‘I use this term [struggle for existence] in a large and metaphorical sense including dependence of one being on another, and including (which is more important) not only the life of the individual, but success in leaving progeny.’

Darwin (1859)

Genetic and functional variability in plant associated bacteria

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Papers

Peer-reviewed

1. Trabelsi D, **Pini F**, Bazzicalupo M, Biondi EG, Aouani ME and Mengoni *Development of a cultivation-independent approach for the study of genetic diversity of Sinorhizobium meliloti populations*. Molecular Ecology Resources (2010) 10, 170–172
2. Mengoni A, **Pini F**, Huang LN, Shu WS, Bazzicalupo M. *Plant-by-plant variations of bacterial communities associated with leaves of the nickel hyperaccumulator Alyssum bertolonii Desv.* Microb Ecol. (2009) Oct;58(3):660-7.
3. Trabelsi D, **Pini F**, Aouani ME, Bazzicalupo M and Mengoni A. *Development of Real-Time PCR assay for detection and quantification of Sinorhizobium meliloti in soil and plant tissue*. Lett Appl Microbiol. (2009) Mar;48(3):355-61.

Submitted

1. **Pini F,** Biondi EG, Bazzicalupo M, Frascella A, Santopolo L, Scotti C and Mengoni A. *Exploring the bacterial community associated with the leguminous plant Medicago sativa*

In-preparation

1. **Pini F**, Ferri L, Frage B, Fioravanti A, Brilli M, Mengoni A, Bazzicalupo M, Becker A and Biondi EG. *The cell cycle kinase DivJ in Sinorhizobium meliloti*

Book chapter

1. Mengoni A, **Pini F**, Bazzicalupo M. *The Bacterial Flora of the Nickel-hyperaccumulator Plant Alyssum bertoloni.* In "Biomanagement of Metal Contaminated Soils", Md. Saghir Khan Ed., Springer-Verlag, The Netherlands (submitted)

Abstract

A wide range of bacteria interact with plants, these microorganisms grow in and around roots, in the vasculature, and on aerial tissues. Plant-associated bacteria can be divided in phyllospheric, rhizospheric and endophytic, the last two being the most studied. Two specific classes of plant-interacting bacteria were objects of my studies: endophytic and symbiotic bacteria. The first part of my work was focused on the variability of endophytic bacterial communities associated with plant tissues of *Alyssum bertolonii*, a Nickel hyperaccumulator plant endemic of serpentine outcrops of Central Italy. In parallel I moved my attention on bacteria associated with alfalfa plants (*Medicago sativa*). Leguminous in their evolution developed novel system of interaction with rhizobia forming special structures on the roots called nodules. Inside nodules, rhizobial cells become elongated and polyploid and are called bacteroids, a differentiated form able to fix nitrogen. These dramatic changes are induced by the plant determining a loss of viability of bacteria. Actually, the current model for life-style of rhizobia is based on the alternation of free-living, and symbiosis. In classical studies, due to the biased bacterial sampling (only strains from nodules are usually isolated), the role and evolutionary significance of free-living and nodule-forming strains in a given *Sinorhizobium meliloti* (the principal symbiont of alfalfa plants) population cannot be satisfactorily clarified. Moreover despite the large number of data on the molecular basis of plant-bacterium interaction, the taxonomic diversity and the ecological roles of bacterial endophytes in leguminous plants are still not clear, especially with relation to the nitrogen-fixing symbiotic partners. During my Ph.D. work on rhizobia-legume symbiosis, first of all I analysed the bacterial community associated with alfalfa plants and the genetic diversity of *S. meliloti* populations associated with plant tissues and soil; for this purpose two specific molecular tools were also developed. Data revealed a great biodiversity of the endophytic community and a high incidence of *Alpha-Proteobacteria* in plant tissue, identifying a clear differential pattern of bacterial community diversity between soil and plant tissues. This pattern was conserved also at the taxonomic level of family revealing the presence of specific group (e.g. *Sphingomonas, Methylobacterium* in shoots). Moreover we found a different pattern also at sub-species level when considering *S. meliloti* populations, resulting in a significant difference between soil and nodulating strains.

Another aspect of this research was to evaluate the endophytic abilities of *S. meliloti*. So we set up an hydroponic system to test four strains: *S. meliloti* Rm1021 wild type strain, a mutant derived from Rm1021 defective for nodulation and two natural strain AK83 and BL225C (all GFP tagged). We demonstrated that the nodulation defective mutant can endophytically colonize the plant and it does it even better than wild type strain, suggesting the existence of an additional life-style, the endophytic, alternative to the symbiosis and to the soil, that could explain the coexistence of strains with very different characteristics.

The fourth part of this work was focused on the investigation of the mechanisms that drive the differentiation of *S. meliloti* in bacteroids. Bacteroids are characterized by an enlargement of cell shape and by endoduplication of the genome uncoupled from cell division; these observations have suggested that the regulation of cell cycle progression may be involved in the differentiation process. Based on previous analyses, I assumed that the *Caulobacter crescentus* model of regulation of cell cycle could be valid also for *S. meliloti*. In *Caulobacter* the principal regulator of the cell cycle is CtrA (1) that is inhibited by another regulator called DivK in a cell cycle dependent fashion. The activation of DivK depends on the histidine kinase DivJ while PleC is its principal phosphatase. I preliminarily analyzed the role of the DivJ ortholog in *S. meliloti* demonstrating that *divJ* is not essential, but the deletion strain (Δ*divJ*) resulted in a reduced growth rate and in a dramatic cell elongation and branching. Moreover *S. meliloti* Δ*divJ* is able to form normal-shaped nodules but inefficient (not fixing nitrogen). This phenotype could be related to a defect in the differentiation process or to the reduced ability to fix nitrogen. I hypothesize that the reduced efficiency of nitrogen fixation of the *divJ* mutant is due to an enhanced activity of CtrA; so DivJ controlling CtrA phosphorylation is indirectly involved in bacteroid differentiation.

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products: *MtNFP; MtDMI1; MtDMI2; MtDMI3; MtNSP1; MtNSP2; MtCRE1;* and *MtNIN*. *MtLYK3/HCL* is required for colonized curled root hair (CCRH) formation, but not for the induction of cortical cell divisions. The required rhizobial genes are boxed in brown and the required plant genes are boxed in light green. From Jones *et al.*, see also ref therein……20

Figure I.9. Root hair invasion by *Sinorhizobium meliloti*. a) *S. meliloti exoY* and *Medicago trunculata* MtLIN and MtNIN are required for infection thread initiation. b) *S. meliloti exoH* and M. trunculata *MtNFP, MtLYK3/HCL, MtBIT1/ERN, MtNIN* and *MtCRE1* are required for infection threads to extend to the base of the root hair cell. c) *MtCRE1, MtBIT1/ERN, MtRIT1* and *MtSLI* are required for infection thread penetration into the underlying cell layers. The required rhizobial genes are boxed in brown and the required plant genes are boxed in light green. a,b,c figure are taken from Jones *et al.*, see also ref therein. d) Invasion of the roots of alfalfa (red) by cells of *S. meliloti* that over-express the green fluorescent protein (GFP). This root hair contains a double strand of rare infection. From Gage *et al*……22

Figure I.10. Endocytosis of bacteria and bacteroid differentiation. Bacterial endocytosis requires the *Sinorhizobium meliloti hem*A gene, the *Medicago truncatula NIP* gene and wild-type expression levels of the *MtDMI2* and *MtHAP2-1* genes. *S. meliloti lpsB* and *bacA* are required for bacterial survival within the symbiosome membrane. *S. meliloti fixJ*, *M. truncatula MtSYM1, MtDNF1, -4, -5* and *-7* , and pea (*Pisum sativum*) *PsSYM13* are required for bacteroid differentiation. The *S. meliloti nifHDK* genes encode nitrogenase and are required for nitrogen fixation. The pea *PsRUG4* gene encodes sucrose synthase and is required to support bacteroid nitrogen fixation. The *M. truncatula MtDNF3* and *-6* genes are required for the maintenance of nitrogen fixation. The required rhizobial genes are boxed in brown and the required plant genes are boxed in light green.From Jones *et al.*, see also ref therein……..…23

Figure I.11. Schematic representation of the rhizobium cell cycle at different stages of symbiosis. (*a*) The *S. meliloti* cell cycle is modeled after that of the alphaproteobacterium *Caulobacter crescentus* (see section I.6). A cell division cycle is comprised of three distinct phases: G1, S, and G2. Chromosome segregation begins during S phase and continues in G2 phase. Cell division begins in G2 phase and is completed before the next DNA replication initiation event. During free-living growth, *S. meliloti* is thought to initiate DNA replication only once per cell cycle and divides asymmetrically to produce daughter cells of different size. In analogy to *C. crescentus*, the small daughter cell likely proceeds into G1 phase while the larger daughter cell directly re-enters S phase. (*b*) *S. meliloti* proliferating in the IT originate from a clonal expansion of founder cells entrapped in the tip of the root hair curl. Cells appear to lack flagella and are loosely associated with one another in a pole-to-pole manner, typically forming two or three columns with a braided appearance. Active propagation of bacteria is observed only in a limited area called the growth zone near the tip of the IT, while bacteria outside of the growth zone do not grow or divide. It seems likely that the restricted growth of bacteria enables synchronization of bacterial growth with extension of the IT. (*c*) Bacteria colonize the cytoplasm of plant cells located in the invasion zone. Bacteria are surrounded by a plant-derived membrane and differentiate into a bacteroid. Orange lines, host plasma membrane; green lines, host cell wall. (*d*) A model of the *S. meliloti* cell cycle in planta has three possible exits from S phase, two of which (*in blue*) represent an exit from the typical free-living cycle (*in red*). Bacteria within the infection thread are thought to progress through the cell cycle in the same manner as free-living cells, and in particular transition from S phase into G2 phase (represented by arrow 1). Bacteria that undergo bacteroid differentiation undertake the process of endoreduplication and therefore re-enter G1 phase after the completion of S phase (represented by arrow 2); the bacteria may cycle from S to G1 multiple times during endoreduplication. Once endoreduplication is complete, the bacteroid enters a

Chapter I

Introduction

I.1 Plant-associated bacteria: who, why, for what?

A diverse range of bacteria, including pathogens, mutualists, and commensals is supported by plants. These bacteria grown in, and around roots, in the vasculature, and on aerial tissues(1). Most bacteria that are associated with plants are saprotrophic and do not harm to the plant itself, and only a small number of them is able to cause disease (2). Plant-associated bacteria can be divided in phyllospheric, rhizospheric and endophytic, the last two being the most studied.

The rhizosphere is the important terrestrial habitat that contains living plant roots and closely associated soil where plant exudates stimulate microbial metabolism and productivity. In turn, the activities of the rhizosphere microbial community significantly influence many aspects of plant physiology and growth, and therefore are important for terrestrial ecosystems and agriculture. Plants provide rhizosphere microbes with carbon sources. In turn, microbes may provide nitrogen and phosphorous and also protect plants from parasites and pathogens. Root–microbial interactions play key roles in several other ecosystem functions, such as decomposition of organic matter, and the maintenance of soil structure and water relationships. The role of root-associated microbes in maintaining soil structure (i.e. aggregate stability) has also been documented (4). There is accumulating evidence that biotic interactions, occurring below ground, play an important role in determining plant diversity above ground by direct feedback on host growth and indirect effects on competing plants (4).

Table I.1. List of recovered endophytic species. Modified from Rosenblueth and Martinez-Romero (8), Rajkumar *et al.* (7) and Ryan *et al.* (6).

Endophytes	Plant species
α-Proteobacteria	
Azorhizobium caulinodans	Rice
Azospirillum brasilense	Banana
Azospirillum amazonense	Banana, pineapple
Bradyrhizobium japonicum	Rice
Devosia sp.	*Thlaspi caerulescens*
Gluconacetobacter diazotrophicus	Sugarcane, coffee
Methylobacterium mesophilicum	Citrus plants; *Thlaspi goesingense*
Methylobacterium extorquens	Scots pine, citrus plants; *Thlaspi goesingense*
Methylobacterium populi BJ001	*Populus deltoides x nigra* DN34
Methylobacterium oryzae sp. *CBMB20*	*Oryza sativa*
Methylobacterium sp.	*Thlaspi caerulescens*
Phyllobacterium sp.	*Thlaspi caerulescens*
Rhizobium leguminosarum	Rice
Rhizobium (*Agrobacterium*) *radiobacter*	Carrot, rice
Sinorhizobium meliloti	Sweet potato
Sphingomonas paucimobilis	Rice
Sphingomonas sp.	*Thlaspi caerulescens; Thlaspi goesingense*
β-Proteobacteria	
Azoarcus sp.	Kallar grass, rice
Burkholderia pickettii	Maize
Burkholderia cepacia	Yellow lupine, citrus plants
Burkholderia sp.	Banana, pineapple, rice
Burkholderia sp. *Bu61 (pTOM-Bu-61)*	Poplar
Chromobacterium violaceum	Rice
Herbaspirillum seropedicae	Sugarcane, rice, maize, banana
Herbaspirillum rubrisulbalbicans	Sugarcane
Herbaspirillum sp.*K1*	Wheat
γ-Proteobacteria	
Citrobacter sp.	Banana
Enterobacter spp.	Maize; *Nicotiana tabacum*
Enterobacter sakazakii	Soybean
Enterobacter cloacae	Citrus plants, maize
Enterobacter agglomerans	Soybean
Enterobacter asburiae	Sweet potato
Erwinia sp.	Soybean
Escherichia coli	Lettuce
Klebsiella sp.	Wheat, sweet potato, rice
Klebsiella pneumoniae	Soybean
Klebsiella variicola	Banana, rice, maize, sugarcane
Klebsiella terrigena	Carrot
Klebsiella oxytoca	Soybean
Pantoea sp.	Rice, soybean
Pantoea agglomerans	Citrus plants, sweet potato
Pseudomonas chlororaphis	Marigold (*Tagetes* spp.), carrot
Pseudomonas putida	Carrot
Pseudomonas fluorescens	Carrot, *Brassica napus*
Pseudomonas citronellolis	Soybean
Pseudomonas synxantha	Scots pine

Pseudomonas viridiflava	Grass
Pseudomonas aeruginosa strain R75	Wild rye (Elymus dauricus)
Pseudomonas savastanoi strain CB35	Wild rye (Elymus dauricus)
P. putida VM1450	Poplar (Populus) and willow (Salix)
Pseudomonas fulva	*Nicotiana tabacum*
Pseudomonas sp	Populus cv. Hazendans and cv. Hoogvorst; *Alyssum Bertolonii, Nicotiana tabacum*
Salmonella enterica	Alfalfa, carrot, radish, tomato
Serratia sp.	Rice
Serratia marcescens	Rice, Rhyncholacis penicillata
Stenotrophomonas sp.	Dune grasses (*Ammophila arenaria* and *Elymus mollis*); *Nicotiana tabacum*
Firmicutes	
Bacillus spp.	Citrus plants; *Alyssum bertolonii; Thlaspi goesingense*
Bacillus megaterium	Maize, carrot, citrus plants
Clostridium	Grass *Miscanthus sinensis*
Clostridium aminovalericum	*Nicotiana tabaccum*
Desulfitobacterium metallireductans	*Thlaspi goesingense*
Paenibacillus odorifer	Sweet potato
Paenibacillus polymyx	Wheat, Lodeg pine, green beans,*Arabidopsis thaliana, Canola*
Paenibacillus sp.	*Alyssum bertolonii*
Staphylococcus saprophyticus	Carrot
Staphylococcus sp.	*Alyssum bertolonii*
Bacteroidetes	
Flavobacterium sp.	*Thlaspi goesingense*
Sphingobacterium sp.	Rice
Sphingobacterium multivorum	*Thlaspi caerulescens*
Actinobacteria	
Arthrobacter globiformis	Maize
Arthrobacter sp.	*Alyssum bertolonii*
Blastococcus sp.	*Thlaspi goesingense*
Curtobacterium flaccumfaciens	Citrus plants
Curtobacterium sp.	*Alyssum bertolonii; Thlaspi goesingense*
Kocuria varians	Marigold
Leifsonia	*Alyssum bertolonii*
Microbacterium esteraromaticum	Marigold
Microbacterium testaceum	Maize
Microbacterium sp.	*Brassica napus, Alyssum bertolonii*
Mycobacterium sp.	Wheat, Scots pine
Nocardia sp.	Citrus plants
Plantibacter flavus	*Thlaspi goesingense*
Propionibacterium acnes	*Thlaspi goesingense*
Rhodococcus sp.	*Thlaspi caerulescens, Thlaspi goesingense*
Streptomyces	Wheat
Streptomyces griseus	Kandelia candel
Streptomyces NRRL 30562	Kennedia nigriscans
Streptomyces NRRL 30566	Grevillea pteridifolia
Streptomyces sp.	*Monstera* sp.
Sanguibacter sp.,	*Nicotiana tabaccum*

Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plants howing no external sign of infection or negative effect on their host (6), they can be classified as 'obligate' or 'facultative' endophytes in accordance with their life strategies. Obligate endophytes are strictly dependent on the host plant for their growth and survival, besides transmission to other plants could occur only by seeds or via vectors, while facultative endophytes could grow outside host plants (7). In these last years there has been a considerable interest towards the potential application of endophytic bacteria for plant growth promotion and for the improvement of phytoremediation.

Phyllospheric (epiphytic) bacteria inhabits the aerial parts of the plant (leaves, stems, buds, flowers and fruits) eventually affecting plant fitness and productivity of agricultural crops (9). Studies on the composition of bacterial communities on leaves have been numerous but rather limited in scope. It is generally believed that populations of culturable aerobic bacteria on leaves are dominated by a few genera. Epiphytes are involved in processes as large in scale as the carbon cycle (intercepting carbon compounds released directly from plants or removed by sucking arthropods) and the nitrogen cycles (nitrification of ammonium pollutants intercepted by plants; nitrogen fixation) to processes affecting the health of individual plants (10).

Bacteria can have in fact a profound influence on plant health and productivity. Several researches have been conducted on the plant growth-promoting abilities of various rhizobacteria (PGPR) and endophytes that increase plant growth through the improved cycling of nutrients and minerals such as nitrogen, phosphate and other nutrients (6). Under N stressed conditions, rhizobia, a paraphyletic group which falls into two classes of *Proteobacteria* (Alpha- and Beta-Proteobacteria), drive the formation of symbiotic nitrogen-fixing nodules on the roots or stems of their leguminous hosts; the converted ammonia is then used by the plant as a N source (11). Moreover, plant growth can be facilitated by endophytes altering the plant hormonal balance. Several bacteria are able to produce phytohormones such as strains of *Pseudomonas, Staphylococcus, Enterobacter, Azotobacter,* and *Azospirillum* which can release auxins and cytokinins. Moreover some bacterial strains, like, *Methylobacterium oryzae* CBMB20, *Pseudomonas fluorescens*, as well as strains of the nitrogen-fixing symbiont *Sinorhizobium meliloti* and *Mesorhizobium loti*, can decrease the level of ethylene cleaving its precursor by production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (7). Endophytic bacteria influence also plant health, decreasing or preventing the pathogenic effects of certain parasitic microrganisms by the production of antimicrobial compounds. For instance, in *Enterobacter* sp. 638, an endophyte of poplar, genes for the synthesis of the antimicrobial 4-hydroxybenzoate and 2-phenylethanol have been found (12). Many endophytes indeed are members of common soil bacterial genera, such as *Pseudomonas, Burkholderia* and *Bacillus (7)*. In Table I.1 a list of bacterial strains found associated with several plant species is reported. Endophytes can also enhance plant growth and increase plant resistance to heavy metal stress in several ways. Indirect mechanisms are

similar to those described for PGPR (7) such as nitrogen fixation, improving of mineral nutrition (for instance the solubilization of phosphorus into plant-available forms), or increasing resistance or tolerance to biotic and abiotic stresses(6). Moreover, bacteria can directly increase heavy-metals mobilization or lessen heavy-metal toxicity by the production of bacterial siderophore that enhance the supply of iron to the plant(13). Siderophores are organic molecules that show high affinity for Fe(III) ions, but they can also form complexes with other bivalent heavy metal ions that can be assimilated by the plant(7). Cd resistant endophytes isolated from *N. tabacum* seeds decrease the Cd toxicity by increasing the uptake of trace elements (Zn and Fe) by plants (14).

