54 and *S. medicae* SS55, with plasmid DNA from different *Sinorhizobium* donors including self DNA. In abscissa there are the recipient strains and each bar differently colored represents a different donor of DNA. Each bar represent the average of three independent replicates with the standard error.

Data obtained demonstrates that *S. meliloti* and *S. medicae* strains analyzed did not show donor-dependent efficiency of acquisition of foreign plasmid DNA and the efficiency of transformation with non-self DNA was never significantly reduced respect to that with self DNA. Results showed also that efficiency of electroporation, in the tested conditions,

represents a variable phenotype among different *S. meliloti* and *S. medicae* strains: the values with self DNA ranged in fact from 10^3 to 10^6 according to the strain. Compared with other DNAs, DNA from *S. medicae* strain SS55 was less efficient in the transformation of the three recipient strains tested: Rm1021, BM7 and the self SS55. Plasmid extraction from strain SS55 was repeated two other times, but each preparation confirmed the data obtained previously for all tree strains Rm1021, BM7 and SS55. The quality of plasmid DNA preparations from SS55 was assessed after gel purification and was apparently similar to other plasmid DNAs (data not shown). Some unidentified aspect of the quality of the DNA preparation from SS55 strain may be responsible of reduced transforming capacity with electroporation.

Table 10. Electroporation of *S. meliloti* and *S. medicae* strains with pMR20 plasmid DNA from different *Sinorhizobium* donors.

Recipient	Species	DNA Donor	Viable title (cfu/ml)	Transformants (cfu/ml)	Efficiency (cfu/µg of DNA)
AK58	*S. meliloti*	Rm1021	$2,64 \times 10^9$	$1,95 \times 10^3$	$3,90 \times 10^4$
AK58	*S. meliloti*	Rm1021	$4,98 \times 10^9$	$1,17 \times 10^3$	$2,34 \times 10^4$
AK58	*S. meliloti*	Rm1021	$3,92 \times 10^9$	$1,45 \times 10^3$	$2,90 \times 10^4$
AK58	*S. meliloti*	LMG18864	$1,12 \times 10^9$	$5,42 \times 10^3$	$1,08 \times 10^4$
AK58	*S. meliloti*	LMG18864	$4,73 \times 10^9$	$2,46 \times 10^3$	$4,09 \times 10^4$
AK58	*S. meliloti*	LMG18864	$4,86 \times 10^9$	$1,91 \times 10^3$	$3,83 \times 10^4$
AK58	*S. meliloti*	AK58	$2,34 \times 10^9$	$1,42 \times 10^3$	$2,86 \times 10^4$
AK58	*S. meliloti*	AK58	$3,88 \times 10^9$	$8,10 \times 10^2$	$1,62 \times 10^4$
AK58	*S. meliloti*	AK58	$3,34 \times 10^9$	$1,10 \times 10^3$	$2,02 \times 10^4$
BL225C	*S. meliloti*	Rm1021	$1,04 \times 10^9$	$2,40 \times 10^2$	$4,80 \times 10^3$
BL225C	*S. meliloti*	Rm1021	$3,01 \times 10^9$	$1,45 \times 10^2$	$2,90 \times 10^3$
BL225C	*S. meliloti*	Rm1021	$9,98 \times 10^8$	$1,95 \times 10^2$	$3,90 \times 10^3$
BL225C	*S. meliloti*	LMG18864	$9,70 \times 10^8$	$1,68 \times 10^2$	$3,36 \times 10^3$
BL225C	*S. meliloti*	LMG18864	$1,31 \times 10^9$	$2,10 \times 10^2$	$4,20 \times 10^3$
BL225C	*S. meliloti*	LMG18864	$1,03 \times 10^9$	$6,00 \times 10^1$	$1,20 \times 10^3$
BL225C	*S. meliloti*	BL225C	$8,95 \times 10^8$	$1,30 \times 10^3$	$2,60 \times 10^3$
BL225C	*S. meliloti*	BL225C	$9,84 \times 10^8$	$1,55 \times 10^2$	$3,10 \times 10^3$
BL225C	*S. meliloti*	BL225C	$1,08 \times 10^9$	$1,20 \times 10^2$	$2,40 \times 10^3$
LMG18864	*S. medicae*	Rm1021	$1,81 \times 10^9$	$2,55 \times 10^5$	$5,10 \times 10^6$
LMG18864	*S. medicae*	Rm1021	$1,11 \times 10^9$	$2,22 \times 10^5$	$4,44 \times 10^6$
LMG18864	*S. medicae*	Rm1021	$1,02 \times 10^9$	$2,01 \times 10^5$	$4,02 \times 10^6$