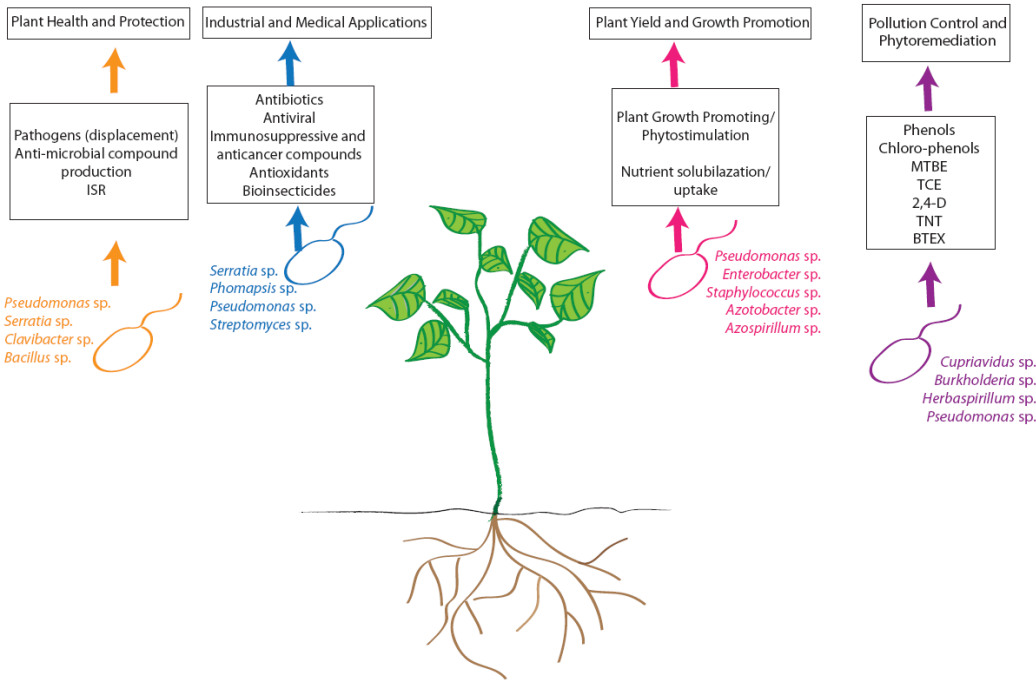


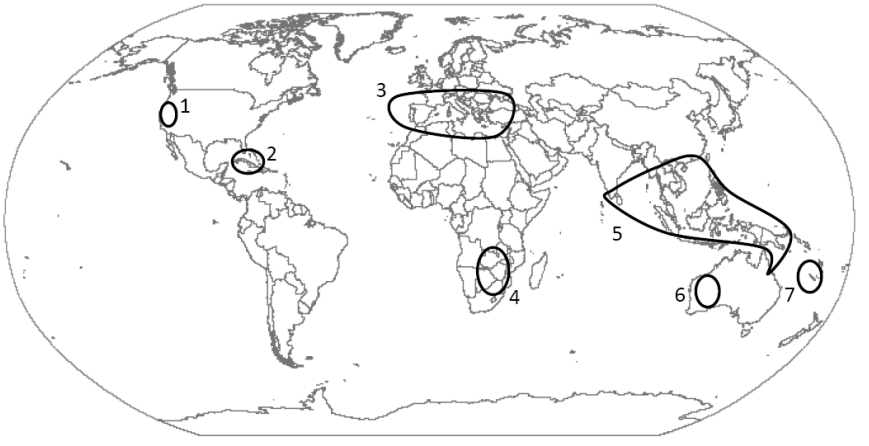
Figure I.1. Possible applications of plant-associate bacteria. Modified from Ryan *et al.* (6)

New challenging goals will be the use of engineered endophytic bacteria to enhance plant growth on polluted soil over phytotoxicity threshold, for instance the pTOM toluene degradation plasmid naturally inserted into the lupine endophyte *Burkholderia cepacia* G4 improve the in planta degradation of toluene and decrease its transpiration to the atmosphere (15). The use of these technologies is at the beginning; stability of the degradation capabilities within the endophytic community (16) and the eventually production of secondary toxic metabolite in the degradation pathway are problems to be fixed well but the use of endophytic bacteria to improve phytoremediation shows great promises (17). A schematic representation of application of plant-associated bacteria is presented in Figure I.1.

I.2 The Nickel-hyperaccumulator Plant *Alyssum bertolonii*

I.2.1 Botany and life history of *Alyssum bertolonii*

Serpentine soils are one of the most famous examples of soils naturally enriched by heavy-metals (Figure I.2).



Number	Area	Main Ni-hyperaccumulating species	References
1	Northern California/Oregon	*Streptanthus polygaloides*, *Thlaspi montanum*	(18)
2	Cuba	*Phyllanthus discolour*, *Phyllomelia coronata*	(19)
3	Southern Europe/Asia Minor	*Alyssum* (several species), *Bornmuellera* (syn. *Ptilotrichum*) *baldaccii*, *Thlaspi goesingense*	(18)
4	Zimbabwe/Zambia/Zaire/South Africa	*Berhkeya coddii*, *Senecio coronatus*	(20, 21)
5	South-East Asia	*Myristica laurifolia*, *Rinorea bengalensis*, *Walsura monophylla*	(18, 22)
6	Western Australia	*Stackhousia tryonii*	(23)
7	New Caledonia	*Sebertia acuminata*, *Xylosma* (several species)	(18)

Figure I.2. Distribution of serpentine outcrops where Ni-hyperaccumulators have been found (modified from Brooks (24)). Areas with serpentine outcrops are encircled with a black line. The table reports the name of the respective geographical areas and of main Ni-hyperaccumulating plant species. Modified from Mengoni *et al.* (25).

They are characterized by high levels of nickel, cobalt and chromium, low levels of N, P, K, Ca, and present a high Mg/Ca ratio, which, in addition, limits plant colonization of these sites (18).

Since the sixteenth century (for a review see Vergnano Gambi (26)), several endemic taxa have been identified within the characteristic flora of serpentine soils throughout the world ((27-29). One of the most interesting features described in serpentine endemic taxa is metal hypertolerance or metal hyperaccumulation (30), a puzzling phenotype

consisting of extremely high foliar metal contents, probably as a defence against herbivory (31). In temperate latitudes the hyperaccumulation trait is mainly found in members of the family *Brassicaceae* (especially in the genera *Alyssum* and *Thlaspi*). The first record of a metal hyperaccumulator was for *Alyssum bertolonii* in which up to 1.2% nickel was found in the leaves (32). Many taxa in genus *Alyssum* have subsequently been shown to accumulate nickel in their aerial parts (see for instance Brooks *et al.* (33)). *Alyssum* is a genus of about 175 species, mainly of Mediterranean Europe and Turkey, with a few species in North Africa, the Near East (Iran, Iraq, and Transcaucasia) and scattered across the Ukraine and Siberia into the northwest of the American continent (Alaska, Yukon). In Europe it is confined to the southern half of the continent and it may well be a pre-glacial relic since its distribution is to the south of areas formerly covered by the ice sheet during the Ice Ages.

One of the most investigated member of this genus is *Alyssum bertolonii* Desv. This is a diploid (2n=16,(34)) perennial plant, living exclusively on serpentine outcrops in Central Italy and particularly in Tuscany (35). *A. bertolonii* is one of the fourteen European species of *Alyssum* that hyperaccumulate nickel (36). The species has been suggested to be a useful indicator plant in prospecting for nickel (37). Moreover, cultivars of *Alyssum* have been proposed for phytoremediation (38) and patented for phytomining practices (39). Phylogeny, population genetics and physiological properties of this species have been deeply investigated (40-43). In particular it has been reported that, tough nickel tolerance and hyperaccumulation are well-known constitutive species-level traits, the extent, or levels, of tolerance and Ni-accumulation are strongly variable among the different populations. Variability of metal-accumulation has been observed on other hyperaccumulating plants also (44, 45). The presence of populations or accessions of the same species having different tolerance and accumulation levels is an important features for improvement of such traits through breeding and for identifying candidate genomic regions or genes responsible for the trait (46). While in *A. bertolonii* these studies are still in progress, for *Arabidopsis lyrata*, a species which present populations locally adapted to serpentine soils, a genome-wide map has recently been provided (47), which identify several candidate loci for serpentine adaptation.

However, it is becoming more and more evident that complex traits, which involves both specific genes and growth features as metal hyperaccumulation, strongly rely under field conditions not only on the genetic background of the plant, but also on the interaction with soil mineral elemental composition and with the indigenous microbial flora. In particular, plant-associated bacteria have been claimed as important factors for the improvement of metal hyperaccumulation and consequently for improving phytoremediation of contaminated soils (48, 49)

I.2.2 Soil and rhizosphere bacteria

Serpentine soil bacteria were described by Lipman in 1926 who, in an attempt to identify the reasons for the low fertility of serpentine soils, wrote: "*there is little diversity, as well as a general paucity, in the bacterial flora of the serpentine soils*" (50). However, it still is not clear if certain bacterial taxonomic groups are inhibited or favored by the serpentine soil conditions (51-53). Moreover, metal-hyperaccumulating plants have been proposed as a selective factor toward soil bacteria, increasing the level of metals near. Actually, it has been found that the presence of some plants (i.e. the Ni-hyperaccumulating tree, *Sebertia acuminata*) positively correlated with the presence of Ni-resistant soil bacteria (54). A hypothetical "nickel cycle", driving the evolution of the bacterial community towards a higher percentage of nickel-resistant strains was suggested for such species. The "nickel cycle" leads to an increased nickel concentration in the upper soil layers in the proximity of the plant due to the "pumping" of nickel from deep soil performed by the roots, followed by the translocation of nickel to leaves and then, after the abscission of the leaves, the release of accumulated nickel from the litter. As a consequence of this cycle, top soil layers near the plant contain higher nickel concentrations than those far away from the plant, and consequently exert a stronger selective pressure for Ni-resistance towards soil bacteria. An increased fraction of Ni-resistant bacteria was also observed in the rhizosphere of the Ni-hyperaccumulators *A. bertolonii* (53) (Figure I.3).

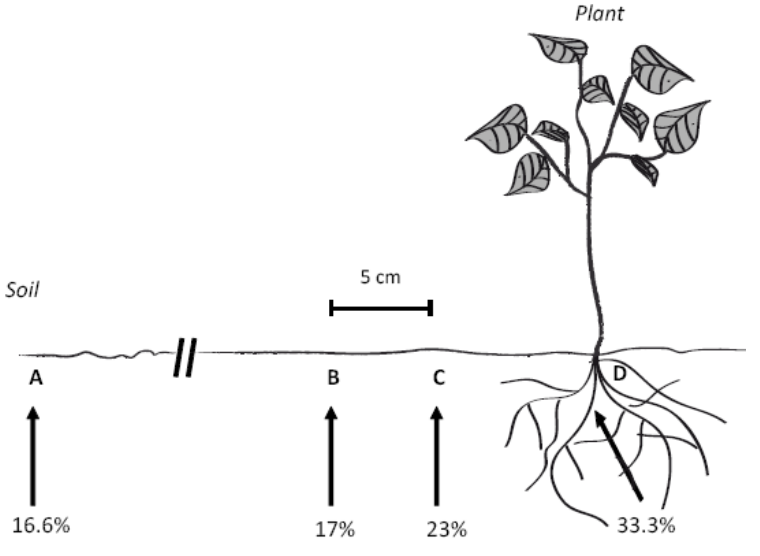


Figure I.3. Proportion of nickel-resistant bacteria at different distances from the Ni-hyperaccumulator *A. bertolonii*. A, bulk soil; B, 10 cm, C, 5 cm, D, rhizosphere soil; Values are percent of resistant bacteria over the total isolates. Adapted from Mengoni *et al.* (53).

This finding was also confirmed in other species as *Thlaspi goesingense* and *A. serpyllifolium* susp. *lusitanicum* and *T. caerulescens* (55-58). However, due to the small size and shallow rooting of plants of these plants (including *A. bertolonii*), it is probably

not correct to invoke a real "metal cycle", that is, an increase of the top soil metal concentration due to the foliar hyperaccumulation of deep-soil metals and subsequent leaf fall. Recently Mengoni, Vangronsveld and Schat (25) proposed a "root-foraging" linked hypothesis, that is a presence of highly tolerant bacteria near the roots of metal hyperaccumulators due to the specific tropism of roots of hyperaccumulating plants toward soil patches rich in metals (59, 60).

Consequently, the presence of highly tolerant bacteria near *A. bertolonii* roots may not be due to plant activity but simply to the chemical parameters of the soil patch that already selected a highly tolerant bacterial flora (Figure I.4). In agreement with such a model (Ni content of soil patches play the main role in the selection of Ni-resistant bacteria) was found in *A. bertolonii* that the proportion of resistant bacteria was different in different outcrops and partially directly related to soil Ni content, that is higher the bioavailable Ni in soil, higher the percentage of Ni-resistant bacteria in bulk soil. From the genetic point of view, despite the selective environment of serpentine soil and rhizosphere, a high genetic diversity was in general found, in contrast with the initial finding by Lipman (50).

However, probably due to the rich culture medium used (LB), mainly copiotrophic species were recovered, and in particular members of genera *Pseudomonas* and *Streptomyces*. Interestingly, *Pseudomonas* isolates were strongly present in the rhizosphere, while *Streptomyces* were predominant in the soil samples, in agreement with a "rhizosphere effect" which favors the presence of genera which include known plant growth promoting bacteria (PGPR). Rhizosphere effect was also shown in an analysis of total bacterial flora by cultivation-independent analysis (61) and the presence of other bacteria groups known to interact with plant roots was detected (i.e. Alpha-Proteobacteria).

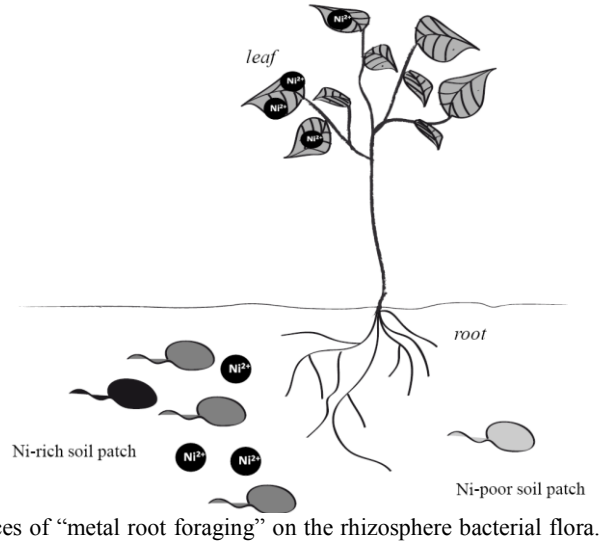


Figure I.4. Consequences of "metal root foraging" on the rhizosphere bacterial flora. Patches of soil rich in metals are already inhabited by a large fraction of Ni-resistant bacteria. Different grey tones suggest possibly different bacterial species. Adapted from Mengoni *et al.* (25).

Another interesting point found in the analysis of serpentine soil bacteria associated with the presence of *A. bertolonii* (53) was the high phenotypic diversity for metal tolerance with the presence of multiple resistance (Ni-Co-Cr) and also resistance to Cu and Zn. The more common phenotypes showed a simultaneous resistance to Ni, Cr and Co. Zn- and Cu-resistant phenotypes were few and associated with resistance to Ni, Cr and Co.
Interestingly, no correlation between genetic groupings and heavy-metal tolerant phenotypes was found. Nevertheless, a higher proportion of *Pseudomonas* strains were resistant to high concentrations of nickel compared to *Streptomyces*, probably reflecting the highest bioavailable Ni present in rhizosphere soil.

I.3 The nitrogen fixation process

The element nitrogen or "azote", meaning "without life", as Antoine Lavoisier called it about 200 years ago, has proven to be anything but lifeless, since it is a component of food, poisons, fertilizers and explosives (62). The atmosphere contains about 10^{15} tonnes of N_2 gas (78% of total volume) and the nitrogen cycle involves the transformation of some $3*10^9$ tonnes of N_2 per year on a global basis (63). However the transformations (e.g., N_2 fixation) are not exclusively biological. Lightning probably account for about 10% of the world's supply of fixed nitrogen (64). For more than 100 years, the biological nitrogen fixation (BNF) has commanded the attention of scientist concerned with plant mineral nutrition, and it is been exploited extensively in agricultural practice (65, 66). However, its importance as a primary source of N for agriculture has diminished in recent decades as increasing amounts of fertilizer-N have been used for the production of food and cash crops (67). The fertilizer industry provides very important quantities of chemically fixed nitrogen. World production of fixed nitrogen from dinitrogen for chemical fertilizer accounts for about 25% of the Earth's newly fixed N_2, and biological process account for about 60%.

The focus of research is based on the use of nitrogen-fixing bacteria as bio-fertilizers in order to respond to an increasing demand of production especially in less developed countries (67). The requirement for fertilizer-N are predicted to increase further in the future (68); however with the current technology the production and the inefficient methods employed for fertilizer application, both the economic and ecological costs (energy consumption and nitrate water contamination) of fertilizer usage will eventually become prohibitive. Moreover, international emphasis on environmentally sustainable development with the use of renewable resources is likely to focus attention on the potential role of BNF in supplying N for agriculture (66, 67).

The entire ecosystem can benefit of nitrogen fixed by rhizobia, which enters in the trophic network through the flow of elements between organisms (69). From an economic point of view these associations are very important: is estimated that the nitrogen fixation in symbiotic association between legumes and rhizobia provides 90 million tons per year of assimilable-nitrogen worldwide. The amount of nitrogen fixed annually by the symbiosis *Sinorhizobium-Medicago*, put in comparison with chemical fertilizers is estimated around $200 million. The economic value of crops of alfalfa in the U.S. is estimated about $ 8.1 billion per year (70). The study of the symbiosis between rhizobia and plants is one of the greatest contributions of microbiology to agricultural applications, designed to improve the yield of leguminous crops and their cultivation as fodder plants, as crops for bioenergy and to recover degraded areas (71-74). Recently, the growth capacity of legumes in association with rhizobia in soils not suited to traditional crops, has also received the attention of biofuels factory. Pilot studies have shown that alfalfa plants could be used as resource for energy production through gasification process and the ashes obtained could be still used as fertilizers (75, 76).

From these data, several research programs targeting the use of alfalfa and other perennial legumes, (or plants that unlike the annual and biennial, continue to live for several years after flowering and withering) on bioenergy production are started. Indeed, one of the major limitations in bioenergy and biofuel production is the great dependence on annual crops of cereals that require an expensive chemical fertilization of the soil to maintain high production.

I.3.1. Alfalfa

Alfalfa (*Medicago sativa*) also called lucerne, or purple medic, is a auto-tetraploid (2n = 4x = 32) (77). perennial, clover-like, leguminous plant of the pea family (Fabaceae), known for its tolerance of drought, heat, and cold; for the remarkable productivity and the quality of its herbage; and for its value in soil improvement. It is widely grown primarily for hay, pasturage, and silage. The plant, which grows 30–90 cm tall, arises from a much-branched crown that is partially embedded in the surface layer of soil. As the plant develops, numerous stems bearing many trifoliolate leaves arise from the crown buds. Racemes of small flowers arise from the upper axillary buds of the stems. With approaching maturity, corkscrew coiled pods containing from two to eight or more seeds develop abundantly in regions with much sunshine, moderate heat, dry weather, and pollinating insects (78). The primary root of alfalfa attains great depths. When 20 or more years of age, this taproot may descend as much as 15 m or more where the subsoil is porous. This accounts for the unusual ability of the plant to tolerate drought. The roots of seedling plants are known to penetrate the soil for 90 cm at two months and for 180 cm with plants five months of age. Not infrequently, newly established fields of alfalfa survive severe summer drought and heat when other leguminous plants with shallower and more branching roots succumb (78). Alfalfa has a remarkable capacity for rapid and abundant regeneration of dense growths of new stems and leaves following cutting. This makes possible from 1 to as many as 13 crops of hay in one growing season. The frequency of harvest and the total seasonal yields are dependent largely on the length of the growing season, the adaptability of the soil, the abundance of sunshine, and especially the amount and distribution of rainfall or irrigation during the growing season. Green leafy alfalfa hay is very nutritious and palatable, containing about 16 percent proteins and 8 percent mineral constituents. In addition it is rich in vitamins A, E, D, and K (78).

This plant exhibits autotoxicity, which means that it is difficult for alfalfa seed to grow in existing stands of alfalfa. Therefore, it is recommended that alfalfa fields be rotated with other species (for example, corn or wheat) before reseeding (79).

Alfalfa is widely grown throughout the world as forage for cattle, and is most often harvested as hay, but can also be made into silage, grazed, or fed as greenchop. Alfalfa has the highest feeding value of all common hay crops, being used less frequently as pasture. When grown on soils where it is well-adapted, alfalfa is the highest yielding forage plant (79).

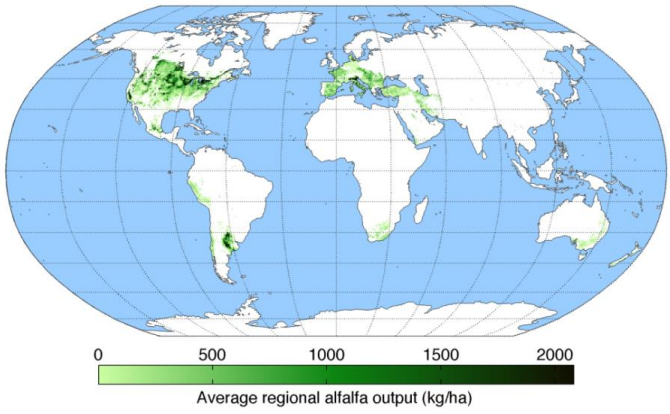


Figure I.5. Worldwide alfalfa production (79).

Alfalfa is the most cultivated legume in the world. Worldwide production was around 436 million tons in 2006. The US is the largest alfalfa producer in the world, but considerable area is found in Argentina (primarily grazed), Australia, South Africa, and the Middle East (79).

Alfalfa in symbiosis with *S. meliloti* can grow in soils low in nitrogen and dry land, where other more demanding plant, such as cereals, can't be cultivated. When used in rotation with other crops, alfalfa increases the diversity of species and interrupt the cycle of pathogens and pests that can affect crops. Furthermore, the different varieties of alfalfa can be used to remove toxins from contaminated soils and aquifers and for the recovery of degraded areas. Moreover, in phytoremediation, alfalfa plants could be used to capture and remove nitrates from depths of soil (80), and as pioneer plants in desertic land, because of their low need of fertilizer and water and their tolerance to salinity. Indeed, while the young alfalfa plants are very sensitive to salts, mature plants are very durable and successfully colonize dry and saline soils (81).