LMG18864	*S. medicae*	LMG18864	$1,27 \times 10^9$	$1,38 \times 10^5$	$2,76 \times 10^6$
LMG18864	*S. medicae*	LMG18864	$1,54 \times 10^9$	$1,38 \times 10^5$	$2,75 \times 10^6$
LMG18864	*S. medicae*	LMG18864	$1,02 \times 10^9$	$1,25 \times 10^5$	$2,50 \times 10^6$
SS54	*S. medicae*	Rm1021	$2,96 \times 10^9$	$2,26 \times 10^3$	$4,50 \times 10^4$
SS54	*S. medicae*	Rm1021	$2,54 \times 10^9$	$2,12 \times 10^3$	$4,24 \times 10^6$
SS54	*S. medicae*	Rm1021	$2,36 \times 10^9$	$2,20 \times 10^3$	$4,40 \times 10^6$
SS54	*S. medicae*	LMG18864	$1,70 \times 10^9$	$1,21 \times 10^3$	$2,42 \times 10^4$
SS54	*S. medicae*	LMG18864	$1,76 \times 10^9$	$1,70 \times 10^3$	$3,40 \times 10^4$
SS54	*S. medicae*	LMG18864	$1,70 \times 10^9$	$1,64 \times 10^3$	$3,28 \times 10^4$
SS54	*S. medicae*	SS54	$1,95 \times 10^9$	$9,30 \times 10^2$	$1,86 \times 10^4$
SS54	*S. medicae*	SS54	$3,10 \times 10^9$	$7,50 \times 10^2$	$1,50 \times 10^4$
SS54	*S. medicae*	SS54	$3,15 \times 10^9$	$8,60 \times 10^2$	$1,72 \times 10^4$
SS55	*S. medicae*	Rm1021	$9,80 \times 10^8$	$4,32 \times 10^3$	$8,64 \times 10^4$
SS55	*S. medicae*	Rm1021	$7,50 \times 10^8$	$4,18 \times 10^3$	$8,34 \times 10^4$
SS55	*S. medicae*	Rm1021	$8,90 \times 10^8$	$4,28 \times 10^3$	$8,56 \times 10^4$
SS55	*S. medicae*	LMG18864	$5,00 \times 10^8$	$1,44 \times 10^3$	$2,88 \times 10^4$
SS55	*S. medicae*	LMG18864	$2,20 \times 10^9$	$1,33 \times 10^3$	$6,16 \times 10^4$
SS55	*S. medicae*	LMG18864	$1,17 \times 10^9$	$1,81 \times 10^3$	$7,24 \times 10^4$
SS55	*S. medicae*	SS55	$6,40 \times 10^8$	$1,12 \times 10^2$	$2,24 \times 10^3$
SS55	*S. medicae*	SS55	$4,60 \times 10^8$	$1,30 \times 10^2$	$2,60 \times 10^3$
SS55	*S. medicae*	SS55	$5,15 \times 10^8$	$1,10 \times 10^2$	$2,20 \times 10^3$

C.12. CONJUGATION OF *S. MELILOTI* Rm1021 AND THE MUTANT BM7 WITH *E. COLI* S17-1 AS DONOR

Strain S17-1 (Simon *et al.*, 1983) is an *E. coli* strain engineered to perform conjugational transfer of plasmid DNA by biparental mating. This kind of mating is simpler and known to be more efficient with respect the triparental mating, that requires an helper strain. Thus we chose this strategy to evaluate the effect of *hsdR* barrier of Rm1021 in conjugal foreign plasmid DNA acquisition. As first step, the plasmid pMR20 was introduced in S17-1 by electroporation. Preparation of electrocompetent cells and transformation by electroporation were performed using the standard protocol for *E. coli* (Dower *et al.*, 1988). Four tetracycline resistant colonies were checked for the presence of plasmid and all of them carried the correct one. Thus one of those transformants was used for subsequent experiments.

Efficiency of conjugation was evaluated for both the wild type *S. meliloti* Rm1021 and its mutant strain BM7 in three independent replicates. Results obtained are reported in Figure 15, while data obtained from each replicas are reported in Table 11.
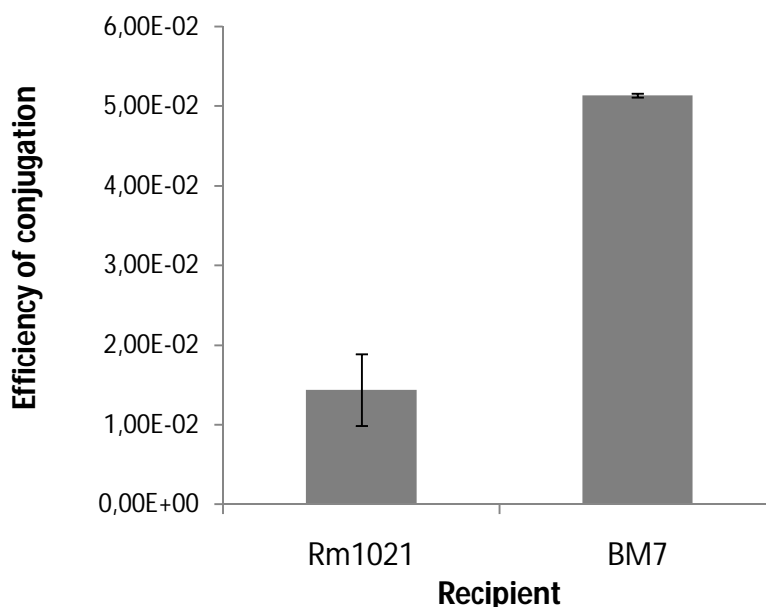

Figure 15. Conjugation efficiencies *S. meliloti* Rm1021 and its mutant BM7 with S17-1 as donor of pMR20 plasmid DNA. Conjugation efficiency is expressed as the number of transconjugants per donor cells. Each bar represent the average of three independent replicates with standard deviation.

Table 11. Conjugation of Rm1021 and BM7 as recipient with *E. coli* S17-1 as donor of pMR20 plasmid DNA.

Recipient	No. of recipients	No. of donors	No. of transconjugants	Efficiency of conjugation*
Rm1021	$1{,}20 \times 10^9$	$1{,}00 \times 10^8$	$1{,}90 \times 10^6$	$1{,}90 \times 10^{-2}$
Rm1021	$1{,}02 \times 10^9$	$2{,}50 \times 10^8$	$2{,}50 \times 10^6$	$1{,}00 \times 10^{-2}$
Rm1021	$1{,}26 \times 10^9$	$1{,}70 \times 10^8$	$2{,}40 \times 10^6$	$1{,}41 \times 10^{-2}$
BM7	$1{,}45 \times 10^9$	$4{,}50 \times 10^8$	$2{,}30 \times 10^7$	$5{,}11 \times 10^{-2}$
BM7	$1{,}42 \times 10^9$	$3{,}50 \times 10^8$	$1{,}80 \times 10^7$	$5{,}14 \times 10^{-2}$
BM7	$1{,}27 \times 10^9$	$3{,}10 \times 10^8$	$1{,}60 \times 10^7$	$5{,}16 \times 10^{-2}$

* number of transconjugants per number of donors.

Data obtained show that inactivation of *hsdR* gene in the mutant BM7 increases significantly, by 3,58 times, the efficiency of conjugation compared to the wild type Rm1021.

In conjugation, the barrier constituted by *hsdR* in strain Rm1021 seems to have a lower effect (in the tested condition), than in transformation, where the effect is of more than 10-fold. However these data demonstrate that *hsdR* (Smc02292) represents a barrier against foreign DNA acquisition by both mechanisms, transformation and conjugation. This finding is actually interesting because sometimes restriction is not considered to be effective against conjugation. In fact during conjugation DNA enters in the cell as single strand and this is considered a protection from restriction since it acts against double strands DNA. Our data, instead, indicate that restriction can act also against DNA acquisition by conjugation, demonstrating its importance as barrier against horizontal gene acquisition in *S. meliloti* Rm1021.