I.3.2. General features of Rhizobia

The BNF is the process by which atmospheric nitrogen N_2 (chemically inert), is enzymatically reduced to ammonia (NH_3), which is metabolically accessible by the plant, through the action of nitrogenase (82). The ability to catalyze the conversion of N_2 to NH_3 evolved only among prokaryotes, a role of particular interest is played by the groups of rhizobia, cyanobacteria, azobacteria, *Frankia* and by some strain of *Archaea* (83). The word *rhizobia* comes from Ancient Greek "*rhiza*" meaning "root" and "*bios*" meaning "life". The rhizobia are Gram-negative bacteria belonging to the rhizosphere microbial community, the region of soil characterized by the presence of plant roots. The rhizobia, a group which comprehend the *Rhizobiaceae* family of the Alpha-proteobacteria subdivision and some genera of Beta-proteobacteria (*Cupriavidus*), are able to fix nitrogen through a symbiosis process with their host plants, belonging to the

Leguminosae family (or *Fabaceae*). The rhizobia-legume symbiosis occurs in nitrogen limiting conditions, this process leads the development of new structures in the plant, the nodules, where atmospheric nitrogen is fixed; it is estimated that the contribution of rhizobia is equal to about half of the total nitrogen biological fixed in the biosphere (84). Thus, the rhizobia, providing reduced nitrogen, helps the growth of the plant. In exchange rhizobia receive nutrients from plant (85), such as sugars and other products of photosynthesis and protection within the structure of the nodule (11). In non-efficient nodules, nitrogen is not fixed, but rhizobia receive the same nutrients, and in this case, rhizobia can be considered as parasites rather than symbionts (86). However, there are metabolic sanctions that plants can apply to non-efficient nodules to limit the development of rhizobial strains which do not fix nitrogen efficiently (87).
The assimilation of nitrogen by plants is of great importance to ecological level because it is an essential process for the growth and the proper development of plants. The plant gets all the nitrogen it needs for the production of proteins and nucleic acids. Symbiotic nitrogen fixation has been used in agriculture to increase growth of leguminous plants used for human food (beans, peas, peanuts, soybeans, etc ...) and legume crops used to produce animal's food or to perform other important functions as balancing the different components of the agrosystem and the maintenance of soil fertility (clover, alfalfa, etc ...). Nodulated plants have higher yield than those of the same species non-nodulated; besides nitrogen fixation is a feature which varies between different species of rhizobia, and crop yields will be greater the more efficient will be its nitrogen-fixing rhizobia. The biological processes that generate this variability can be controlled in order to obtain certain characteristics useful in bacteria (88). These specific features could be optimized to inoculate plants to use in phytoremediation of degraded areas and poor soils.

I.3.3 Taxonomy of Rhizobia

Beijerinck (1888) had first isolated a bacterium from root nodule, which he named *Bacillus radicicola*. The taxonomy, and the nomenclature of the root nodule bacteria, has been in constant review ever since. After Franck (1889) named the bacterium *Rhizobium leguminosarum*, all subsequent species were initially placed in the genus Rhizobium. Then, thanks to more advanced methods of analysis, classification has been revised according to the latest version of the taxonomy, the rhizobia are divided into 13 genera, for a total of 76 specie (89). However, the word rhizobium is still often used as a singular of rhizobia without reference to the taxonomy. The rhizobia belong to two classes of proteobacteria: the Alpha and Beta-proteobacteria. Most of these bacterial species are in the Rhizobiacae family in the Alpha-proteobacteria and are in either the *Rhizobium*, *Mesorhizobium*, *Ensifer*, or *Bradyrhizobium* genera.
The *Sinorhizobium* genus was described by Chen *et al*. in 1988 (90). However some recent studies show that *Sinorhizobium* and the genus *Ensifer* (91) belong to a single taxon. *Ensifer* is the earlier heterotypic synonym (it was named first) and thus takes priority (92). This means that all *Sinorhizobium* spp. must be renamed as *Ensifer* spp.

according to the Bacteriological code. The taxonomy of this genus was verified in 2007 by Martens et. al. (93). The genus currently consists of 15 species.

However recent research has shown that there are many other rhizobial species in addition to these. In some cases these new species have arisen through lateral gene transfer of symbiotic genes (94, 95). Among rhizobia the species *Azorhizobium caulinodans*, represent one interesting exception (96). In fact, beyond to be the only species able to grow to the free state with N_2 as sole nitrogen source, it is also the only one that induces the formation of nodules, on *Sesbania rostrata* plant, not on the roots but on the stem, characteristic from which its name derives.

I.4 The nodulation process

Nodulation is a multistep process in which, following an initial exchange of signal molecules between bacteria and plants (Figure I.6), developing programs of differentiation in both the bacterium and the plant lead to the formation of root nodules and bacteroids (differentiated forms of rhizobia inside the nodule). The whole process is tightly regulated at the genetic level and is developed in several stages, described in the following paragraphs.

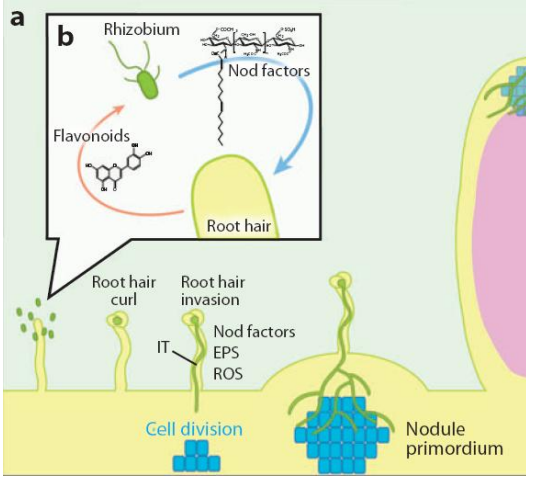


Figure I.6. Schematic model of nodule development (a,b). Host flavonoids exuded into the soil trigger bacterial Nod Factor production. Nod factor is perceived by host receptors and elicits various host responses, such as root hair curling and root hair invasion. Root hair invasion also requires bacteria EPS and host ROS production. Nod factors induce mitotic cell division in the root cortex (represented in *blue*), leading to formation of the nodule meristem. An indeterminate nodule originates from the root inner cortex and has a persistent meristem (Zone I). The nodule also contains an invasion zone (Zone II) and a nitrogenfixing zone (Zone III). In older nodules, a senescent zone (Zone IV) develops in which both plant and bacterial cells degenerate.From Gibson *et al* (97)

I.4.1. Evolution of nodulation

Current evidence suggests that legumes evolved about 60 million years ago. What could the older plant groups provide that legumes could capitalize on to produce nodules? One of the first prerequisites was the ability of the two partners in the symbiosis to recognize each other. This is generally agreed to have developed from the ancient symbiosis between fungi and land plants, arbuscular mycorrhizas (AM) (98). In extant legumes, many of the signals are common between these symbioses. Another process that may have been 'hijacked' is that leading to pollen tube growth. This has much in common with the growth of infection threads down root hairs of both legumes and actinorhizal plants. Recent evidence suggests that gene duplication may have preceded this modification in function (98).

Knowledge on the leguminous evolution and on the onset of nodulation indicates that the first event of symbiosis (both ontogenetically and phylogenetically) is related to bacterial invasion of the host roots through breaks in the epidermis, due to the emergence of lateral roots (often referred to as crack infection) (98). Then were evolved mechanisms of development, often linked to the appearance of new gene functions on paralogous genes, which led to the current symbiosis highly selective and organized. In particular, the

emergence of a structure due to the guest infection could be related to the need to control the process of bacterial infection by plant, to prevent the spread of the bacteria in other tissue causing a pathogenic phenomena. The symbiont, in this context is seen as a "domesticated" pathogen (99).

There are also clear evidence that the genomic capacity of symbiotic rhizobia has in part evolved through horizontal gene transfer events. Among the symbiotic rhizobia, it is estimated that *Sinorhizobium* diverges from *Bradyrhizobium* approximately 500 million years (Mya) (100), which is well before the appearance of legumes, dated to around the end of the Cretaceous (60 Mya) (98). The rhizobia tend to have large genomes subdivided in several replicons consisting of a chromosome integrated with one or more independent plasmids (101), which contribute to the dynamic evolution of the genome through the process of horizontal gene transfer. Furthermore, genes involved in the symbiosis of rhizobia are often placed within chromosomal islands or on plasmids: the genes of *Sinorhizobium meliloti* involved in the biosynthesis of Nod factor (*nod, nol* and *noe*) and nitrogen fixation (*nif* and *fix*) are located on megaplasmid pSymA, while genes involved in biosynthesis of exopolysaccharides (*exo*) and use of C4-dicarboxylic acids (*dct*), which is extremely important in the interaction with plant structures and plant metabolism, are located on chromid (102) pSymB (103). The horizontal transfer of these genomic elements has been observed between bacteria in the rhizosphere and allows the conversion of a non-symbiont in a symbiont by a single transfer event (104-106). In addition regarding symbiosis genes, there is no significant synteny between plasmids of different species of rhizobia or between chromosomes (107, 108), suggesting an origin by transfers, mergers and independent gene rearrangements. A confirmation of the high mobility of genetic elements required for symbiotic nitrogen-fixing, was recently discovered, indeed some *Beta-proteobacteria* are able to establish symbiotic nitrogen fixation with certain species of tropical legumes (109), in addition, phylogenetic analysis support the idea that genes required for symbiosis (*nod* and *nif*), which are located on plasmids of these strains belonging to the genus *Burkholderia* and *Cupriavidus* could be derived from horizontal gene transfer events (110).

Several studies have also revealed a striking similarity between the molecular mechanisms underlying the perception of nodulation factors of rhizobia and molecular structures that are associated with bacterial pathogens of plants (Figure I.7). In fact, many bacteria are able to regulate gene expression in response to changes in population density, a process known as quorum sensing (QS). The QS is mediated by small diffusible signal molecules, as a population of quorum-sensing bacteria grows, a proportional increase in the extracellular concentration of the signaling molecule occurs. When a threshold concentration is reached, the group detects the signaling molecule, called autoinducer, and responds to it with a population-wide alteration in gene expression (111). The most common signal of QS is the N-acetilhomoserin lactone (AHL), which contains a conserved homoserin ring tied to a variable acyl chain. These molecule enter in the cytoplasm where they enable the dimerization of a transcriptional activator of the LuxR type turning it on. Several AHL were identified in both the

rhizobia and in plant pathogens (112, 113). In these bacteria, the QS regulation is also mediated by signals different from AHL. It seems that these signals are detected through two-component regulatory systems. Shortcomings in QS lead to a reduction or a loss of virulence in plant pathogenic bacteria and to an alteration in the efficiency of nodulation and nitrogen fixation in rhizobia (112-114). The two-component regulatory systems, which consist of a sensor kinase and a response regulator, enable bacteria to regulate gene expression in response to environmental changes, enabling them to quickly adapt to new conditions (For details see Box 1 in section I.6.2). Two-component regulatory systems (2-CR) were found in the same plant pathogens and rhizobia, and are essential for successful interaction with their host plants. The plants, however, in response to microbial invasion, can set up a complex defense responses, mediated by signal molecules such as salicylic acid, reactive oxygen species (ROS), nitric oxide, jasmonic acid or ethylene (115). Therefore, the Nod factors of rhizobia are able to avoid the accumulation of salicylic acid and the production of ROS, when they are recognized by the legume host (116-118).

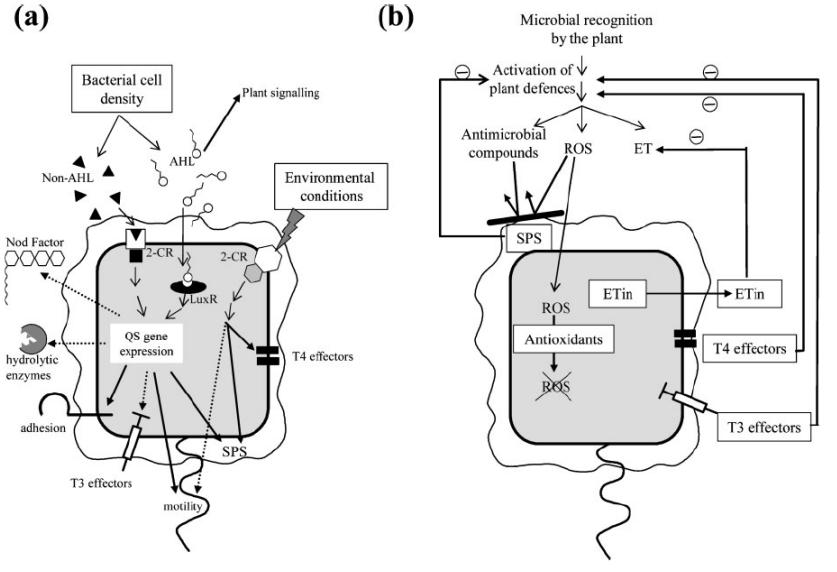


Figure I.7. Common strategies used by plant-interacting bacteria to establish compatible associations with their hosts. (a) Coordination of gene expression for host colonization and invasion mediated by quorum sensing (QS) signals and twocomponent regulatory (2-CR) systems. Detection of N-acylhomoserine lactones (AHL, loop and tail) by cytoplasmic LuxR-type transcriptional activators (black oval), and non-AHL (black triangles) by 2-CR systems (white and black squares), allow plantinteracting bacteria to coordinate the expression of important genes for host colonization and invasion in response to cell density. AHLs play an additional role in plant signalling (see text for details). Regulation of bacterial factors required during the infection process is also accomplished in plant-interacting bacteria by 2-CR systems (white and grey hexagons) which are activated by environmental conditions usually encountered during the invasion process. Common rhizobial and pathogenic bacterial responses are shown by bold arrows whereas responses observed only in one or the other are represented by dotted arrows. (b) Bacterial components used to control plant defence responses. Surface polysaccharides (SPS) are able to suppress microbial-induced defence reactions and/or to act as shields protecting the bacterium against toxic compounds. Additionally, active suppression of the defence reaction is achieved with ethylene (ET) inhibitors (ETin) and virulence factors such as type III and IV secretion systems (T3 and T4). Antioxidant systems protect bacteria against reactive oxygen species (ROS). From Soto *et al*. (120).

On the other hand, several strains of *P. syringae* produce a phytotoxin (coronatine), which suppresses plant defenses based on salicylic acid inducing the jasmonic acid signaling pathways (119). In addition to these strategies to control or actively suppress plant defences, rhizobia and plant pathogens use similar components, such as surface polysaccharides (EPS), antioxidant systems, ethylene inhibitors and specific virulence factors.

For example, strategies to limit the synthesis of ethylene by the plant in response to microbial invasion are taken by some rhizobia and plant pathogens. *Bradyrhizobium Elkana* and the plant pathogen *Burkholderia andropogonis* produce rizobitoxine an inhibitor of ethylene synthesis (121, 122), while several rhizobia produce the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase , which degrades the immediate precursor of ethylene (122). In rhizobia, each strategy leads to an increase in efficiency of nodulation. Moreover, an analysis of available genomes of rhizobia revealed the presence of hundreds of genes homologous to pathogens virulence factors (123). It's interesting to note that the functional characterization of some of these genes, such as those that encode for type III and IV secretion systems (respectively, T3SS and T4SS) indicate a similar role in rhizobia-legume interaction.

Thus, in plant pathogens and rhizobia are present factors such as surface polysaccharides, quorum sensing signals and secretion proteins, which play an important role modulating the plant defense response and in the outcome of the interaction. Therefore, studying these factors, it will be possible to design specific strategies to create pathogens resistant plants and rhizobial strains with improved symbiotic properties (120).

I.4.2. Genes involved in nodulation

The genes involved in nodulation can be divided into four main groups, without considering other auxiliary genes.

***nod* genes**

The nod genes are divided into two groups (124):

- Common *nod* genes are *nodABC*, *nodIJ*, were discovered long ago in studies of *Azorhizobium*, *Rhizobium* and *Bradyrhizobium* (125, 126). These genes are called common because they are structurally conserved and functionally interchangeable between different species without altering the host range (126).
- Host specific *nod* genes are not conserved between the rhizobia. These genes are required for the nodulation of specific host plants (124). In many cases a host-specific mutation in the genes can not be fully complemented by the introduction of orthologous genes from other rhizobia and often causes an alteration or extension of the host specificity (127).

***nif* genes**

It's a group of about twenty genes, and all of them are involved in the synthesis, operation and regulation of the nitrogenase enzyme complex.

***fix* genes**

fix genes are genes essential for the proper functioning of the nodule because coordinate and regulate the process of nitrogen-fixation inside symbiosome.

***enf* genes**

Affect the kinetics and efficiency of nodulation.

I.4.3. Pre-infection

Nitrogen-fixing rhizobia and leguminous plants have developed complex mechanisms for the exchange of signals needed to a specific bacterial species to induce the development of an intrusion structure in its host plant, through which rhizobia can enter in the root. The symbiosis is highly specific: each bacterial species could interact with one or few plant species, this specificity is mainly due to biochemical signals produced by the two symbionts. The rhizobia respond to root exudates and move by chemotaxis toward specific sites localized on the roots (128, 129). Apparently chemotaxis is not a necessary requirement for nodulation, because mutants lacking the flagellum are still able to nodulate normally, although this has an influence on competition and organization in the rhizosphere (130). Flavonoids released by plants are the key signals for the beginning of the root nodules formation (131, 132).

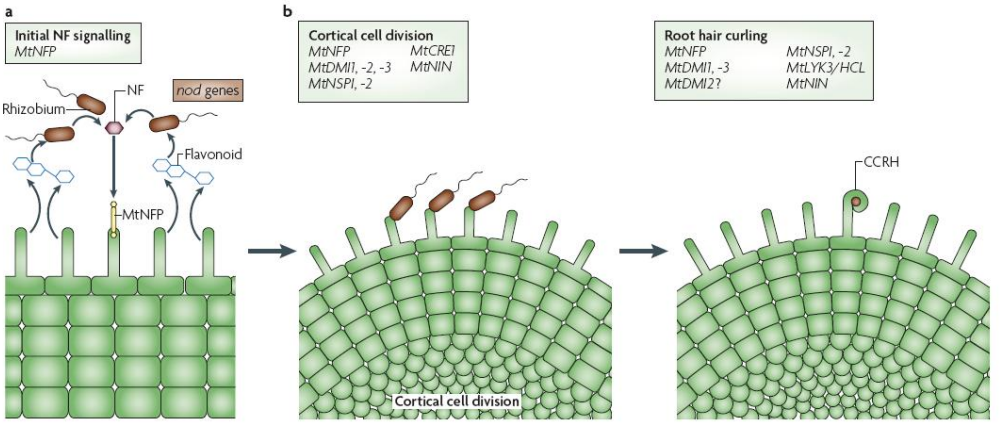


Figure I.8. The initial signalling dialogue between *Sinorhizobium meliloti* and *Medicago truncatula*. a) The induction of rhizobial nod genes requires plant flavonoids. The nod gene products produce Nod factor (NF), which is initially perceived by the *M. truncatula* MtNFP receptor. b) Root hair curling and cortical cell divisions require many *M. truncatula* gene products: *MtNFP; MtDMI1; MtDMI2; MtDMI3; MtNSP1; MtNSP2; MtCRE1;* and *MtNIN*. *MtLYK3/HCL* is required for colonized curled root hair (CCRH) formation, but not for the induction of cortical cell divisions. The required rhizobial genes are boxed in brown and the required plant genes are boxed in light green. From Jones *et al* (136), see also ref therein.

Flavonoids are a class of different compounds produced by secondary metabolism of the plant. They are aromatic compounds with a skeleton of 15 carbon atoms and can be divided into various subclasses based on their structure. Flavonoids, penetrate into the bacterium cytoplasm and interact with NodD a transcriptional activator, which binds DNA in an area upstream the *nod* operons (*nod* box) inducing their transcription. Nodulation genes encode enzymes responsible for the biosynthesis of Nod factor, a lipochito-oligosaccharide. The Nod factor, once produced and secreted by the bacterium, induces deformation of root hairs and guide the entrace of rhizobia during infection thread development (133). *nod*ABC operon genes encode proteins needed to construct the basic body structure of the Nod factor. The products of other *nod* genes (and also *noe* and *nol* genes) produce changes in the Nod factor in order to make it specific to certain host (134, 135).

Multiple receptors containing extracellular domains are essential for a complete response by the plant to Nod factors. For example, in absence of functional gene *MtNFP* (*M. truncatula* Nod factor perception), *Medicago truncatula* can not respond to the signal (Figure I.8).

There are no known specific mechanisms that characterize the secretion of Nod factor, but recent studies have shown that *nodI* and *nodJ* genes produce proteins involved in the secretion of lipo-oligosaccharides(137).

I.4.4. Infection

For many rhizobia primary sites of infection, although not exclusive, are young root hairs (138). The host lectins play an important role for the adhesion of rhizobia to the plant. These lectins are located in root hair apex and is believed could help to maintain the host-symbiont specificity by binding simultaneously the plant cell wall and the carbohydrate portions of compatible bacteria outer surface. The latest studies suggest that cell-cell contact and specific binding of compatible bacteria to root hairs are important for early infection and formation of the infection thread because of an high localized concentration of Nod factors is needed to stimulate the curling of the root hair and the formation of the infection thread (139, 140).When the bacteria adhering to the wall produce the Nod factor and these one is absorbed by root cells, cell growth is stimulated both in roots and root hairs, which undergoes into a curled development followed by the invagination of cell wall trapping rhizobia (Figure I.9a).

After the entrapment, a local lesion of the cell wall will be formed by hydrolysis (Figure I.9b). Inside these tubular structures, formed by the ingrowth of the root hair cell walls from the point of penetration of rhizobia, bacterial invasion proceed to the root cortical cells, developing its growth from internal apex (Figure I.9c,d) .

Although size and shape of the nodules is very different depending on the species of legume, they can be distinguished from an histological point of view, in indeterminate nodules and determinate nodules.

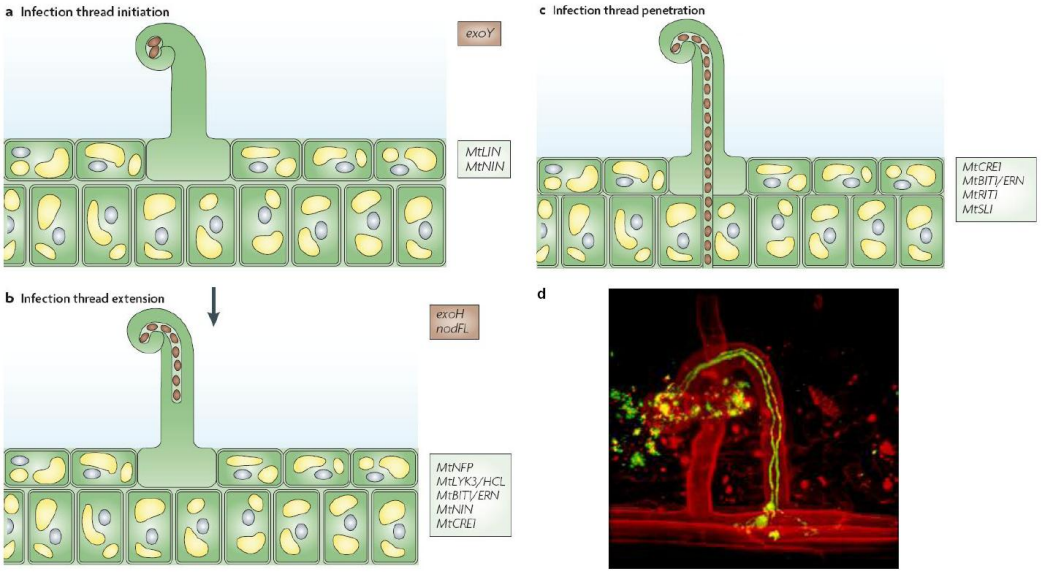


Figure I.9. Root hair invasion by *Sinorhizobium meliloti*. a) *S. meliloti exoY* and *Medicago trunculata* MtLIN and MtNIN are required for infection thread initiation. b) *S. meliloti exoH* and M. trunculata *MtNFP, MtLYK3/HCL, MtBIT1/ERN, MtNIN* and *MtCRE1* are required for infection threads to extend to the base of the root hair cell. c) *MtCRE1, MtBIT1/ERN, MtRIT1* and *MtSLI* are required for infection thread penetration into the underlying cell layers. The required rhizobial genes are boxed in brown and the required plant genes are boxed in light green. a,b,c figure are taken from Jones et al (136), see also ref therein. d) Invasion of the roots of alfalfa (red) by cells of *S. meliloti* that over-express the green fluorescent protein (GFP). This root hair contains a double strand of rare infection. From Gage *et al.* (141).

I.4.5. Nodule development

When the bacteria reach their target tissue, which is the inner bark of the plant, they must be internalized in the cell cortex. Each bacterial cell undergoes endocytosis by a target cell in an individual vesicle in which the membrane is formed by the plasmalemm of plant cells. The entire unit, which consists of a single bacterium and the endocytic membrane that surrounds it, is called symbiosome (142). At this point rhizobia (in indeterminate nodules) undergo into a series of changes and develop into bacteroids: bacteroids are surrounded by the plant membrane, greatly increase their size, assuming a club shape, lose the ability to replicate, moreover their membrane contains many invaginations to improve the metabolic exchanges between the two symbionts, and their cytoplasm is rich in nitrogenase and has more than one nucleoid. Bacteroids are the active form of rhizobia able to fix nitrogen. New lipidic and proteic material attached to the symbiosome membrane assigns a new chemical identity to this compartment (143).
Transcriptional changes in bacteroids consist in downregulation of many metabolic processes in conjunction with an increased expression of gene products involved in nitrogen fixation (144). Bacteroids begin to reduce nitrogen using the ATP-dependent enzyme nitrogenase, to provide to the plant easily assimilable nitrogen. The concentration of O_2 in the infected cells must be strictly controlled because it's a great

inhibitor of nitrogenase, but on the other side the O_2 is required for cellular respiration to provide ATP to the enzyme in addition to the normal metabolic activities of bacteria. Controlling the concentration of oxygen in the nodule is mediated by a globin, the leghaemoglobin. The leghaemoglobin is formed by a heme synthesized by the rhizobia and a globinic part synthesized by the plant cell an example that well simplifies the mutual symbiotic relationship. The bacteroids, throught the action of nitrogenase, produce ammonia, which is released by diffusion through the membrane that surrounds them in the cytoplasm of the host cells, where it enters in the metabolic synthesis of the glutamine. The energy used to reduce a molecule of atmospheric nitrogen into two molecules of ammonia is about 16 molecules of ATP. (145)

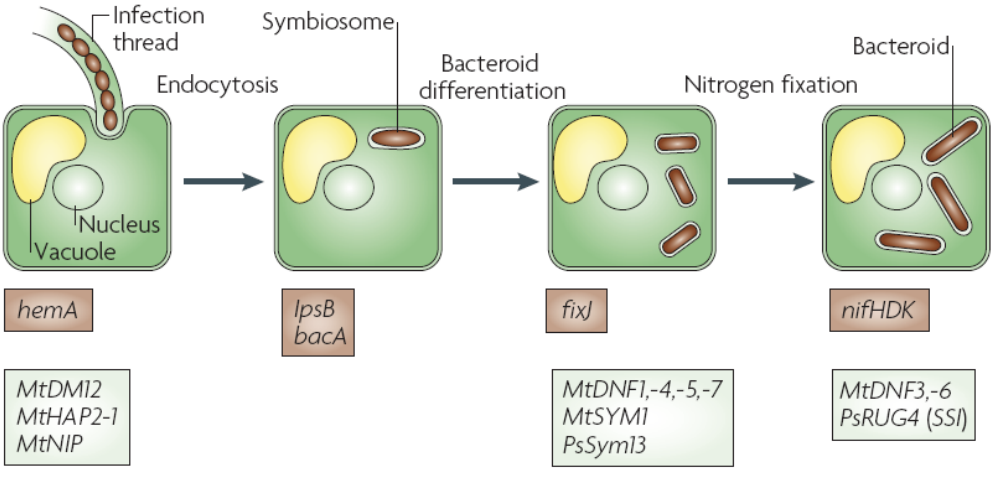


Figure I.10. Endocytosis of bacteria and bacteroid differentiation. Bacterial endocytosis requires the *Sinorhizobium meliloti hem*A gene, the *Medicago truncatula NIP* gene and wild-type expression levels of the *MtDMI2* and *MtHAP2-1* genes. *S. meliloti lpsB* and *bacA* are required for bacterial survival within the symbiosome membrane. *S. meliloti fixJ*, *M. truncatula MtSYM1*, *MtDNF1*, *-4*, *-5* and *-7* , and pea (*Pisum sativum*) *PsSYM13* are required for bacteroid differentiation. The *S. meliloti nifHDK* genes encode nitrogenase and are required for nitrogen fixation. The pea *PsRUG4* gene encodes sucrose synthase and is required to support bacteroid nitrogen fixation. The *M. truncatula MtDNF3* and *-6* genes are required for the maintenance of nitrogen fixation. The required rhizobial genes are boxed in brown and the required plant genes are boxed in light green.From Jones *et al.* (136) see also ref therein.

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 (146). Differentiation of infected cells occurs synchronously and the mature nodule contains symbiotic bacterial cells with a homogenous population of nitrogen-fixing bacteroids (147). Bacteroids in determinate

nodules (nongalegoid legumes) are comparable to free-living bacteria in their genomic DNA content, cell size, and viability (148).

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 (149, 150).

The bacteria object of this work, *Sinorhizobium meliloti*, forms indeterminate nodules, thus the following description will be focused on this kind of 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) (151).

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.

I.4.6. Host sanction

Why rhizobia should maintain a large number of genes for mutualism with their legume hosts (152)? This issue has become particularly important following the recent comments on the bacteroids (see section I.5). An indeterminate nodule bacteria can contain from 10^5-10^{10} located within the zone of invasion and not yet differentiated. It is assumed that a single symbiotic rhizobia have a greater fitness if successfully colonize a nodule, compared with a relative non-symbiont that resides in the soil, where growth may be severely limited by nutrients availability. But while this may be a gain for the fitness of rhizobia, it is also clear that the host has developed mechanisms to prevent the parasitism of the nodule by non-fixers rhizobia. While the host controls the infection process and the morphology of the nodule, rhizobia controls the efficiency of nitrogen fixation. A mathematical model predicts that if the plant apply the same selective pressure on fixers and non-fixing rhizobia that are within the nodule, the non-fixing rhizobia were most successful in the competition compared to fixing rhizobia. Maybe for this reason, host plant impose effective sanctions on non-fixing rhizobia (153). So far, sanctions on non-fixing bacteria involve oxygen limitation inside the nodule, inhibiting their growth and survival (153, 154). Thus, the host legume is able to impose a selective pressure on rhizobia favouring the evolution of bacterial populations able to fix nitrogen (152). But if the penalties are so effective, why in nature can we find less efficient strains? Possible explanations for the persistence of these strains despite of sanctions, could be the presence of mixed population inside nodules, systems of balanced selection, biochemical manipulations of the host by some rhizobial strains and differences in sanctions by different host genotypes (155). The frequency of mixed nodules has rarely been measured in field. More than 32% of the nodules of soybeans grown in field contains two strains (156), which allow to maintain the total nitrogen fixation per nodule high enough to avoid sanctions if one strain fix less nitrogen. In addition, the rhizobia that are descended from clones of undifferentiated bacteroids that undergo to terminal differentiation (such as *S. meliloti* with alfalfa), which are not capable of reproduction, may evolve differently from those that derive directly from undifferentiated bacteroids in terminal mode (such as those of *R. leguminosarum* with the pea). Indeed selection of bacteroids that fix more or less nitrogen acts indirectly through effects on survival and reproduction of their undifferentiated clones. Probably, this difference in the evolution of rhizobia has long-term consequences for the species than to rhizobia in legume nodule. However, natural selection is driven by the immediate benefits to the individual plant, not by future consequences for the entire specie. In general, the plant's effects on the evolution of rhizobia are preferentially on benefits to a single plant, although there are some exceptions. For example, plants where nodulation occurs more than one time during their life (eg perennial plants) could benefit from the evolution of a preferential mutualism with those rhizobia which could re-infect their roots. In any event, any benefit resulting from an optimization of mutualism, populations of soil rhizobia, however, should be shared with nearby competitors. It is therefore not yet clear, partly because of

large gaps in the available experimental data, a real advantage in the optimization of mutualism. Another question, related to the lifecycle of species such as *S. meliloti*, is: how can a plant (and a bacterium) benefit from the removal of the reproduction of bacteroids? (87) In this regard, Mergaert *et al.* (148) suggested that limiting the reproduction of bacteroids we can avoid that they become pests and infect other tissues of the plant. However, it was found that the endophytic rhizobia of the leaves of rice (*Oryza sativa*) have beneficial physiological effects on the plant (157). From this brief review of the literature, it is evident a strong evolutionary gap in the issues related to the rhizobia-legume symbiosis: the bacteroids differentiate and lose their reproductive capacity in some host species, while in other they are similar to free-living ones and continue to reproduce. This difference in the lives of symbiotic rhizobia could be significant in the evolution of mutualism. Indeed, it seems that the suppression of the reproduction of bacteroids from the host plants appear to be arise or has been abandoned at least twice during the evolution of legumes. Second, the evolutionary transition toward (or away from) the dimorphism of rhizobia induced by the host was probably driven by the immediate benefits for the single plant of legume, not by subsequent evolutionary changes in the rhizobia. For example, the bacteroids can fix nitrogen more efficiently or may be more easily lysed during senescence of the nodule, thereby facilitating the recovery of nutrients from the host. The bacteroids, then, usually do not reproduce, thus diverting resources from nitrogen fixation to the reproduction does not occur. In addition, bacteroids do not have direct benefits to accumulate reserve substances (such as polyhydroxybutyrate, PHB) if they can not have descendants. Moreover the accumulation of PHB in two ways may damage their clones present within the same nodule and able to reproduce. First, the synthesis of PHB by bacteroids may reduce the total amount of carbon available for clones capable of replication, and second, diverting resources from nitrogen fixation to the synthesis of PHB could trigger sanctions at the nodule that may damage undifferentiated clones. However, some bacteroids divert resources from nitrogen fixation toward their clones capable of replication by the rhizopine, compounds synthesized by bacteroids within the nodule and catabolised by undifferentiated rhizobia (158). Has been suggested that these rhizopine foster the rhizobia mutualism through parental selection, increasing the flow of root exudates to rhizospheric rhizobia able to reproduce (159, 160). This mechanism is based on the assumption that the rhizobia that receive benefits are closely related at the genetic level with rhizobia found in root nodules, due to limited dispersal (161). This form of parental selection to be effective, however, has very strict requirements of spatial distance of the other bacterial populations that are outside the nodule (160). Finally, to add more variables to the ecological and evolutionary dynamics of rhizobia, has recently reported that other organism such as the soil nematode *Caenorhabditis elegans*, can mediate a positive interaction between roots and rhizobia, favouring the nodulation process (162). *C. elegans* could transfer bacteria of the *Sinorhizobium meliloti* species to the roots of *Medicago truncatula* in response to volatile compounds released by plants that attract the nematode. This discovery, together with those reported by van Borm *et al.(163)*, which

have found rhizobia symbionts inside the ant *Tetraponera binghami*, shows that there are multitrophic interactions within the rhizosphere largely unknown and of great biological relevance, and indicates how these bacteria are able to colonize many different environments, and not only soil and nodules.

I.5 Free living bacteria versus bacteroids

During free-living growth, and presumably within the IT, *S. meliloti* grows as a rod-shaped bacterium with no greater than a 2N complement of its genome (Figure I.11*a*,*b*) , which implies that these bacteria initiate DNA replication only once per cell cycle (97).
A recent a study Mergaert *et al.* (148) 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.

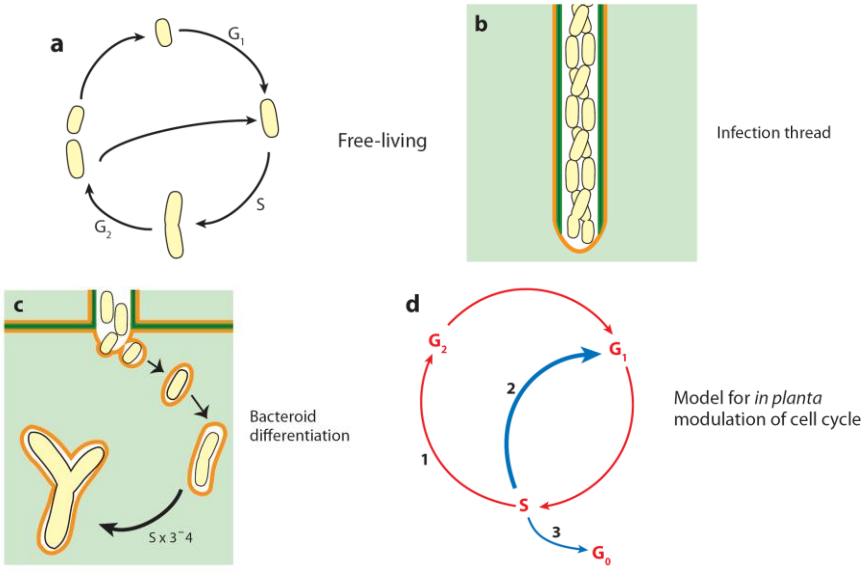


Figure I.11. Schematic representation of the rhizobium cell cycle at different stages of symbiosis. (*a*) The *S. meliloti* cell cycle is modeled after that of the alphaproteobacterium *Caulobacter crescentus* (see section I.6). A cell division cycle is comprised of three distinct phases: G1, S, and G2. Chromosome segregation begins during S phase and continues in G2 phase. Cell division begins in G2 phase and is completed before the next DNA replication initiation event. During free-living growth, *S. meliloti* is thought to initiate DNA replication only once per cell cycle and divides asymmetrically to produce daughter cells of different size. In analogy to *C. crescentus*, the small daughter cell likely proceeds into G1 phase while the larger daughter cell directly re-enters S phase. (*b*) *S. meliloti* proliferating in the IT originate from a clonal expansion of founder cells entrapped in the tip of the root hair curl. Cells appear to lack flagella and are loosely associated with one another in a pole-to-pole manner, typically forming two or three columns with a braided appearance. Active propagation of bacteria is observed only in a limited area called the growth zone near the tip of the IT, while bacteria outside of the growth zone do not grow or divide. It seems likely that the restricted growth of bacteria enables synchronization of bacterial growth with extension of the IT. (*c*) Bacteria colonize the cytoplasm of plant cells located in the invasion zone. Bacteria are surrounded by a plant-derived membrane and differentiate into a bacteroid. Orange lines, host plasma membrane; green lines, host cell wall. (*d*) A model of the *S. meliloti* cell cycle in planta has three possible exits from S phase, two of which (*in blue*) represent an exit from the typical free-living cycle (*in red*). Bacteria within the infection thread are thought to progress through the cell cycle in the same manner as free-living cells, and in particular transition from S phase into G2 phase (represented by arrow 1). Bacteria that undergo bacteroid differentiation undertake the process of endoreduplication and therefore re-enter G1 phase after the completion of S phase (represented by arrow 2); the bacteria may cycle from S to G1 multiple times during endoreduplication. Once endoreduplication is complete, the bacteroid enters a terminally differentiated state (G0) and is no longer able to initiate cellular growth or DNA replication (represented by arrow 3). From Gibson *et al. (97)*

The differentiation includes an important elongation of the cells, free-living cells were 1–2 µm long, whereas the bacteroids were 5–10 µm (148) (Figures, I.11c, I.12a). 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 (148). 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 (148) (Figure I.12b). 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 Rm1021 is close to 1 for all genes as well as for the control comparing two samples of cultured Rm1021 bacteria (148) as seen in figure . 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 I.11d).

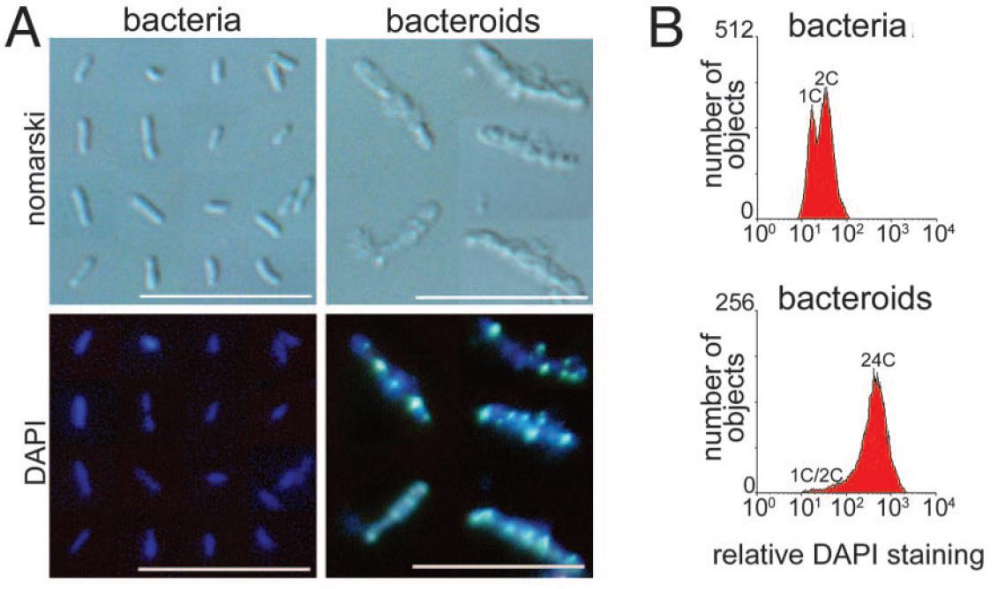


Figure I.12. Size, shape, and DNA content of free-living, cultured *S. meliloti* bacteria and *S. meliloti* bacteroids isolated from nitrogen-fixing *M. truncatula* nodules. (A) Nomarski (*Upper*) and fluorescence (*Lower*) microscopy of DAPI stained bacteria and bacteroids (*B*)DNAcontent of DAPI-stained bacteria and bacteroids measured by flow cytometry. From Mergaert *et al.* (148).

The viability of bacteroids (ability to resume growth and to produce descendants) is a long controversy in the literature (164). 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(148).

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 (148). This is also impossible in

endoreduplicated, highly polyploid eukaryotic cells. Moreover, the membranes of the bacteroids of galegoid legumes became permeable for diffusion (148), which could also compromise the capacity of bacteroids to reproduce.
The meaning of differentiation process from living form to bacteroid is under discussion. It could be either, the differentiated bacteroids have a better symbiotic performance, e.g. 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.
Taken together, these observations imply that the *S. meliloti* cell cycle has at least three branch points subject to in planta regulation (Figure I.11d), and it will be of great interest to understand how the cell decides which path to choose under different host conditions.
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.