D. DISCUSSION

Sinorhizobium populations are characterized by a high degree of genetic diversity (Carelli *et al.*, 2000; Biondi *et al.*, 2003; Silva *et al.*, 2007) both between species and also within each species. Those observations suggest the presence of barrier to genetic exchange blocking the spreading of the DNA horizontally transferred. In this work horizontal transfer of plasmid DNA between different strains of both species was studied by electroporation to investigate the presence of cytoplasmic barriers in different *S. meliloti* and *S. medicae* strains. Published data on electroporation for *S. medicae* and for *S. meliloti* optimization is missing. Thus the first step was the optimization of the protocol of electroporation for both *S. meliloti* (Rm1021) and *S. medicae* (LMG18864), demonstrating, for the first time, that high efficient plasmid transformation (more than 10^6 cfu/μg of DNA) with gel-purified plasmid DNA from *E. coli* in *S. medicae* is possible and that it is 10^3 times more efficient than the *S. meliloti* transformation. Low efficiency of transformation with plasmid DNA from *E. coli* was already known in *S. meliloti* strains (Hayashi *et al.*, 2000; Vincze & Bowra, 2006), however the efficiency obtained here by electroporation is higher, nearly 30 times, than that obtained using other techniques like freeze-thaw transformation (Vincze & Bowra, 2006).

Transforming *S. meliloti* and *S. medicae* strains with plasmid DNA from *Sinorhizobium* donors gave results leading to different conclusions. Firstly, concerning the efficiency of transformation, in the tested conditions, it represents a variable phenotype among different *S. meliloti* and *S. medicae* strains and this observation could be related to the high level of genetic diversity known in *Sinorhizobium* populations (Carelli *et al.*, 2000; Biondi *et al.*, 2003; Silva *et al.*, 2007). Concerning barriers to gene transfer, we found that only the *S. meliloti* strain Rm1021 discriminates self DNA from foreign DNA. *In silico* analysis of the genome of this strain showed the presence of one type I restriction-modification system (*hsdR* Smc02292). From a previous comparative genomic hybridization experiment (Giuntini *et al.*, 2005), it emerged that strains BL225C and AK58 do not carry the Smc02292 gene; this observation was also confirmed by gene-specific PCR amplification. The same polymorphism was recently observed in another *S. meliloti* strain that also lacked this gene (Stiens *et al.*, 2008). Genes homologous to Smc02292 have not been found in the genome of the *S. medicae* sequenced strain WSM419 and no PCR products were obtained after amplification of the DNA of the *S. medicae* strains tested in this work with Smc02292 specific primers (see

material and methods). This polymorphism of *hsdR* has been previously reported also in other bacterial species as *Helicobacter pylori* (Nobusato *et al.*, 2000) and *Xylella fastidiosa* (Picchi *et al.*, 2006) where it was correlated with the transformability phenotype.

The involvement of Smc02292 gene in the donor selectivity of strain Rm1021 was tested constructing and the mutant strain BM7. Strain BM7 derives from Rm1021 but the *hsdR* gene was disrupted by plasmid integration. Testing mutant BM7 by electroporation showed that the efficiency of transformation with non-self DNA was increased, demonstrating an important role of the gene Smc02292 as cytoplasmic barrier to foreign DNA acquisition in this strain. Furthermore mutant BM7 showed an increase in the efficiency of transformation respect to Rm1021 also when transformed with plasmid DNA from *E. coli* DH5α obtaining $3,73 \times 10^4 \pm 1,19 \times 10^4$ instead of $3,41 \times 10^3 \pm 2,66 \times 10^2$. Mutant strain BM7, however, was transformed less efficiently by foreign DNA than self-DNA. This observation indicates that *hsdR* gene does not represent the sole barrier mechanism of strain Rm1021 toward foreign DNA. Residual barrier could have different possible causes. One possibility could be DNA methylation not related to restriction: as known in enterobacteria, the plasmid replication initiation can depend on the methylation status of the sequences of the origin of replication (as Dam methylation). Another possibility is the presence of more than one restriction-modification system in the strain Rm1021. Data reported here and genome annotation however do not allow to hypothesize which could be the genetic determinants of this further barrier. Conjugation experiments involving *E. coli* S17-1 as donor and the mutant BM7 and the wild type Rm1021 as recipients show that inactivation of Smc02292 increases the efficiency more than three times. This data suggest an involvement of the *hsdR* barrier in horizontal gene acquisition in *S. meliloti* strain Rm1021.