I.5.1 Eukaryotic control on bacterial cell cycle

Margaert *et al* (148) has demonstrated that plant factors present in nodules of galegoid legumes but absent from nodules of nongalegoid legumes block bacterial cell division and trigger endoreduplication cycles, thereby forcing the endosymbionts toward a terminally differentiated state. Hence, *Medicago* and related legumes have evolved a mechanism to dominate the symbiosis. To demonstrate it a bacterial strain able to nodulate both a legume forming determinate nodules such as bean or lotus and a legume of the galegoid clade forming indeterminate nodules was used. In nature no known *Rhizobium* strain is able to do so. Nevertheless, some recombinant laboratory strains can cross this barrier. It was shown that the same bacterial species can enter in two entirely different differentiation processes to form nitrogen fixing bacteroids, being highly differentiated in nodules of galegoid legumes and visibly ‘‘undifferentiated’’ in lotus or bean nodules.
Moreover in two recent works (165, 166) was found that this process is driven by nodule-specific cysteine-rich (NCR) peptides.
These peptides are targeted to the bacteria and enter the bacterial membrane and cytosol. Obstruction of NCR transport in the dnf1-1 signal peptidase mutant correlated with the absence of terminal bacterial differentiation. On the contrary, ectopic expression of NCRs in legumes devoid of NCRs or challenge of cultured rhizobia with peptides provoked symptoms of terminal differentiation (165).

I.6 Cell cycle regulation in bacteria

Although the cell cycle of eukaryotes is now understood in molecular details, the bacterial cell cycle remains poorly understood. Genome sequencing projects have demonstrated that the major cell cycle regulators in eukaryotes, such as cyclin-dependent kinases, are not found in bacteria. Therefore how do bacteria regulate cell cycle progression? Early studies improved the knowledge in the field of bacterial cell cycle using as model organisms the Gram negative, γ-proteobacterium *E. coli* and Gram positive bacterium *B. subtilis.* However, only 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 (167, 168) 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).

I.6.1 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 (169). 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 (170) (Figure I.13). The two daughter cells inherit a different developmental program. Stalked cell, immediately after cell division, enter 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. Coincidentally with 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.

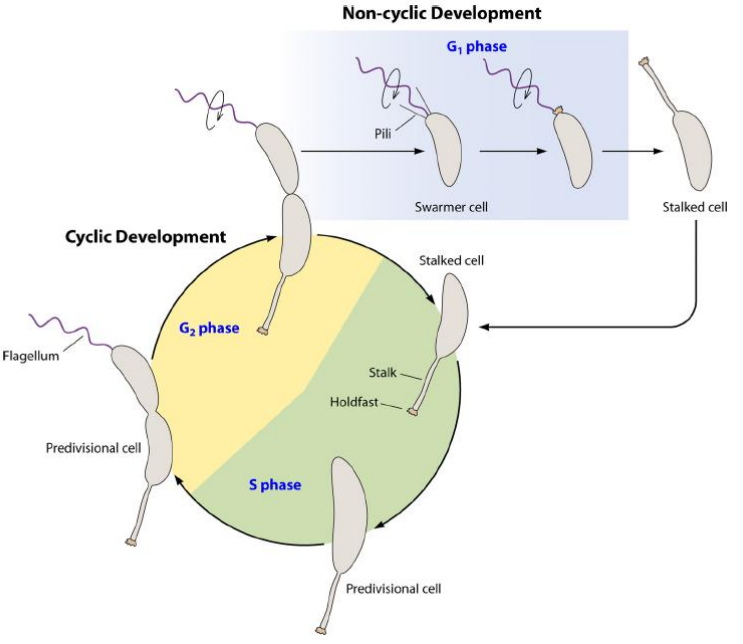


Figure I.13. Life cycle of *Caulobacter crescentus*. The cyclic developmental program begins with a stalked cell with an adhesive holdfast at the tip of the stalk. The stalked cell enters S phase, a cell state where it is competent for DNA replication. As the cell grows and replicates its DNA, it becomes a predivisional cell. During this time the cell becomes incompetent for DNA replication, entering the G2 phase. In the late predivisional stage, a flagellum is formed at the swarmer cell pole. After compartmentalization, flagellar rotation is activated (circular arrow) and pili are extruded. Cell separation leads to two different cell types. One cell is a stalked cell which reenters the cyclic developmental program and S phase, completing the circle. The other cell is a swarmer cell. The swarmer cell cannot replicate its chromosome yet is distinct from the predivisional cell and therefore is in a separate phase, referred to as G1. The holdfast is formed predominantly during the swarmer cell stage. Later the swarmer cell differentiates into a stalked cell. This differentiation comprises the noncyclic developmental program. From Curtis and Brun (171).

I.6.2 Regulation of cell cycle progression

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 (172). A DNA microarray analysis of 90% of all predicted genes showed that 19% significantly change their expression at the mRNA level as a function of the cell cycle (173). 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 (173).
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 (173). 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 (173).

Grunenfelder *et al*. (174) 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.

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 (3). Signal transduction is achieved by the transfer of the phosphoryl group onto 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 (5). 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.

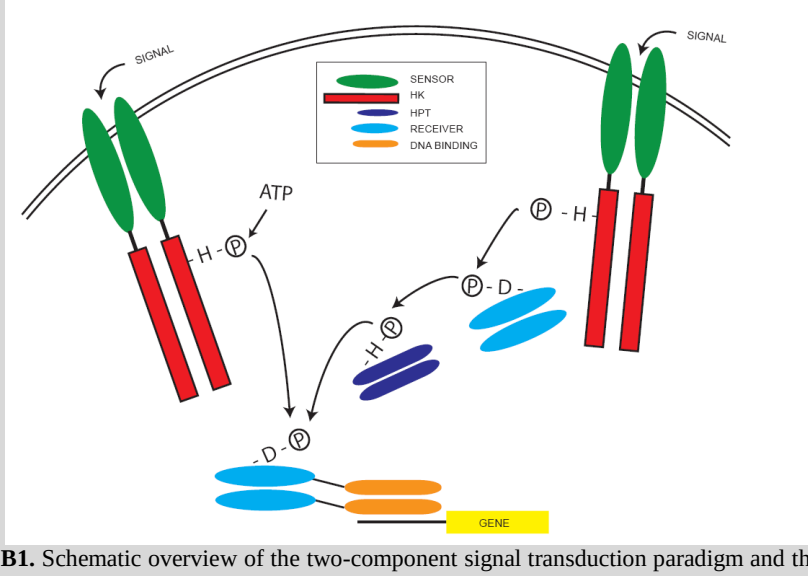


Figure I.B1. Schematic overview of the two-component signal transduction paradigm and the domain structure of each component.

I.6.3 A master response regulator controls global regulation of cell cycle

Many factors are known to regulate cell cycle progression and most of them are members of the family of two component signal transduction proteins, comprised of histidine kinases and their response regulator substrates (168). Among those proteins CtrA is the master regulator of the *Caulobacter* cell cycle, an essential response regulator whose activity as a transcription factor varies as a function of the cell cycle (175-177). CtrA controls various functions during cell cycle progression by activating or repressing gene expression. CtrA also blocks the initiation of DNA replication through binding of the replication origin (175). Among genes regulated by CtrA we can find those involved in cell division (*ftsZ, ftsA, ftsQ* and *ftsW*), the protease encoding gene *clpP* which is essential in *Caulobacter*, the DNA methylase gene *ccrM*, flagellar biogenesis genes, stalk biogenesis regulatory genes, pili biogenesis genes such as *pilA*, and chemotaxis genes (178-184). CtrA activity and stability varies during the cell cycle. Oscillation of CtrA levels, peaking at the predivisional stage before cell division, is achieved by different mechanisms (Figure I.14): transcription, proteolysis and phosphorylation control as discussed in detail below. DnaA and GcrA, and the DNA methyltransferase CcrM are involved in controlling *ctrA* transcription (180, 185). DnaA is a key element in cell cycle regulation because it is required for the initiation of DNA replication; it also controls the transcription of about 40 genes involved in nucleotide biogenesis, cell division, and polar morphogenesis (186, 187), and it activates the transcription of the *gcrA* gene (188). GcrA controls the transcription of *ctrA* and genes involved in DNA metabolism and chromosome segregation, including those encoding DNA gyrase, DNA helicase, DNA primase, and DNA polymerase III (188). As a consequence of this genetic circuit, CtrA accumulates out-of-phase with GcrA (188). The transcriptional loop of *ctrA* is closed by CcrM. CtrA activates the transcription of *ccrM*, which encodes for a DNA methyltransferase whose abundance is cell cycle dependent. CcrM is able to activate *dnaA* promoter region through methylation, closing the positive feedback composed by CtrA, DnaA and GcrA.

A second essential regulatory control on CtrA is carried out by phosphorylation. In fact, CtrA must be phosphorylated to bind DNA and its phosphorylation depends on cell cycle progression. An essential phosphorelay, composed of the hybrid histidine kinase CckA and the histidine phosphotransferase ChpT, is responsible for CtrA phosphorylation (189, 191). DivK, which is a response regulator, plays an essential role as a positive regulator of cell cycle progression because when phosphorylated, it indirectly inactivates CtrA and thus promotes DNA replication. Two histidine kinases are known to interact with DivK: PleC and DivJ (184, 192-194). Bacterial histidine kinases can have alternatively both kinase and phosphatase activities and these opposite activities are modulated by conformational changes of the protein (195). A null *Caulobacter pleC* mutant produces almost symmetric cells at the division and displays abnormal polar development.

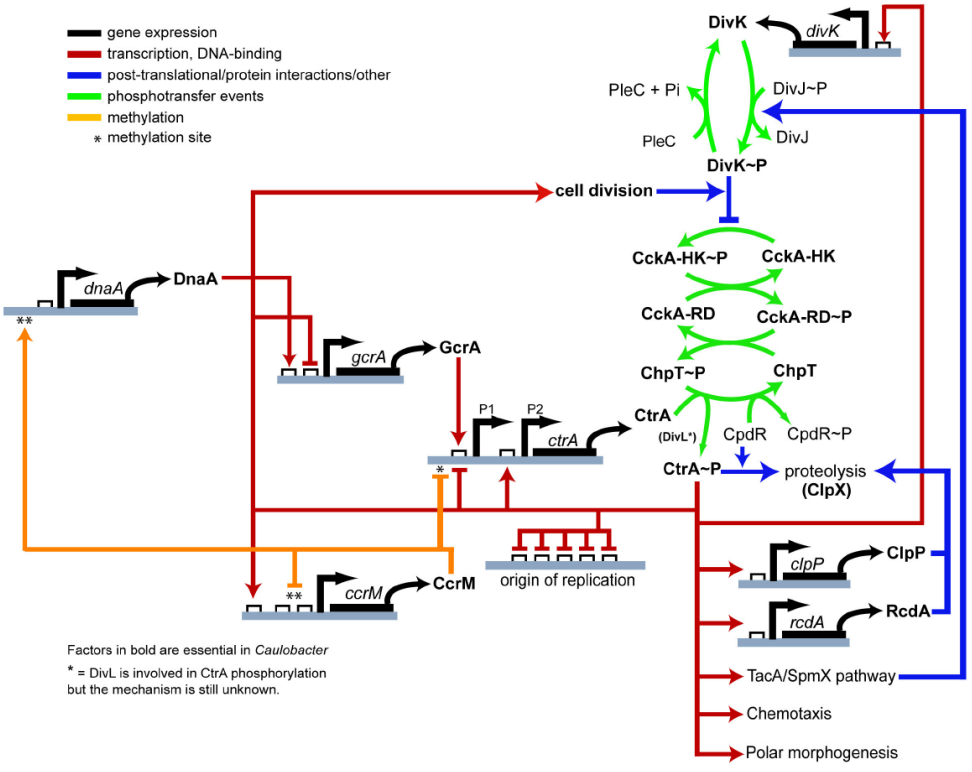


Figure I.14. Two main oscillators are working during cell cycle progression: (i) the transcriptional and epigenetic circuit (CtrA-DnaA-GcrA-CcrM); (ii) the phosphorylation/ proteolysis and transcription circuit (CckA-CtrADivK). The latter also involves coordination of CtrA proteolysis and cell division through regulation of DivK activity. Several of these regulatory mechanisms are at least partially redundant, and it has been demonstrated that only phosphorylation of CtrA is indispensable during cell cycle progression; in fact, cell cycle regulated transcription of *ctrA* can be substituted by constitutive transcription (189) and proteolysis can also be removed. From Brilli *et al. (190).*

The DivJ histidine kinase plays a role in controlling the length and location of the stalk and cell division. PleC and DivJ are considered the principal phosphatase and kinase, respectively, of DivK and they are in opposite locations during cell cycle progression (196, 197). DivJ activity is also positively controlled by the TacA/SpmX pathway, which is transcriptionally activated by CtrA (179, 198). ChpT also transfers the phosphate to a second response regulator named CpdR, which, together with RcdA, is a factor involved in CtrA proteolysis mediated by ClpPClpX protease (199-201). CtrA is degraded at the stalked pole at the G1/S transition when the origin of replication needs to be cleared and also in the stalked compartment, where initiation of DNA replication occurs immediately after cell division (202, 203). All these regulations are schematized in Figure I. where are illustrated the multilevel regulation of the *Caulobacter* cell cycle.

I.7 Cell cycle in the Alpha-proteobacteria group (from Brilli *et al.* (190))

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 (204, 205). 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.

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* (206), indicating that at least some of the features governing cell cycle progression in *Caulobacter* might also be present in other species.

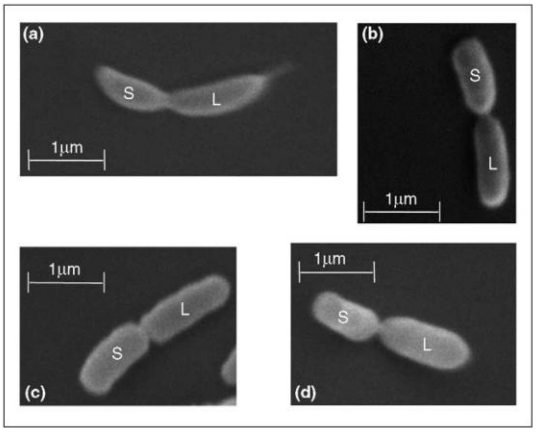


Figure I.15. Scanning electron micrographs of a-proteobacterial cells just before septation. The species observed are (a) Caulobacter crescentus, (b) Brucella abortus, (c) Sinorhizobium meliloti and (d) Agrobacterium tumefaciens. The large and small cells are called L and S, respectively. From *Hallez et al. (206).*

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 (207). CtrA in *Brucella* controls cellular events similar to those controlled by CtrA in *Caulobacter*, but through a direct effect on different targets (207). Moreover CtrA from *Caulobacter* is able to bind the *B. abortus ccrM* promoter in vitro(208). 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*)(207).

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) (209). In *A. tumefaciens ccrM* is essential and cell-cycle regulated (210). Also in *Brucella ccrM* is essential and that its overexpression impairs proper intracellular replication in murine macrophages (208). A yeast two hybrid in *Brucella* with DivK as a bait returned DivL, DivJ, PleC and PdhS (211). In *S. meliloti ctrA* is essential (212) and the closest PdhS homolog, called CbrA is not essential and it appears to be involved in succinoglycan production (213). 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.

In Brilli *et al.* (190) the regulatory cell cycle architecture was identified in all representative alpha-proteobacteria, revealing a high diversification of circuits but also a conservation of logical features.

Probably the regulation of cell cycle progression in *Caulobacter* has evolved in order to respond to a lifestyle in nutrientpoor environments but other Alpha-proteobacteria occupy different ecological niches, suggesting that cell cycle regulation must respond to different requirements; from an evolutionary perspective this means that features found in *Caulobacter* should not be completely conserved in other Alpha-proteobacteria, especially those experiencing different environments. Conversely, similarities between closer organisms were expected due to common phylogenetic ancestries (190). Gupta and Mok (214) proposed that *Rhodospirillales* and *Novosphingomonadales* branched earlier than the other alphas and after *Rickettsiales.* Two schemes appeared to be evolved: a complex circuit in *Caulobacterales* and *Rhizobiales* and a simpler one in *Rhodobacterales* (190).

Those differences, in particular the architecture in *Rhizobiales* will be discussed more in details in section VII.

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(references are reported at the end of every chapter/work)

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Chapter II

Aim and development of the work

My PhD work has been focused on plant-associated bacteria. It is known that large numbers of bacteria grow in and around roots, in the vasculature, and in the aerial tissues of plants, collectively referred to as endophytes; however, many questions about driving forces and ecological rules underlying the relationships between these microbes and plants remain unanswered (1-3). Most of the studies conducted so far were focused on rhizosphere bacteria, while, the microbial communities of the above-ground plant tissues have been far less analyzed particularly by the culture-independent methods; that in spite of the importance of shoot-associated bacteria, which are known to affect the growth and development of plants (1). In fact, to our knowledge, only two reports have been published so far on the diversity of stem-associated bacteria at the whole community level: in *Thlaspi goesingense* (4) and poplar (5) by using universal bacterial primers for ribosomal rRNA amplification. More recently Ikeda *et al.* (6) have shown that in legumes the endophytic bacteria are somehow related to the symbiosis, in soybeans indeed a subpopulation of proteobacteria is controlled through the regulation systems of plant-rhizobia symbiosis and by the plant nitrogen signalling pathway.

The aim of this work is to shed more light on the specificity of plant colonization patterns by bacteria using two different model plants: *Alyssum bertolonii* a nickel-hyperaccumulator plant living in metal contaminated soils and *Medicago sativa* a legume crop commonly used as forage or in crop rotation practices to contribute organic nitrogen to the soil via its symbiosis with the nitrogen fixing bacterium *Sinorhizobium meliloti.*

Metal hyperaccumulators such as *Alyssum bertolonii* have received much attention for their potential biotechnological exploitation in phytoremediation processes (7). Recently, metal hyperaccumulators have been explored in a new perspective, not only as plants, but

also as unusual, extreme habitats for the associated bacterial flora, which could reveal novel details concerning bacterial adaptation to metal, providing possible new models for addressing questions in microbial ecology, and possibly contributing to exploit the plant remediation abilities (7).

The first part of this work was then aimed at the characterization, by total DNA fingerprinting, of the leaf-associated bacteria of three different populations of *A. bertolonii* plants trying to answer the question whether and to what extent plant genotype can influence the phyllospheric bacterial community.

In the second part of this work the plant selected was *Medicago sativa*: alfalfa could indeed represent a conjunction point where to study both endophytic and symbiotic bacteria, their mutual interactions and the interaction of both of them with the plant. In fact the model for life-style of rhizobia includes the alternation of free-living in soil, symbiosis within the nodules, and also endophytic location (8) where rhizobia share the same habitat with all other endophytic bacteria. Actually, almost nothing is known about the distribution of rhizobia (more specifically *S. meliloti*) populations among the free living in soil and the plant colonizers; many rhizobial cells stay in soil and does not participate to nodulation or endophytic colonization but it's not clear if there are differences among these sub-populations and what is their possible ecological meaning.

Data regarding the whole bacterial community of alfalfa are still missing despite the importance of this legume, particularly considering that this plant could be a good resource to investigate plant-bacteria association pattern at different taxonomic levels, from that of classes (i.e. overall analysis of the bacterial community associated with alfalfa plants) to the single species level (i.e. analysis of genetic diversity across *S. meliloti* populations present in plant tissues).

The first steps of the present research on bacteria associated with alfalfa plants were focused on the development of molecular markers to trace and quantify *S. meliloti* cells in plant tissues and soil. The use of such markers, together with established molecular markers for the analysis of bacterial community composition, allowed to study the genetic diversity of the whole bacterial community on soil and plant tissues (nodules, stem and leaves).

The endophytic abilities of *S. meliloti* were studied by an *in-vitro* experiment infecting *Medicago sativa* with *S. meliloti* 1021 wild type strain or with a mutant defective for nodulation, in order to investigate a possible connection with nodulation. Moreover the endophytic ability of two natural strains (AK83 and BL225C, recently sequenced in our lab in collaboration with JGI) were tested.

It is well known that in nodules, where *S. meliloti* is able to fix atmospheric nitrogen, bacterial cells become elongated and polyploid, incapable of further division and are called bacteroids. These dramatic changes are induced by the plant (9-11), however at which level the plant acts on bacteria is not known. To try to look at this interesting differentiation event, the final part of my Ph.D. has been focused on the molecular analysis of factors involved in the establishment of symbiosis, in particular on the mechanisms that drive the differentiation of *S. meliloti* in bacteroids. Bacteroids are

characterized by an enlargement of cell shape and by endoreduplication of the genome (9), suggesting the involvement of a differential regulation of cell cycle progression. A model of regulation of cell cycle that could be valid also for *S. meliloti* was proposed by Biondi and co-workers in the alpha proteobacterium *Caulobacter crescentus (12)*. In *Caulobacter* the master regulator of the cell cycle is CtrA that is inhibited by another regulator called DivK, in a cell cycle dependent fashion. The activation of DivK depends on the histidine kinase DivJ while PleC is its principal phosphatase. I preliminarily analyzed, the role of the DivJ ortholog in *S. meliloti* (13) constructing the deletion strain for *divJ* and analyzing it in terms of growth rate, phenotypic features, nodulation capability and its interconnection with the expression of *ctrA* gene.