This is the first time that a genetic bases of sexual isolation between bacterial strains of the same species is described in *S. meliloti*, although it was previously observed in other species as *Pseudomonas stutzeri* (Lorenz and Sikorski, 2000; Berndt *et al.*, 2003). Barriers to horizontal gene transfer, able to separate different species and different strains of the same species, can have important consequences at the population level. The ability of *S. meliloti* strain Rm1021 to discriminate self from non-self DNA suggests the occurrence of sexually isolated subpopulations within the *S. meliloti* species. This observation could partially explain the high degree of genetic diversity between S. *meliloti* strains (Carelli *et al.*, 2000; Biondi *et al.*, 2003; Giuntini *et al.*, 2005; Bailly *et al.*, 2006; Silva *et al.*, 2007).

Since restriction-modification systems work to preserve genetic identity and can even support speciation (Jeltsch, 2003), a possible consequence of the results presented here is that Rm1021, with its ability to restrict DNA originated from other *S. meliloti* strains, could be considered the representative of one particular *S. meliloti* subpopulation. Accordingly, it will be essential to study more strains and their phenotypes to increase the understanding of the biology of the whole *S. meliloti* species.

E. MATHERIALS AND METHODS

E.1. MATHERIALS

E.1.1. Bacterial strains and plasmid

Strain or plasmid	Relevant properties*	Reference
S. meliloti		
Rm1021	SU47 *str-21*	Meade *et al.*, 1982
BM7	Smc02292:: pK19mob2ΩHMB kanamycin resistant, Rm1021 *hsdR*⁻ derivative	This work
AK58	isolated from Kazakhstan	Giuntini *et al.*, 2005
BL225C	isolated from Italy	Giuntini *et al.*, 2005
S. medicae		
LMG18864	Isolated from Syria	de Lajudie *et al.*, 1998
SS54	Isolated from Tunisia**	This work
SS55	Isolated from Tunisia**	This work
E. coli		
DH5α	F_ *supE44 _lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (_80*lacZ*_M15)	Hanahan, 1983
S17-1	RP4-2, Tc::Mu,Km-Tn7, for plasmid mobilization	Simon *et al.*, 1983
Plasmid		
pMR20	RK2-derived low copy number broad host range vector. tc^R	Roberts *et al.*, 1996
pK19mob2ΩHMB	pK19mob2 derivative suicide plasmid, for plasmid integration mutagenesis in *S. meliloti*. km^R	Luo *et al.*, 2005
pK19mob2-*hsdR*	pK19mob2ΩHMB with cloned a PCR fragment of 300bp from Smc02292 of Rm1021. km^R	Anke Becker

* abbreviations: km^R, kanamycin, tc^R, tetracycline.
**Both strains were isolated from agricultural Tunisian soil in the vicinity of Soliman by alfalfa trapping (*Medicago truncatula*)

E.1.2. Primers

Name	Sequence (5′-3′)
pSMc02292-full-fv	CTGCCGTCTCCAGTGAAGGT
pSMc02292-full-rv	TTGCCGAGGCTGCGGAATAG

E.1.3. Growth media

All the media were dissolved in distilled water and autoclaved.

LB medium (Luria-Bertrani Broth, (Sambrook *et al.*, 1989))

> 10 g/l Tryptone
> 5 g/l Yeast Extract
> 5 g/l NaCl

TY-medium (Beringer, 1974)

> 5 g/l Tryptone
> 3 g/l Yeast extract
> 0.4 g/l CaCl2

E.1.4. Supplements for growth media

Agar (Oxoid)

For plates 16 g/l were added.

Antibiotics

> Kanamycin (km)
>
> For the selection of kanamycin-resistant *E. coli* clones 50 mg/l of antibiotic were added to solid media; 30 mg/l were added to liquid media to prevent the loss of plasmids.
>
> For the selection of kanamycin-resistant *S. meliloti* clones 200 mg/l of antibiotic were added to solid media; 200 mg/l were added to liquid media.
>
> Tetracycline (tc)
>
> For the selection of tetracycline-resistant *E. coli* clones and *S. meliloti* AK58, BL225C and *S. medicae* SS54 and SS55, 10 mg/l of antibiotic were added to solid media; 10 mg/l were added to liquid media to prevent the loss of plasmids.
>
> For the selection of tetracycline-resistant *S. meliloti* Rm1021 2 mg/l of antibiotic were added to solid media; 1 mg/l were added to liquid media.
>
> For the selection of tetracycline-resistant *S. medicae* LMG18864 4 mg/l of antibiotic were added to solid media; 4 mg/l were added to liquid media.

Streptomycin (Sm)

For the selection of *S. meliloti* strains Rm2011, 600 mg/l of antibiotic were added to both solid and liquid media.

E.1.5. Buffers and solutions

TE-buffer

10 mM Tris-HCl

1 mM EDTA

pH 7.5

10 mM dNTP-mix

10 mM dATP

10 mM dCTP

10 mM dGTP

10 mM dTTP

TEA 50X

242 g TRIS
57,1 g Acetic Acid
100ml EDTA pH.8 (0,5M)
H_2O up to a final volume of 1 liter

Glycerol 10%

100ml of Glycerol 99,9% (Sigma)

900ml distilled water

Physiological solution

0,85 % (W/V) of NaCl.

E.2. METHODS

E.2.20. Cultivation of bacteria

Bacteria were grown using solid media, as well as liquid media. In special cases, the media were supplemented with additives listed above (D.1.4). *E. coli* cells were grown at 37°C in LB. *S. meliloti* cells were grown at 30°C in TY. Liquid cultures for both species were also shaken at 180 RPM.

E.2.21. Storage of bacterial strains

Bacteria were grown in the liquid medium overnight until the optical density (OD_{600}) was more than 1 OD, then 500 µl of bacterial culture were mixed with 500 µl of glycerol 50% and stored at -80°C.

E.2.22. Determination of viable title

Sinorhizobium cells viable titles were determined by serial 10-fold dilutions. 100 microliter of the appropriate dilutions were then plated on non selective plates using sterile handles. Plates were then incubated at 30°C until colonies were grown. Each title was determined from the average of the number of colonies of at least two equivalent plates.

Percentage of survival was determined as the ratio between the title of total viable cells after treatment and that of viable cells without treatment. Efficiency of transformation was expressed as the number of transformants per microgram of DNA.

E.2.23. Preparation of *Sinorhizobium* electrocompetent cells

The following protocol was applied as described by Dower *et al.* (1988) modified increasing the washing steps, to ensure removal of salts.

- From an over-night culture of *Sinorhizobium* cells grown in TY medium at 30°C. Inoculate 500 ml (two 1L-flasks with 250ml each) of TY medium to an optical density of 0,2 OD.
- Incubate the culture at 30°C to an optical density of 0.6-0.7 OD.
- Chill the culture in ice for 15 min.
- Pellet the cells centrifuging 5 min at 8000 RPM at 4°C and discard the supernatant.

- Resuspend the pellet with 500ml of sterile distilled H_2O by vortexing and centrifuge again.

- Repeat the last step.

- Resuspend the pellet with 500 ml of sterile 10 %glycerol solution by vortexing and centrifuge again.

- Resuspend the pellet in a final volume of 2 ml of 10 % glycerol and aliquot (50 µl) the suspension in eppendorf tubes. Store at -80°C.

  NB. Cell number of each 50 µl aliquot will range between 10^9 and 10^{10}.

E.2.24. Electroporation of *S. meliloti* and *S. medicae*

All electroporations were performed using sterile electroporation cuvette (inter-electrode distance of 0.1 cm) supplied by Molecular BioProducts and a Gene Pulser® Apparatus connected to the Pulse controller, version 2-89 supplied by Biorad.