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Chapter III

The Nickel-hyperaccumulator Plant *Alyssum bertolonii*

Recent years have witnessed a considerable growth of microbiological researches in serpentine soils in relation to the presence of hyperaccumulating plants. Nickel-hyperaccumulating plants accumulate huge amounts of heavy-metals in shoots, and therefore, provide a specific environment for bacterial populations and in particular for endophytic bacteria. Bacterial endophytes have been studied in many different plant species and in some cases they have been shown to promote plant growth or to confer the plant higher tolerance to biotic and abiotic stress. The increasing interest in the use of endophytic bacteria, has opened up new perspectives on the study of metal-hyperaccumulating plants. Endophytes may colonize plant-internal environments that are less toxic than soil (that is with lower available metal content), or environments, such as xylem vessels, where toxic metals might be available at higher concentration than in soil. The Ni-hyperaccumulator *Thlaspi goesingense* was the first species to be investigated for its endophytic bacterial community composition. Obtained results showed that the endophytic community was rich in members of the *Proteobacteria* division and a high number of sequences related to the genus *Sphingomonas* were found. Moreover, members of the genus *Methylobacterium* were recovered and a new species, namely *Methylobacterium goesingense*, was found to be associated with *T. goesingense*. Previous studies on cultivable fraction of *A. bertolonii* endophytic community show that most of the diversity was represented by Gram-positive bacteria. In particular, genera as *Bacillus*, *Paenibacillus*, *Leifsonia*, *Curtobacterium*, *Microbacterium*, *Micrococcus* and *Staphyloccoccus* were found. Only few members of *Proteobacteria* (mainly belonging to the genus *Pseudomonas*) were found. In this work, by using cultivation-independent analysis (Terminal-Restriction Fragment Length Polymorphism, T-RFLP), we characterized the leaf-associated bacterial flora of *A. bertolonii* plants collected from

three different populations. Obtained results showed for the very first time that a large variability, between plants belonging to the same species, is present at the taxonomic level in the associated bacteria. This variability account for over 93% of the overall variability detected, suggesting that the notion of “endophytes”, as bacterium recovered in a plant tissue, may not be related to any specificity of plant-bacterium interaction. However, a fraction, tough small, of the taxa is found in all analyzed plants, suggesting the presence of specific bacterial groups, which may really behave as “endophytes”.

Plant-by-Plant Variations of Bacterial Communities Associated with Leaves of the Nickel Hyperaccumalator *Alyssum bertolonii* Desv,

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PLANT MICROBE INTERACTIONS

Plant-by-Plant Variations of Bacterial Communities Associated with Leaves of the Nickel Hyperaccumulator Alyssum bertolonii Desv.

Alessio Mengoni & Francesco Pini & Li-Nan Huang & Wen-Sheng Shu & Marco Bazzicalupo

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Abstract Bacteria associated with tissues of metal-hyperaccumulating plants are of great interest due to the multiple roles they may play with respect to plant growth and resistance to heavy metals. The variability of bacterial communities associated with plant tissues of three populations of Alyssum bertolonii, a Ni hyperaccumulator endemic of serpentine outcrops of Central Italy, was investigated. Terminal-restriction fragment length polymorphism (T-RFLP) analysis of bacterial 16S rRNA genes was applied to DNA extracted from leaf tissues of 30 individual plants from three geographically separated serpentine outcrops. Moreover, T-RFLP fingerprinting was also performed on DNA extracted from the same soils from which the plants were collected. Fifty-nine unique terminal-restriction fragments (TRFs) were identified, with more than half of the taxonomically interpreted TRFs assigned to Alpha- and Gamma-Proteobacteria and Clostridia. Data were then used to define the extent of variation of bacterial communities due to single plants or to plant populations. Results indicated a very high plant-by-plant variation of leaf-associated community (more than 93% of total variance observed). However, a core (numerically small) of plant-specific TRFs was found. This work demonstrates that plant-associated bacterial communities represent a large reservoir of biodiversity and that the high variability existing between plants, even from the same population, should be taken into account in future studies on association between bacteria and metal-hyperaccumulating plants.

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Introduction

In recent years, there has been an increased interest in the bacterial communities associated with plants [28, 29]. More and more bacterial species have been found associated with plant aerial parts and their interactions with roots and soil have been explored (for a partial list of species, see Ref. [28]). These studies found that leaves harbor a complex bacterial flora, which is thought to have originated from ascending migration of bacteria through the stem from roots and, in turn, from soil [12]. Even if the ecological importance of such bacteria for plants is in most of the cases unknown, bacteria inside plant may provide fixed nitrogen [9], reduce susceptibility to pathogens or abiotic stress [4, 31, 34], and improve phytoremediation operated by plants [8, 25, 26].

Metal-hyperaccumulating plants are found on highly mineralized, heavy-metal-rich serpentine outcrops [1] and present the unusual physiological ability to transfer the heavy metals (Ni, Zn, etc.) from soil to leaf tissues where they are stored in the cell vacuoles [13]. Since the amount of metals accumulated in leaves may reach very high levels (generally higher than 10,000 μg/g dry weight) [1], the leaf environment could be far more toxic than soil, thus possibly contributing to shape structure and function of the associated bacterial community. Recent works have investigated taxonomic composition and phenotypic features of isolates from bacterial communities associated with metal hyperaccumulators [2, 11, 24]. In particular, a new Methylobacterium species tolerant to high levels of Ni was recently described in the Ni hyperaccumulator Thlapsi goesingense [10], which could have evolved through the

selective pressure provided by the plant Ni content. As for bacteria associated with other plant species, bacteria associated with plant tissues of metal hyperaccumulators may play multiple roles with respect to plant growth and resistance to heavy metal stress [16]. Thus, the identification of bacterial strains specifically associated with such plant species could help promote a more efficient use of plants in phytoremediation practices via the exploitation of their bacterial flora.

The Ni hyperaccumulator Alyssum bertolonii subsp. bertolonii Desv. is an endemic plant colonizing serpentine outcrops of Central Italy [36] and containing in its aerial part from 7,000 to 12,000 μg of Ni/g dry weight [7]. We have previously investigated the cultivable and uncultivable bacterial communities present in serpentine soil and in the rhizosphere of A. bertolonii [22, 24]. Moreover, we also studied the cultivable bacterial communities present inside A. bertolonii tissues and found that leaf tissues harbor a quite diverse heterotrophic bacterial flora displaying high phenotypic variability with respect to heavy-metal tolerance [2]. However, the uncultured fraction of the leaf-associated community in this hyperaccumulator and the possible variability of the bacterial community in different plant populations remain unknown. In fact, despite the large amount of data available up to now of endophytic and epiphytic bacteria on different plant species (see for instance Ref. [29]), no investigation has been done to estimate the variability of bacterial communities among different individual plants or between plant populations. The definition of the relative contribution of individual plants and soil bacteria to the establishment of the plant-associated bacterial community is a relevant issue in order to assess the species specificity of plant-associated bacteria. Thus, we conducted a microbial community fingerprinting survey, using T-RFLP [17], of three plant populations, previously characterized for their physiological and population genetics features [6, 7, 19].

Methods

Plant and Soil Sampling

Leaf samples of A. bertolonii subsp. bertolonii Desv. were collected from plants of approximately the same age present in the populations of the three serpentine outcrops of Galceti (G), Impruneta (I), and Pieve Santo Stefano (P) in Central Italy during the sampling for a plant population genetic study [19] in winter 2001/2002. Three leaves per plant were collected. The distance between the three outcrops was in the range 25–90 km, the lowest one being between Galceti and Impruneta outcrop, and the highest one between Galceti and Pieve Santo Stefano. Previous physiological analyses [6] showed different levels of tolerance to Ni and different Ni accumulation features of plant populations (Table 1). In particular, the population from the outcrop of Impruneta was the most tolerant (highest value of plant root length at 100 μM Ni), while the population from Pieve Santo Stefano had the capacity to accumulate the highest Ni content. Leaves were stored at 4°C immediately after collection and transported to the laboratory in a few hours for DNA extraction. Soil samples were collected in winter 2001/2002, during the sampling for a previous study [22], at more than 10 cm distance from A. bertolonii plants. The serpentine soils studied had very high Ni, Co, Cr, and Mg concentrations, a low Ca concentration, and a Ca/Mg ratio lower than 1 (Table 1). Organic matter and cation exchange capacity were highest in Galceti soil. All three soils had slightly basic pH values. Ten individual plants and three soil samples were taken from each serpentine outcrop for a total of 30 plants and nine soil samples.

DNA Extraction, PCR Amplification, and T-RFLP Analysis

Whole leaves were washed with sterile distilled water to remove dust and small soil particles, and DNA was extracted immediately with a CTAB method [20]. DNA from soil samples (0.5 g) was extracted with the FastDNA kit for Soil (QBiogene). Partial bacterial 16S rRNA genes were amplified by using the primers 799f (labeled with FAM) and PH, following the protocol reported by Idris et al. [11]. This primer set amplifies a fragment of about 722 bp without targeting chloroplast ribosomal operons [11]. Purified amplification products were digested separately with restriction enzymes AluI and MspI and digestions were resolved by capillary electrophoresis on an ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using LIZ 500 (Applied Biosystems) as size standard. T-RFLP analysis was performed on two technical PCR replicates from each DNA extract as previously reported [23]. Only peaks present in both duplicate runs were considered for successive analyses. Taxonomic interpretation of bands was done by MiCA web tool (http://mica.ibest.uidaho.edu/) and by cloning and sequencing, following a previously described protocol [21].

Statistical Analyses

Chromatogram files from automated sequencer sizing were imported into GeneMarker ver. 1.71 software (SoftGenetics LLC, State College, PA, USA) by filtering with the default options of the module for AFLP analysis. Peaks above 70 fluorescence units and whose size ranged from 35 to 500 nt were considered for profile analysis. Statistical analyses were performed on a binary matrix obtained by linearly

Table 1 Chemical parameters of soils and main Ni-hyperaccumulation features of plant populations in three different serpentine outcrops[a]

Outcrop	pH	CEC[b]	OM %[c]	Ca/Mg ratio	Ni (mg kg^{-1} d.w.)	Mg (mg kg^{-1} d.w.)	Co (mg kg^{-1} d.w.)	Ca (mg kg^{-1} d.w.)	Cr (mg kg^{-1} d.w.)	Ni tolerance[d]	Ni hyperaccumulation (mg kg^{-1} d.w.)[d]
Galceti	7.26±0.04	38.1±0.2	8.26±1.26	0.042±0.003	1,490±55	105,038±2,632	84±4	4,358±206	1,357±81	3.93±0.10	7,210±26
Impruneta	7.52±0.09	24.5±4.9	4.00±0.74	0.035±0.006	979±64	97,135±6,488	63±5	3,353±351	984±69	6.02±0.19	4,804±146
Pieve Santo Stefano	7.15±0.06	28.1±5.1	1.66±0.04	0.239±0.106	1,212±61	75,874±2616	107±17	18,139±1,971	898±59	4.67±0.10	8,368±50

[a] Data from Refs. [6, 7, 22]. For pH, CEC, and OM, values are means±standard deviation. For the other parameters, values are means ± standard error. The Ca/Mg ratio and metal contents in soil are shown: d.w. dry weight

[b] CEC cation exchange capacity, in meq 100 g^{-1} dry weight

[c] OM organic matter

[d] Tolerance was estimated as root length (cm) in hydroponic culture with 100 µM Ni. Ni hyperaccumulation was estimated as Ni content in plants from hydroponic experiments with 175 µM Ni

combining data from the two restriction enzymes as previously reported [23]. NTSYS-pc ver. 2.02 [27] software package was used to compute a Pearson (product moment) correlation matrix from the binary matrix and for performing correspondence analysis (CA) and UPGMA (unweighted pair group method with arithmetic mean) clustering of the binary vectors for each sample. To test the distribution of the variance of T-RFLP profiles within plant populations and among populations and sites, analysis of molecular variance (AMOVA) [5, 18] was applied using Arlequin 3.11 software (http://cmpg.unibe.ch/software/arlequin3/). AMOVA was used as a statistical methodology alternative to the classical analysis of variance (ANOVA). AMOVA is more flexible for biological data than classical ANOVA because it does not require a prior assumption of normality of the dataset and statistical significance is computed through a permutation test [5]. Pairwise F_{ST} distances [32] between T-RFLP profiles of plant populations and soils, computed with Arlequin 3.11, were used to infer a Neighbor-Joining dendrogram with the software MEGA4 [35]. Pairwise F_{ST}, originally developed to measure the differentiation among populations [32], was here applied as an estimator of bacterial community differentiation.

Results

T-RFLP Community Diversity

T-RFLP analysis with AluI and MspI restriction enzymes on the nine soil samples and 30 individual plants, yielded a total of 59 polymorphic markers (TRFs, terminal-restriction fragments) ranging in size from 37 to 419 bp. Within these TRFs, seven were common to all plant populations and all serpentine outcrops, as they were present in at least one plant for each population and one soil sample for each outcrop. Ten TRFs were exclusively present in plant populations (absent from soils). No TRFs were unique to soil samples and absent from plants. Taxonomic interpretation of T-RFLP profiles was accomplished using MiCA web tool (http://mica.ibest.uidaho.edu/) and the sequences from four of the larger sized TRFs. Out of 59 TRFs, 43 (72.9% of the profiles) were putatively assigned a taxonomic classification by "unambiguous in silico matching" of the Ribosomal Database Project (release 9.6 which comprises 511,847 bacterial 16S rRNA entries) performed with MiCA. All 43 of these TRFs were present in plants while only 29 were found in soil samples. The putative taxonomic distribution of TRFs in plant and soil samples is reported in Fig. 1. TRFs attributed to members of Alpha- and Gamma-Proteobacteria subdivisions and Actinobacteria were highly present in plant and soil profiles (25 TRFs out of 43 and 16 out of 29 TRFs, respectively). TRFs attributed

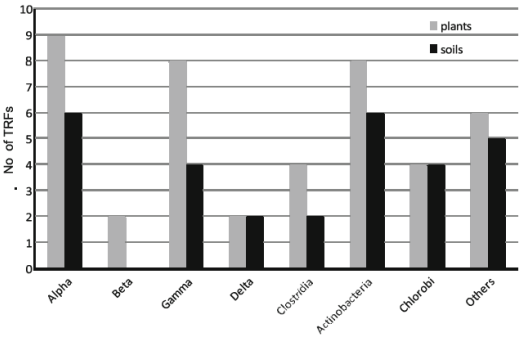


Figure 1 Distribution of taxonomically interpreted TRFs in plants and in soil. "Others" indicates TRFs taxonomically interpreted by MiCA as due to members of Chloroflexi, Aquificae, Gemmatimonadetes, and Spirochaetes

to Beta-Proteobacteria were found only in plant samples. Out of the ten plant-exclusive TRFs, three belonged to Alpha-, two by Beta-, two by Gamma-Proteobacteria, and one by Clostridium, Actinobacteria and Aquificales, respectively. In order to validate the taxonomic putative assignments done by MiCA, four of the larger sized TRFs (at 219, 282, 284, and 377 bp after MspI digestion) were cloned and sequenced. Results from a BLAST analysis of the TRF sequences using GenBank database confirmed the putative taxonomic assignments of the MiCA software. In particular, TRFs at 219, 284, and 282 bp (GenBank accession numbers FJ345396, FJ348229, and FJ348230, respectively), found in both soils and plants T-RFLP profiles, were most similar to sequences of Methylobacterium (EU879041, 100% identity), Sphingomonas (EU723166, 98% identity), and Flexibacteriaceae (DQ847443, 94% identity), respectively. The TRF at 377 bp (GenBank accession number FJ345397), exclusively found in plants, matched with a 16S rRNA gene sequence of Methylobacterium (AM910535, 100% identity).

Variance of Bacterial Community Among Plant Populations and Serpentine Outcrops

To evaluate the relationships between the bacterial community in different plant populations and different soil samples, correspondence analysis (CA), UPGMA clustering, and AMOVA were carried out. Figure 2a reports CA results from the first two components, which account for about 21% of total inertia. Individual plants from the same population did not show clear clustering. On the contrary, soil samples from the same locality grouped together. Moreover, even if the amount of displayed inertia is low, it is possible to recognize on the second dimension two loose groups representing the plant and the soil bacterial communities, respectively. In particular, P soil samples appeared the most different from plant samples. UPGMA clustering of Pearson correlations between T-RFLP binary profiles (Fig. 2b) confirmed the observed pattern of CA, with clustering of P soil samples apart from plants and a clustering of I and G soil samples within plants. As with CA, no obvious clusters of plants according to the population were observed. To statistically test the differentiation between plant populations and soils, pairwise F_{ST} distances were computed and a Neighbor-Joining dendrogram was drawn from them (Fig. 3). Results, reported in a radiation dendrogram, showed the three soils scattered apart and the plant populations closer to each other. Statistical support for the pairwise differences was obtained through a permutation test whose results (Fig. 3b) showed that pairwise F_{ST} values were statistically supported for all soil vs. plant population comparisons and for the comparisons between G vs. P and P vs. I plant populations. Soil vs. soil pairwise F_{ST} values though high were not significant in the permutation test due to the low number of samples (three replicates) within each outcrop. The AMOVA test was then used to quantitatively evaluate the contribution to the variance of bacterial community fingerprinting due to: (1) the outcrop locality, (2) the population, and (3) the single plants (Table 2). In Table 2a, the contribution of the specific serpentine outcrop in determining soil bacterial community differences is reported. More than 48% of total variance (i.e., differences in TRF profiles) was related to the outcrop the soil samples came from, although soil samples within the same outcrop were variable and accounted for 51% of total variance. Locus-by-locus AMOVA sorted out seven TRFs with statistically significant among-outcrops variance component (TRF at 42, 58, 75, 200, 216, 239, 372 bp, not shown). The role of plant populations in determining plant bacterial community differences is reported in Table 2b. The majority of the variance was related to plant-by-plant differences within the same population (93.14% of total variance). However, a significant, but little, amount of variance (6.86%) was able to discriminate bacterial communities from different plant populations; in particular, "locus-by-locus" AMOVA showed that seven TRFs at 40, 55, 68, 84, 97, 102, 153 bp have a statistically significant among-plant-populations variance (not shown), and consequently mostly contribute to differentiation of bacterial communities in relation to different plant populations. Among these TRFs, three of them (at 68, 84, and 102 bp) were not found in soil samples.

Discussion

In a previous study, A. bertolonii was shown to harbor a highly diverse cultivable endophytic bacterial flora [2],

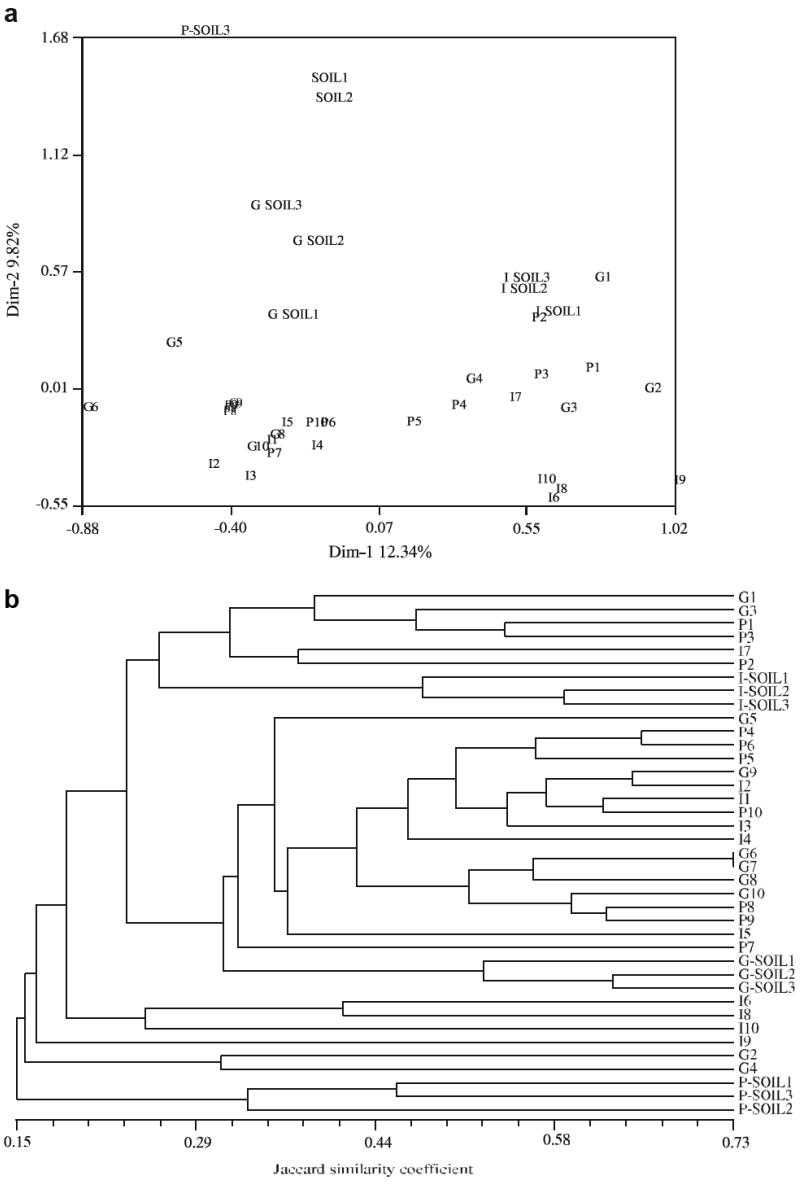
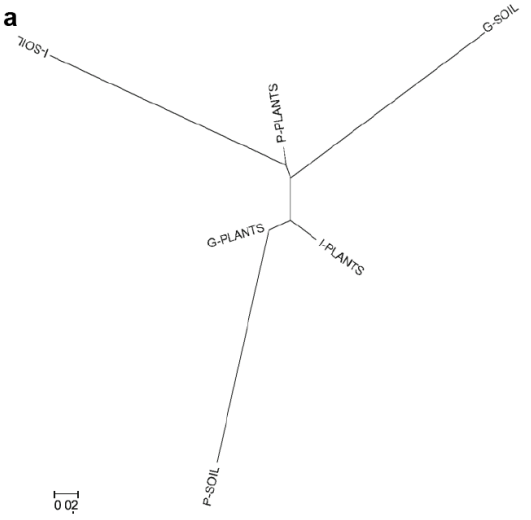