- Thaw in ice electrocompetent cells, about 10 min.

- Add DNA in a volume of no more than 4-5 µl and mix well by vortexing few seconds. Keep in ice.

  All the following steps were performed quickly.

- Transfer the suspension in a new electroporation cuvette.

- Place immediately in the Gene Pulser apparatus and apply to the sample the appropriate impulse.

- Immediately after pulse application resuspend the cells with 1 ml of TY medium and transfer in a 13 ml tube.

- Incubated at 30°C, with shaking at 200 RPM, for 4h without any antibiotic.

- Proceed by plating aliquots from serial dilutions on non-selective and selective medium.

- Incubate at 30°C until colonies are grown.

E.2.25. Targeted mutagenesis of *S. meliloti* Rm1021.

This method was used to generate the mutant strain BM7. Smc02292 gene was mutagenized by plasmid insertion using a suicide plasmid through homologous recombination, as described by Luo *et al.* (2005). The plasmid, kindly provided by A. Becker (Molekulare Genetik, Institut für Biologie III, Albert-Ludwigs-Universität Freiburg), was derived from the

suicidal plasmid pK19mob2ΩHMB (Luo *et al.*, 2005) and carries a PCR fragment of 341 nucleotides of the middle part of Smc02292 gene (position 1041-1382 nucleotides downstream the start codon) inserted into the *Hind*III-*BsrG*I site. The resulting plasmid is not self replicable in Rm1021 and confers the resistance to kanamycin. Plasmid was moved into the wild-type strain *S. meliloti* Rm1021 by biparental mating using the *E. coli* strain S17-1 (Simon *et al.*, 1983).

Conjugation to obtain plasmid integration (biparental mating)

- The *E. coli* S17-1 cells containing the plasmid for disruption of the *hsdR* gene were grown overnight in liquid LB medium supplemented with kanamycin.
- *S. meliloti* Rm1021 cells were grown overnight in liquid TY supplemented with streptomycin.
- Dilute the cultures to an OD_{600} of 1.0 and take 4 ml of *S. meliloti* cells suspension and 2 ml of *E. coli* cells suspension.
- Pellet the cells centrifuging 8000 RPM for 2 min.
- Wash twice with 1 ml of physiological solution.
- Resuspend, mixing together, the pellets with 100 µl of physiological solution and apply the drop in a TY plate without antibiotic.
- Incubate overnight at 30 °C.
- Resuspend cells in 1ml of physiological solution using a sterile handle.
- Proceed by plating.

Selection of conjugants was done on TY plates plus streptomycin, to counter-select *E. coli* donor strain, and plus kanamycin to select the targeted integration of the whole plasmid by single crossing-over in the genome of Rm1021.

E.2.26. Isolation and purification of plasmid DNA.

Isolation

The system used to obtain cell lysis and plasmid extraction is the NucleoSpin® Plasmid kit supplied by Macherey-Nagel. Supplier's instruction were followed for *E. coli*, while for *Sinorhizobium* the following changes were applied:

5) Each pellet of cells was obtained from 5ml of a dilution to 1.0 OD_{600} of an overnight culture.

   N.B. Only one pellet was sufficient to check the presence of the plasmid from Sinorhizobium. 20 pellets were instead produced for large scale preparations of plasmid and subsequent gel purification.

6) Time for Lysis (after addition of buffer A2 and before addition of buffer A3) was extended to 10 min.

7) Time of centrifugation to pellet cell debris (after added buffer A3) was 10 min.

8) Each single column was used to bind DNA from two pellets

   N. B. step 4 only for large scale preparations.

9) Elution of DNA was performed with 55 µl of nuclease free dH_20 (Ambion) incubating 1 min at room temperature and then centrifuging as indicated. After centrifugation 50 µl of the eluate was recovered and loaded again in the same column incubating 1 min at room temperature and then centrifuging again.

Purification

- The product of 20 pellets extractions was concentrated by Microcon® YM-30 (Millipore) in a volume of about 20µl.

- Concentrated DNA was all loaded and run on 0.6 % agarose gel (D.2.8.).

- Covalently closed circular DNA was excised from gel and DNA purified from agarose using QIAquick® Gel Extraction kit (Quiagen) according with manufacturer's instructions.

- DNA was then visualized and quantified as described in D.2.8. on 0,8 % agarose gel.

- DNA was stored at 4 °C and not frozen to avoid breaking of circular DNA.

E.2.27. Visualization and quantification of DNA

Agarose gel electrophoresis

This method is used for visualization, as well as quantification of double-stranded DNA. The agarose gels were prepared at 0.6 - 1 % (W/V).

- Mix the agarose with TEA 1X buffer and boil till dissolved.

- Chill the agarose-TEA to 50 °C, add Ethidium Bromide solution to a final concentration of 1mg/L and pour in a horizontal chamber with a comb.

- After the gel becomes solid, cover it with TEA buffer and remove the comb.

- Mix the DNA with BBF loading buffer 6X and load into the wells of the gel.

- Run the gel using the voltage of 100 V.

- Image of gel was collected making a photograph over UV light transilluminator with red filter.

Quantification of DNA

The concentration and purity of DNA was determined calculating the UV_{260} absorbance using spectrophotometer (BioPhotometer, Eppendorf). Analysis was performed using a programmed method as recommended by the manufacturer.

E.2.28. Bacterial cells lysate

From a fresh ON colony cells were resuspended in 20 µl of dH_2O (alternatively 20 µl of fresh ON liquid culture), heated for 10 min at 99°C and then placed in ice for at least 3 min. Thus, 80 µl of dH_2O were added. Bacterial lysates were stored at – 20°C until use.

E.2.29. Polymerase chain reaction

Polymerase chain reaction is a technique for enzymatic replication of DNA *in vitro.* PCR uses two primers that anneal to the forward and reverse strands at the ends of the DNA fragment which has to be amplified. The cycles of DNA denaturing - primer annealing – and primer extension, permit amplification of the targeted DNA fragment. AccuPrime Pfx DNA polymerase (Invitrogen) was used in all PCR reactions in this work.

PCR mixture:
(Prepared in ice)
1X AccuPrime Pfx reaction mix
10 µM of each primer
1U AccuPrime Pfx DNA polymerase
2 µl of bacterial lysate (see D.2.9.)
dH_2O up to 25 µl

PCR amplification program
- 95°C for 2 min
- 35 cycles as follow:
    95°C 15 sec
    58°C 30 sec
    68 °C 3,5 min
- Hold to 8°C

E.2.30. Conjugation

- Grow ON *S. meliloti* strains in TY medium. Grow ON *E. coli* S17-1 containing the plasmid pMR20 in LB medium supplemented with tetracycline.

- Direct count the titer of the cells with Burker's chamber, under microscope.

- Take at least 10^9 *Sinorhizobium* cells and 0,5 x 10^9 cells of *E. coli* cells (ratio 2:1) for each single mating sample.

- Centrifuge separately donor and recipient 8,000 RPM for 5 min to pellet the cells and discard the supernatant.

- Wash the pellets twice with 0,85% NaCl solution, centrifuging as above.

- Mix recipient and donor cells and pellets again centrifuging as above.

- Resuspend the pellet in a final volume of 0,1 ml of 0,85% NaCl solution.

- Transfer the mating cells on a TY plate well dried and incubate at 30°C for 4 hours.

- Recover the cells from the plate with a sterile handle and resuspend in 1 ml of 0,85% NaCl solution.

- Prepare serial dilutions and spread-plate different aliquots on selective and non-selective medium to estimate the titers of donor cells, recipient cells, and transconjugants.

- Incubate plates at 30°C until colonies are grown.

- Evaluate efficiency of conjugation as the ration between the number of transconjugants and the number of donor cells.

N.B. Usually three days are sufficient to grow colonies of S. meliloti Rm1021, but incubating four-five days assure to estimate correctly the real titers after conjugation.

E.2.31. Statistical analysis

All treatments were performed in triplicate. The significance of difference among means was assessed by a non-parametric Kruskal-Wallis test, followed by Bonferroni error protection with a significance level at $p<0.0001$ by using Analyse-it for Microsoft Excel (Analyse-it Software, Ltd.).

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