Figure 2 a Correspondence analysis of ribotypic variation in bacterial communities from 30 plants and nine soil samples. Values for Dim-1 and Dim-2 indicate the percentage of inertia accounting for the first and second principal dimension, respectively. b UPGMA dendrogram of Pearson (product moment) correlation between samples. G, I, and P are DNA samples from plants of the populations of Galceti, Impruneta, and Pieve Santo Stefano, respectively. G-SOIL, I-SOIL, and P-SOIL indicate soil DNA from the same locality of the plants

which also displayed a wide range of heavy-metal tolerance phenotypes. However, it remained unclear if there were peculiar taxonomic features of the bacterial flora associated with A. bertolonii. To address this issue, three geographically isolated populations of A. bertolonii were sampled in different serpentine outcrops and T-RFLP profiles of leaf-associated (endophytic and residual phyllospheric) and soil bacterial communities were obtained. TRFs present in plants and in soil were taxonomically interpreted as mainly related to the Alpha- and Gamma-Proteobacteria and Actinobacteria. A high presence of Proteobacteria (especially Alpha-Proteobacteria) was also found in the Ni hyperaccumulator Thlaspi goesingense shoot-associated microflora [10] as well as in other plant species [30, 33]

a



b

	I-SOIL	P-SOIL	G-SOIL	G-PLANTS	P-PLANTS	I-PLANTS
I-SOIL	0.000					
P-SOIL	0.489	0.000				
G-SOIL	0.442	0.450	0.000			
G-PLANTS	0.256*	0.185*	0.243*	0.000		
P-PLANTS	0.236*	0.274*	0.228*	0.101*	0.000	
I-PLANTS	0.295*	0.248*	0.273*	0.033	0.081*	0.000

Figure 3 Neighbor-Joining dendrogram (a) of pairwise F_{ST} distances (b) between plant populations and soil. Scale bar indicates pairwise F_{ST} values. Asterisks indicate statistically significant values ($P < 0.05$) after permutation test. G-PLANT, I-PLANT, and P-PLANT are plants of the populations of Galceti, Impruneta, and Pieve Santo Stefano, respectively. G-SOIL, I-SOIL, and P-SOIL indicate the soil from the same locality of the plants

and on the rhizosphere of the same A. bertolonii populations [22]. In contrast, the cultivable endophytic community of A. bertolonii previously surveyed [2] showed a major contribution to community diversity of taxa related to the genera Staphylococcus, Curtobacterium, Bacillus, and Microbacterium, and only a minor contribution of Proteobacteria (mainly Pseudomonas). These results suggest that the plate isolation conditions applied might have introduced strong biases, confirming that methods based on cultivable and uncultivable bacteria capture distinct diversity and can give complementary information. Two TRFs, taxonomically assigned to the Alpha-Proteobacteria division, were sequenced and matched with 16S rRNA gene sequences of Methylobacteria. Interestingly, in the Ni hyperaccumulator T. goesingense [10, 11] as well as in the Zn hyperaccumulator T. caerulescens [15], strains belonging to the genus Methylobacterium were found to be associated with rhizosphere and plant tissues. In some cases [11], such strains displayed tolerance to high Ni concentration leading to speculation that Methylobacteria could play a role in metal tolerance and metal hyperaccumulation in Brassicaceae. Actually, Methylobacteria are known to produce phytohormones that could also help plant growth [14]. Here, we cannot provide evidence that Methylobacteria are particularly exclusive to A. bertolonii, and consequently their role in plant biology and especially Ni tolerance and hyperaccumulation is far from being elucidated. However, the occurrence in A. bertolonii, T. goesingense, and T. caerulescens of Methylobacteria could address future microbiological research on the relationships between metal hyperaccumulators and bacteria from this group. Multivariate analysis was then applied to the T-RFLP data looking for an uneven distribution of the variance of presence/

Table 2 Analysis of molecular variance (AMOVA) for 30 plants and nine soil samples belonging to three different serpentine outcrops by using 59 TRF polymorphic markers[a]

Source of variation	df	Sum of squares	Variance components	Percentage of variation	P value
(a) Soil samples					
Among outcrops	2	12.00	1.48148	48.78	<0.0054
Within outcrops	6	9.33	1.55556	51.22	<0.0001
Total	8	21.33	3.03704	F_{ST} =0.48780	
(b) Plant samples					
Among-plants populations	2	14.33	0.30407	6.86	<0.026
Within-plant populations	27	111.40	4.12593	93.14	<0.0001
Total	29	125.73	4.43000	F_{ST} =0.06864	

[a] AMOVA was performed attributing the following two types of groupings: Computation (a) is made with T-RFLP profiles from soil samples grouped into the three respective outcrops. Computation (b) is made with plant T-RFLP profiles grouped according to the respective plant population. For each grouping, the percent of the total variance observed was attributed to the two hierarchical partitions. Data show the degrees of freedom (df), the sum of squared deviation, the variance component estimate, the percentage of total variance contributed by each component, the F_{ST} statistics (fixation index), and the probability (P) of obtaining a more extreme component estimate by chance alone estimated computing
10,000 permutations

absence of TRFs in plants and in soil samples. Correspondence analysis (CA) and UPGMA clustering showed that soil bacterial communities are in some way different from leaf-associated communities, in particular for P soil, and that soils from the different outcrops could be sorted out according to their origin. Leaf-associated communities formed a more complex, mixed pattern, difficult to interpret by CA. Pairwise F_{ST} and AMOVA were then used to estimate the differentiation between plant populations and soil samples and to hierarchically divide the variance due to intra-population differences and to among-population differences [5, 18]. In this way, we determined that serpentine soil bacterial communities are differentiated and that the locality plays an important role in shaping diversity (about half, 48.78%, of the variance is due to among-outcrops bacterial community differences). On the contrary, for leaf-associated bacteria, most of the contribution to the variance came from plant-by-plant differences inside the same population, and only a minor contribution (6.86% of total variance) was due to among-plant-populations differences. Actually, pairwise F_{ST} differentiation values were statistically significant for two out of three possible pairings (P-PLANTS vs. I-PLANTS and P-PLANTS vs. G-PLANTS) which correspond to the more geographically and genetically different populations [19]. Overall, these data suggest that the variability of plant bacterial communities is so high that any functional study of the association of bacteria with the plants should seriously take into account the possibility that most of the bacteria may occur in plant only by chance. The ability to associate with plants could actually be present in many free-living bacteria that can occasionally be recruited and take advantage of staying inside and on the surface of leaf tissues. However, the significant level of among-plant-populations variance could lead to the hypothesis that an influence (numerically small but present) exists by different plant populations on the recruitment of some different bacterial strains. In this view, the locus-by-locus AMOVA showed that the TRFs which mostly contribute to differentiation among soils do not contribute to differentiation among plant populations. Influence of plant population on associated bacteria has been shown for legume–rhizobia symbiosis in which an effect of plant genotypes over associated rhizobial was reported [3]. Presented data could suggest that a small effect of plant genotype may also be working at the bacterial community level. However, controlled experiments should be performed to evaluate the actual presence of a plant-genotype effect over associated bacterial communities.

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Chapter IV

Molecular tools

Leguminous (*Fabaceae*) plants are the specific partners of the symbiotic association with nitrogen-fixing root nodule forming rhizobia. *Medicago sativa* L. (alfalfa) and the diploid relative *M. truncatula* Gaertn. (barrel medic) are among the most studied species for the molecular issues related to their symbiotic bacterial partner, the nitrogen fixing bacterium *Sinorhizobium* (syn. *Ensifer*) *meliloti*. *S. meliloti* is present in most temperate soils, and, when conditions are suitable, it forms specialized structures, called nodules, in the roots of host plants where it differentiates to bacteroids, which carry on nitrogen fixation. It is assumed that a fraction of the bacterial cells is released from dehiscent nodules to soil, giving rise to new free living rhizobial clones. *S. meliloti* population genetics has been extensively investigated in the past years; however, due to the lack of efficient selective culture media, most of the population genetics studies on *S. meliloti* have been performed only on bacteria isolated from nodules with a few early studies done on bacteria directly recovered from soil and no reports of direct analysis from nodules (or even other plant tissues) without cultivation. These earlier studies suggested that the composition, in genetic terms, of *S. meliloti* population sampled in root nodules by traditional cultivation techniques may not be representative of the actual population in soil, allowing to hypothesize the co-existence, in a theoretical *S. meliloti* metapopulation, of different populations (i.e. residing in soil and nodulating).

In this scenario we developed two molecular tools to shed more light on composition, variability and relationships of sinorhizobial populations present in soil and tissues of *M. sativa* : a) T-RFLP analysis targeting the 16S-23S rDNA intergenic gene spacer (IGS) sequence to specifically investigate the genetic polymorphism of *S. meliloti* population in

DNA extracted fromenvironmental samples; b) two S. meliloti-specific Real-Time PCR markers for direct enumeration of bacterial cell.
The tools were tested on a large collection of *S. meliloti* and on DNA extracted from microcosm and natural samples.

IV.1 Development of a cultivation-independent approach for the study of genetic diversity of *Sinorhizobium meliloti* populations

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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Development of a cultivation-independent approach for the study of genetic diversity of *Sinorhizobium meliloti* populations

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Abstract

The development of a species-specific marker for the analysis of the genetic polymorphism of the nitrogen-fixing symbiotic bacterium *Sinorhizobium meliloti* directly from environmental DNA is reported. The marker is based on terminal-restriction fragment length polymorphism (T-RFLP) methodology targeting specifically the 16S-23S Ribosomal Intergenic Spacer of *S. meliloti*. Species-specificity and polymorphism of the marker were tested on DNA extracted from soil samples and from a collection of 130 *S. meliloti* bacterial isolates. These primers and the T-RFLP approach proved useful for the detection and analysis of polymorphism of *S. meliloti* populations.

Keywords: bacterial population, IGS, *Sinorhizobium meliloti*, T-RFLP

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Sinorhizobium meliloti is a nitrogen-fixing bacterial species which forms symbiotic nodules on the roots of leguminous plants. This species has been extensively used as model for bacterial population genetics (Carelli *et al.* 2000; Jebara *et al.* 2001; Bailly *et al.* 2006). However, due to the lack of efficient selective culture media, most of the population genetic studies on *S. meliloti* have been performed only on bacteria isolated from nodules with a few studies conducted on bacteria directly recovered from soil (Bromfield *et al.* 1995; Barran *et al.* 1997; Hartmann *et al.* 1998). These studies indicated that there is a fraction, possibly genetically differentiated, of the rhizobial population that cannot be detected on nodules and whose ecological role remains mostly unknown. To overcome the limitation due to isolation and cultivation of *S. meliloti* from soil, we developed a terminal-restriction fragment length polymorphism (T-RFLP; Marsh 1999) marker targeting the 16S-23S rDNA intergenic gene spacer (IGS) sequence to detect specifically *S. meliloti* DNA in soil samples and allow the analysis of its genetic polymorphism. IGS is known to be highly polymorphic among *S. meliloti* strains (Biondi *et al.* 2003). Marker specificity and ability to detect the polymorphism were tested on DNA extracted from soil and on DNA from a collection of *S. meliloti* isolates.

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Primer pairs for selective amplification of *S. meliloti* IGS region were developed based on comparison of nucleotide sequence of the IGS region of *S. meliloti* (and its closest phylogenetic relative, *Sinorhizobium medicae*. From the alignment (data not shown), a set of three primers were designed based on the sequence of *S. meliloti* Rm1021 chromosome (accession number NC_003047): IGS-mel-57f, 5′-CTCGAGTGCATGAAGTTGGA-3′ (nt positions 83029–83048); IGS-mel-r, 5′-CTGGCCTTGCGAAGCCTTA-3′ (nt positions 84586–84604); IGS-mel-1392r, 5′-AACCTATCCTCCTCGCTTGC-3′ (nt positions 84345–84364). These primers were used for a semi-nested, two-step amplification reaction involving a first polymerase chain reaction (PCR) reaction with IGS-mel-57f/IGS-mel-r pair followed by a re-amplification of 1 μL of the first PCR product with primer pair IGS-mel-57f/IGS-mel-1392r. Due to the low abundance of *S. meliloti* cells in soil (usually estimated around 10^3–10^4 cells/g of soil out of 10^8–10^9 total bacteria/g of soil as reported in Trabelsi *et al.* 2009), one-step amplification reaction produced faint or no amplification products on soil DNA, while the semi-nested reaction with

Table 1 Number of different terminal-restriction fragments (TRFs) obtained from the analysis of soil DNA and of a collection of 130 *Sinorhizobium meliloti* isolates*. TRFs sizes (in nt) obtained with (a) forward primer (IGS-mel-57f) and (b) reverse primer (IGS-mel-1392r)

*Msp*I	*Alu*I	*Hha*II
(a)		
32 (14)	36	36 (3)
40	46 (9)	38
48 (114)	50	41 (87)
116	52	46 (36)
120	53 (4)	48
282 (1)	55	55 (4)
392 (1)	57	81
	60	148
	63	289
	73	358
	94	
	100	
	103 (65)	
	105 (52)	
	138	
	161	
	170	
	213	
	218	
	442	
Total TRFs = 7	20	10
(b)		
44 (4)	35	39 (3)
49 (52)	37	41
64 (74)	45 (25)	51
125	49	53
132	55	44 (85)
	57 (1)	49 (37)
	96	54
	102	57 (5)
	116	64
	121	69
	140 (104)	169
	165	359
	170	
	204	
	232	
Total TRFs = 5	15	12

*The size of the TRFs obtained by each enzyme on forward and reverse primer is reported. All TRF sizes reported were retrieved in the analysed soil samples. When a particular TRF was found also in the collection of strains, the number of isolates scored with that TRF size is reported in parentheses. No TRFs was exclusively scored in the strain collection.

Table 2 Number and composition of unique T-RFLP profiles scored in 130 *Sinorhizobium meliloti* isolates*

		Profile definition					
Profile code	Occurrence	*Msp*I-Fw	*Msp*I-Rv	*Alu*I-Fw	*Alu*I-Rv	*Hha*II-Fw	*Hha*II-Rv
SM1	23	48	64	103	140	46	49
SM2	22	48	64	105	140	41	44
SM3	17	48	64	103	140	41	44
SM4	14	48	49	105	140	41	44
SM5	6	32	64	105	140	41	44
SM6	5	48	49	103	45	46	49
SM7	5	48	49	103	45	41	44
SM8	5	32	49	105	140	41	44
SM9	4	48	49	46	45	41	44
SM10	3	48	64	103	140	36	39
SM11	3	48	49	103	140	41	44
SM12	2	48	49	53	45	46	49
SM13	2	48	49	103	140	46	49
SM14	2	48	49	103	45	55	57
SM15	1	48	49	103	140	55	57
SM16	1	48	49	46	45	55	57
SM17	1	48	49	46	45	46	49
SM18	1	48	64	103	140	46	44
SM19	1	48	44	53	45	41	44
SM20	1	32	44	46	45	41	44
SM21	1	48	49	103	45	41	49
SM22	1	48	49	103	140	41	49
SM23	1	48	44	53	57	46	49
SM24	1	48	64	103	140	41	57
SM25	1	48	49	105	45	41	44
SM26	1	282	49	105	140	41	44
SM27	1	32	49	46	140	41	44
SM28	1	48	64	46	45	41	44
SM29	1	48	44	105	140	41	44
SM30	1	392	49	105	140	41	44
SM31	1	32	49	105	140	46	49
Total	130						

T-RFLP, terminal-restriction fragment length polymorphism.
*Profile codes, occurrence of the profile and sizes of TRFs obtained by each enzyme combination with forward (Fw) and reverse (Rv) primers are reported.

IGS-mel-57f/IGS-mel-1392r yielded abundant amplicons. Both first and second PCR were run in the same conditions in a total volume of 25 μL containing 10 ng (or 1 μL) of template DNA, 2 U of *Taq* DNA polymerase (Polymed), 10 μg of bovin serum albumine, 10 pmol of each primer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$, and 1X concentration of *Taq* DNA polymerase buffer. Cycling conditions were as follows: 3′ 95 °C, followed by 35 cycles composed by 30′ 94 °C, 30′ 55 °C, 90′ 72 °C and final extension step of 5′ 72 °C.

Species-specificity of primer pairs was tested on strains of the family *Rhizobiaceae* and on ten *S. meliloti* natural strains (Table S1) and results showed that only DNAs from strains of the species *S. meliloti* gave amplification products. Moreover, to exclude the possibility that, when used on soil DNA, primer pairs might amplify DNA from other species, a clone library of IGS amplification products from soil DNA was constructed. All sequenced IGS regions from twenty randomly chosen clones (accession numbers EU718183–EU718202) showed

high similarities (98–99% of identity) to the *S. meliloti* IGS. The genetic polymorphism of IGS in natural populations was assayed by T-RFLP with both forward and reverse primers (fluorescently labelled with FAM and HEX respectively), following previously reported procedures (Mengoni *et al.* 2005). T-RFLP was performed on IGS semi-nested amplified from DNA extracted from two soil samples taken in Soliman (Tunisia) and from individual DNA of a laboratory collection of 130 *S. meliloti* strains isolated from nodules of plants grown on the same soils (Table S2). After restriction enzyme digestion, labelled forward primer (IGS-mel-57f) was found to be slightly more informative than the reverse primer (IGS-mel-1392r), allowing the recognition of 37 and 32 TRFs respectively (Table 1). Among the three different restriction enzymes, *Alu*I produced more TRFs than *Hha*II and *Msp*I. The discriminative power on the isolate's collection (see Table S2 for T-RFLP profiles of each isolate) was high and in line with previous analyses on full length IGS PCR-RFLP (Biondi *et al.* 2003), producing a total of 31 different T-RFLP profiles over 130 isolates (Table 2).

In conclusion, we demonstrated that this new cultivation-independent protocol for the analysis of *S. meliloti* genetic polymorphism could complement and improve current population genetic investigations based on *S. meliloti* nodule isolates.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Species-specificity tests on DNA from selected bacterial strains

Table S2 T-RFLP profiles for each strain tested

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Supplementary material - Table S1. Species-specificity tests on DNA from selected bacterial strains*.

Strain	**Presence of amplification products**
S. medicae LMG18864	-
S. medicae WSM419	-
S. fredii USDA205	-
S. terangae USDA4101	-
S. saheli USDA4102	-
Rhizobium leguminosarum bv. viciae USDA2370	-
Bradyrhizobium japonicum USDA110	-
Mesorhizobium huakuii USDA4779	-
Rhizobium etli CFN42	-
Rhizobium tropici CIAT899	-
Azorhizobium caulinodans USDA4892	-
S. meliloti Rm1021	+
S. meliloti USDA1002	+
S. meliloti BL225C	+
S. meliloti BO21CC	+
S. meliloti AK83	+
S. meliloti AK58	+
S. meliloti SA1	+
S. meliloti SA2	+
S. meliloti SA3	+
S. meliloti SA10	+
S. meliloti SA11	+
S. meliloti SA12	+
S. meliloti SA13	+
S. meliloti SA27	+
S. meliloti SA40	+
S. meliloti SA45	+

* Strain name and PCR result after semi-nested amplification reaction are reported. +, positive amplification; - no amplification.

Supplementary material - Table S2. T-RFLP profiles for each strain tested.*
*Isolate's code and fragment sizes (in nt) per each enzyme combination for forward and reverse primers (Fw and Rv, respectively) are reported.

	***Msp*I-Fw**	***Msp*I-Rv**	***Alu*I-Fw**	***Alu*I-Rv**	***Hha*II-Fw**	***Hha*II-Rv**
SA-01	48	49	103	45	46	49
SA-02	48	49	103	140	55	57
SA-03	48	49	53	45	46	49
SA-04	48	49	103	140	46	49
SA-05	48	49	103	45	55	57
SA-06	48	49	46	45	55	57
SA-07	48	49	103	45	46	49
SA-08	48	49	103	45	46	49
SA-09	48	49	103	45	55	57
SA-10	48	49	103	45	46	49
SA-11	48	49	53	45	46	49
SA-12	48	49	46	45	46	49
SA-13	48	64	103	140	36	39
SA-14	48	64	103	140	46	49
SA-15	48	64	103	140	46	49
SA-16	48	64	103	140	41	44
SA-17	48	64	103	140	46	49
SA-18	48	64	103	140	41	44
SA-19	48	64	103	140	46	49
SA-20	48	64	103	140	41	44
SA-21	48	64	103	140	41	44
SA-22	48	64	103	140	46	44
SA-23	48	64	103	140	41	44
SA-24	48	64	103	140	36	39
SA-25	48	64	103	140	46	49
SA-26	48	64	103	140	46	49
SA-27	48	64	103	140	46	49
SA-28	48	64	103	140	41	44
SA-29	48	64	103	140	46	49
SA-30	48	64	103	140	46	49
SA-31	48	64	103	140	46	49
SA-32	48	64	103	140	41	44
SA-33	48	64	103	140	46	49
SA-34	48	64	103	140	46	49

SA-35	48	44	53	45	41	44
SA-36	48	64	103	140	46	49
SA-37	48	64	103	140	41	44
SA-38	48	64	103	140	46	49
SA-39	48	64	103	140	46	49
SA-42	48	64	103	140	46	49
SA-43	48	64	103	140	36	39
SA-44	48	64	103	140	46	49
SA-45	48	64	103	140	46	49
SA-46	48	64	103	140	46	49
SA-47	48	64	103	140	46	49
SA-48	48	64	103	140	46	49
SA-49	48	64	103	140	46	49
SA-50	48	49	103	45	46	49
SA-51	48	64	103	140	46	49
SA-52	32	44	46	45	41	44
SA-53	48	64	103	140	41	44
SA-54	48	49	103	45	41	49
SA-55	48	64	103	140	41	44
SA-56	48	64	103	140	41	44
SA-57	48	49	103	140	41	49
SA-58	48	64	103	140	41	44
SA-59	48	44	53	57	46	49
SA-60	48	49	103	45	41	44
SA-61	48	49	103	45	41	44
SA-62	48	49	103	45	41	44
SA-63	48	49	103	140	41	44
SA-64	48	49	103	140	46	49
SA-65	48	64	103	140	41	44
SA-66	48	64	103	140	41	44
SA-67	48	49	103	45	41	44
SA-68	48	49	103	140	41	44
SA-69	48	64	103	140	41	57
SA-70	48	64	103	140	41	44
SA-71	48	64	103	140	41	44
SA-72	48	64	103	140	41	44
SA-73	48	49	103	45	41	44
SA-74	48	49	46	45	41	44
SA-75	48	49	103	140	41	44

SS-07	48	64	105	140	41	44
SS-08	48	49	105	45	41	44
SS-09	48	64	105	140	41	44
SS-10	48	64	105	140	41	44
SS-11	48	49	105	140	41	44
SS-12	48	64	105	140	41	44
SS-13	48	64	105	140	41	44
SS-14	282	49	105	140	41	44
SS-15	48	49	105	140	41	44
SS-16	48	49	105	140	41	44
SS-17	48	64	105	140	41	44
SS-18	48	64	105	140	41	44
SS-19	48	49	46	45	41	44
SS-22	48	64	105	140	41	44
SS-24	48	49	105	140	41	44
SS-25	32	64	105	140	41	44
SS-26	48	64	105	140	41	44
SS-27	48	49	105	140	41	44
SS-28	32	64	105	140	41	44
SS-29	32	49	46	140	41	44
SS-30	48	64	46	45	41	44
SS-31	48	49	105	140	41	44
SS-32	32	64	105	140	41	44
SS-35	32	49	105	140	41	44
SS-36	48	49	105	140	41	44
SS-37	48	64	105	140	41	44
SS-38	48	64	105	140	41	44
SS-43	48	64	105	140	41	44
SS-45	48	44	105	140	41	44
SS-46	48	64	105	140	41	44
SS-47	48	64	105	140	41	44
SS-48	48	64	105	140	41	44
SS-49	48	49	105	140	41	44
SS-50	48	49	105	140	41	44
SS-51	48	64	105	140	41	44
SS-52	48	64	105	140	41	44
SS-53	48	64	105	140	41	44
SS-54	32	49	105	140	41	44
SS-56	32	49	105	140	41	44

SS-57	48	49	46	45	41	44
SS-58	48	64	105	140	41	44
SS-59	48	49	46	45	41	44
SS-61	48	49	105	140	41	44
SS-62	48	49	105	140	41	44
SS-63	32	64	105	140	41	44
SS-64	48	49	105	140	41	44
SS-65	392	49	105	140	41	44
SS-66	48	64	105	140	41	44
SS-67	48	64	105	140	41	44
SS-68	48	64	105	140	41	44
SS-69	32	64	105	140	41	44
SS-70	32	49	105	140	46	49
SS-71	32	49	105	140	41	44
SS-72	32	49	105	140	41	44
SS-73	32	64	105	140	41	44
SS-74	48	49	105	140	41	44
SS-75	48	49	105	140	41	44

IV.2 Development of Real-Time PCR assay for detection and quantification of Sinorhizobium meliloti in soil and plant tissue

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ORIGINAL ARTICLE

Development of real-time PCR assay for detection and quantification of *Sinorhizobium meliloti* in soil and plant tissue

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Abstract

Aims: *Sinorhizobium meliloti* is a nitrogen-fixing alpha-proteobacterium present in soil and symbiotically associated with root nodules of leguminous plants. To date, estimation of bacterial titres in soil is achieved by most-probable-number assays based on the number of nodules on the roots of test plants. Here, we report the development of two real-time PCR (qPCR) assays to detect the presence of *S. meliloti* in soil and plant tissues by targeting, in a species-specific fashion, the chromosomal gene *rpo*E1 and the pSymA gene *nod*C.

Methods and Results: *rpo*E1 and *nod*C primer pairs were tested on DNA extracted from soil samples unspiked and spiked with known titres of *S. meliloti* and from plant root samples nodulated with *S. meliloti*. Results obtained were well in agreement with viable titres of *S. meliloti* cells estimated in the same samples.

Conclusions: The developed qPCR assays appear to be enough sensitive, precise and species-specific to be used as a complementary tool for *S. meliloti* titre estimation.

Significance and Impact of the Study: These two novel markers offer the possibility of quick and reliable estimation of *S. meliloti* titres in soil and plant roots contributing new tools to explore *S. meliloti* biology and ecology including viable but nonculturable fraction.

Introduction

Sinorhizobium meliloti is a soil bacterium, belonging to the alpha subdivision of proteobacterium, able to colonize the roots of leguminous plants where it gives rise to the formation of specialized root structures called nodules where bacterial nitrogen-fixation occurs (Rose 2008), thus increasing plant agronomic yield without the need for nitrogen fertilization. Besides the important role as a model for symbiotic nitrogen-fixation, *S. meliloti* is a model species also for bacterial population genetics (see, for examples, Carelli *et al.* 2000; Bailly *et al.* 2006). The actual population size of *S. meliloti* in the environment is due to both the fraction of free-living cells in soil and to the symbiotically associated cells in roots. Despite its prominent role among environmental and agricultural bacteria, to date no selective culture media have been developed allowing the direct estimation of *S. meliloti* titres in soil. Consequently, quantification of *S. meliloti* in soil samples is currently performed via plant trapping, that is by estimating the nodulation potentiality of the soil (that is the presence of *S. meliloti* cells) using different *Medicago* species as host plant in green-house controlled conditions (Thompson and Vincent 1967). This technique, though giving reliable results, is labour intensive and requires facilities and equipments to be performed, hampering large-scale studies about the presence and the abundance of *S. meliloti* in different soils and habitats. Moreover, plant trapping is ineffective with the viable but nonculturable fraction (Basaglia *et al.* 2007) and in the last years there has been an increasing interest towards the possibility to analyse rhizobial populations

without the need of cultivation (Zézé *et al.* 2001; De Oliveira *et al.* 2005).

Quantitative PCR (real-time PCR, qPCR) is a polymerase chain reaction technique which measures PCR amplification kinetics allowing the quantification of template DNA present in a sample. The qPCR assay has been used in many studies for direct quantification of bacteria in different environmental samples by targeting either the 16S ribosomal RNA or other specific gene sequences in the soil (see, for examples, Smits *et al.* 2004; Duodu *et al.* 2005; Sauvage *et al.* 2007; Selleck *et al.* 2008). The development of a qPCR assay for direct estimation of *S. meliloti* cells in soil could represent an advantage over the current labour-intensive and time-consuming nodulation-based protocol.

Here, we present the development and the application of two *S. meliloti*-specific qPCR assays to estimate bacterial titres on soil and plant samples.

Materials and methods

Soil samples, bacterial strains and MPN estimation of *Sinorhizobium meliloti* titres in soil

Three soil samples were taken from Soliman, Tunisia and Grezzano, Italy. Bacterial strains used in this study were *S. meliloti* Rm1021 (fully genome-sequenced type strain); *S. meliloti* BL225C, *S. meliloti* BO21CC, *S. meliloti* AK83 and *S. meliloti* AK58 strains (Giuntini *et al.* 2005); *S. meliloti* USDA1002, *S. medicae* LMG18864, *S. medicae* WSM419, *S. fredii* USDA205, *S. terangae* USDA4101, *S. saheli* USDA4102, *Rhizobium leguminosarum* bv. viciae USDA2370, *Bradyrhizobium japonicum* USDA110, *Mesorhizobium huakuii* USDA4779, *Rh. etli* CFN42, *Rh. tropici* CIAT899 and *Azorhizobium caulinodans* USDA4892. Moreover, 10 *S. meliloti* (SA1, SA2, SA3, SA10, SA11, SA12, SA13, SA27, SA40 and SA45) and 10 *S. medicae* (SS1, SS2, SS21, SS23, SS33, SS34, SS42, SS54, SS55 and SS60) isolates collected from Soliman soil were also included in the study. The most probable number (MPN) estimate of *S. meliloti* bacteria in soil was carried out by using a plant infection method (Thompson and Vincent 1967) with *Medicago truncatula* as trapping plant.

Soil spiking, *in-vitro* nodulation of *Medicago truncatula*

For soil spiking experiment, 0·5 g aliquots of samples from a clay soil in Grezzano (Italy) sampled with a caterpillar at ~3-m depth, previously shown to be free from *S. meliloti* by the application of the present qPCR assay, were spiked with 100 μl of *S. meliloti* Rm1021 serial dilutions of a logarithmic-phase grown culture in TY (Trypone Yeast) medium (to obtain titres from $3{\cdot}4 \times 10^6$ to $3{\cdot}4 \times 10^1$ CFUs g^{-1} of soil). Immediately after spiking, soil aliquots were stirred to homogenize soil particles with bacteria and DNA was extracted as described below.

Nodulation of *M. truncatula* cv. Jemalong plants was performed as reported in Wais *et al.* (2002), allowing plants to grow up to 20 days after *S. meliloti* Rm1021 inoculation. *S. meliloti* titres in plant roots were estimated as CFU g^{-1} wet weight as in Barzanti *et al.* (2007) with slight modification (surface sterilization was performed with 0·1% HClO for 3′). Serial dilutions of the samples were plated on TY plates containing 100 mg l^{-1} streptomycin and incubated at 30°C for 2 days.

DNA extraction, cloning and sequencing

DNA was extracted from soil samples, surface sterilized root tissues and bacterial strains by using standard bead-beating protocols (FastDNA Kit, QBiogene, Illkirch Cedex, France). Quantification of DNA was performed by UV spectrophotometric reading (Biophotometer, Eppendorf).

Two libraries of putative *nod*C and *rpo*E1 amplification products from Soliman soil 1 DNA were constructed after cloning in pGEM-T Easy Vector (Promega Italia, Milano, Italy). Fifteen randomly chosen clones per library were sequenced and sequences were compared with those present in GenBank database by using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al.* 1997).

Primer design, qPCR conditions and data analysis

Primer for amplification of *rpo*E1 and *nod*C gene fragments were designed by using the CLONE MANAGER SUITE ver. 6 software (Sci-Ed, Cary, NC). Primer pairs were tested for similarities with other known sequences present in GenBank database by using BLASTN (Altschul *et al.* 1997). The primer pair specific for *S. meliloti rpo*E1 gene (rpoE1-fw/rpoE1-rv) was designed to amplify a 100-bp fragment between nt positions 2267138 and 2267237 of *S. meliloti* Rm1021 chromosome, whereas the primer pair specific for *S. meliloti nod*C gene (nodC-fw/nodC-rv) was designed to amplify a 149-bp fragment between nt positions 480262 and 480133 of *S. meliloti* Rm1021 pSymA megaplasmid (Table 1, Fig. 1). Alignments between *S. meliloti* Rm1021 sequences and the most similar sequences of *S. medicae* WSM419 were performed with the CLUSTALW module implemented in BIOEDIT ver. 7·0·9·0 (Ibis Biosciences, Carlsbad, CA) (Hall 1999).

Real-time PCR was performed in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) programmed with the following temperature profile: 2 min 94°C, followed by 40 cycles composed by 15 s 94°C, 15 s 65°C, 30 s 72°C. Fluorescence data acquisition was done during the extension step at 72°C. A final

Table 1 Primers developed in this study*

Primer name	Sequence (5′->3′)	Length	Nucleotide positions	Tm	GC%
rpoE1-fw	CGAGGAAGAGGTCCTGGAAT	20	2267138–2267157	60·59	55·0
rpoE1-rv	GACGCAGTCCTGCAACAGAT	20	2267218–2267237	61·44	55·0
nodC-fw	GCCGCTATCTCAATCTACGC	20	480133–480152	59·97	55·0
nodC-rv	TTGAAGCTGGGGACGATAAC	20	480243–480262	60·07	50·0

*Name, sequence, length of primer, nucleotide position in *S. meliloti* Rm1021 genome, Tm and GC% of each primer are reported. rpoE1 primers positions are referred to the *S. meliloti* Rm1021 chromosome (GenBank accession number NC_003047); nodC primers positions are referred to the *S. meliloti* Rm1021 pSymA megaplasmid (GenBank accession number NC_003037).

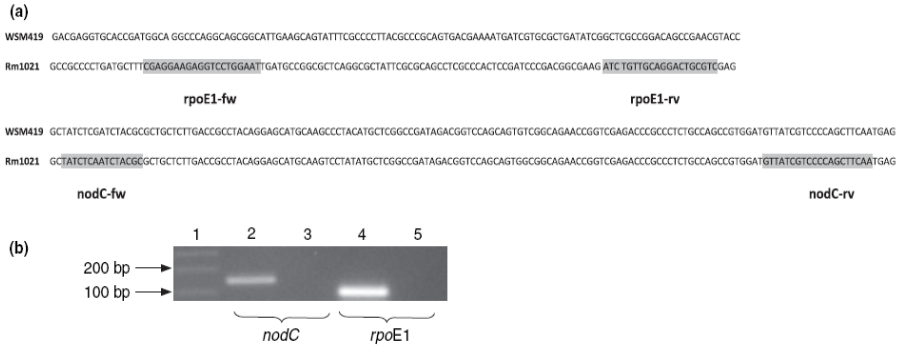

Figure 1 (a) Alignment of *rpo*E1 and *nod*C primer pairs with the corresponding most homologous regions in *S. meliloti* Rm1021 and *S. medicae* WSM419 genomes (see Table 2 for nucleotide positions); (b) Gel electrophoresis stained with ethidium bromide of amplification products on *S. meliloti* Rm1021 DNA with *rpo*E1 and *nod*C primer pairs. Lane 1, size marker; lanes 2 and 4, Rm1021 DNA; lanes 3 and 5, *S. medicae* WSM419 DNA. Sizes of band markers are indicated.

melting curve was performed to check for product specificity. Reactions were performed in 20 μl final volume containing 10 μl of SYBR Green mix (SYBR Green JumpStart Taq Ready Mix, Sigma-Aldrich), 0·5-μl ROX solution (included in the kit SYBR Green JumpStart Taq Ready Mix) and 10 pmols of primers. Template DNA from spiked and unspiked soil samples was 1 μl out of 100 μl of total DNA solution from 0·5 g of soil. All reactions were done in triplicate. Data were analysed with the software SDS version 1·2 (Applied Biosystems). PCR efficiency was calculated as efficiency = $-1 + 1 \times 10^{(-1/\text{slope})}$ (Ståhlberg *et al.* 2003).

Results

Development of *Sinorhizobium meliloti*-specific markers

For the development of *S. meliloti*-specific qPCR markers, a list of loci supposed to be conserved in *S. meliloti* strains based on population genetic (Bailly *et al.* 2006) and genomic data (Giuntini *et al.* 2005), was prepared. The loci were selected to be located on both chromosome and on megaplasmids (pSymA and pSymB) of *S. meliloti* Rm1021 genome to allow the development of a possible multi-marker system based on independent replicons. On the chromosome, the intergenic region named IGS_{RKP} (Bailly *et al.* 2006) and *rpo*E1 (Smc01419 encoding for a putative RNA polymerase sigma factor protein) and 16SrRNA (SMc03222) genes were chosen. For megaplasmid loci, intergenic regions IGS_{NOD}, IGS_{GAB}, IGS_{EXO} (Bailly *et al.* 2006) and genes *nod*A (SMa0869), *nod*B (SMa0868), *nod*C (SMa0866) involved in Nod Factor biosynthesis and required for nodulation were selected.

Primer pairs of all the *IGS* regions were tested in qPCR with Soliman soil DNA as template. Obtained amplification plots and melting curves indicated poor efficiency of amplification and the presence of spurious amplicons. We consequently focussed on protein-coding genes and 16SrRNA, comparing *S. meliloti* Rm1021 *nod*ABC, *rpo*E1 and 16SrRNA sequences with sequences present in GenBank database and in particular with the genome of the sister species *S. medicae* WSM419. Only the chromosomal

gene *rpo*E1 and the pSymA gene *nod*C showed sequence divergence between *S. meliloti* Rm1021 and *S. medicae* WSM419 (87% and 98% of identity, respectively) and were consequently selected for the design of *S. meliloti*-specific primer pairs (Fig. 1, Table 1).

To check the selectivity of *rpo*E1 and *nod*C primer pairs for PCR amplification of *S. meliloti* DNA, DNAs from a collection of *S. meliloti* and other rhizobial strains were tested (see section 'Materials and methods'). The collection was composed of both laboratory strains and environmental isolates of *S. meliloti* and *S. medicae* from Soliman soil (10 isolates each species) and included also *S. meliloti* strains BL225C, BO21CC, AK83 and AK58 for which comparative genomic data are available (Giuntini *et al.* 2005). Both *rpo*E1 and *nod*C primer pairs gave amplification products only with DNA from *S. meliloti* strains, confirming the selectivity of the designed pairs on pure culture DNA. To assay the selectivity on DNA extracted from soil and plant, the presence of spurious amplification products was checked in real-time PCR by performing qualitative analysis of melting curves on the amplicons obtained from DNA extracted from the Soliman soil samples and from *M. truncatula* root nodules. Only single melting peaks with the same Tm (87·5°C for *rpo*E1, 88·6°C for *nod*C) were scored from all assays (Fig. 2), suggesting the absence of spurious amplification products from soil microbial communities and plant DNA. Moreover, PCR products obtained after amplification on Soliman soil 1 DNA with *rpo*E1 and *nod*C primer pairs were cloned and 15 randomly chosen colonies for *rpo*E1 and *nod*C libraries were sequenced. Obtained sequences resulted identical to those of *rpo*E1 and nodC gene fragments of *S. meliloti* Rm1021 present in GenBank database, thus confirming the species specificity also on the DNA of a soil bacterial community.

qPCR assays of soil and plant root DNA

External standard curves were obtained using serial dilutions of *S. meliloti* Rm1021 genomic DNA. Because of *rpo*E1 and *nod*C genes present in single copies in the *S. meliloti* Rm1021 genome, the copy number of the gene

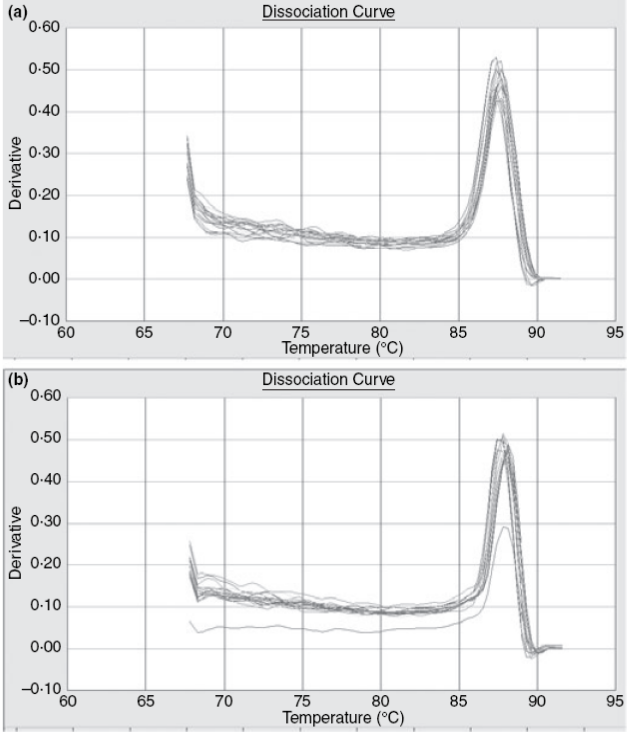

Figure 2 Melting curves for (a) *rpo*E1 and (b) *nod*C amplicons from soil DNA. The different curves represent different soil DNA samples. The intensity of the peak is proportional to the amount of amplicon present in the PCR tube.

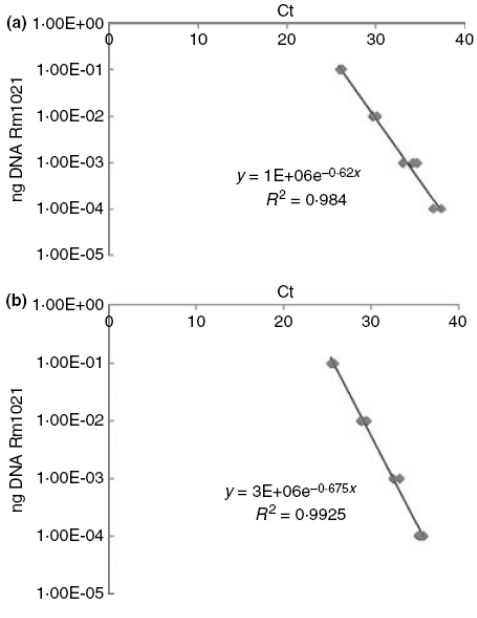

Figure 3 Calibration plots on *S. meliloti* Rm1021 DNA for (a) *rpo*E1 and (b) *nod*C. The threshold cycle (Ct) with respect to the amount of *S. meliloti* 1021 template DNA is reported. Equations and linear correlation coefficients (R^2) are reported.

can be directly related to the number of copies of the genome (whose size is 6·68 Mbp) and, consequently, to the number of cells present in the sample.

Standard curves showed a linear range from 0·1 to 1×10^{-4} ng of Rm1021 genomic DNA corresponding to $1{\cdot}39 \times 10^4$ to 1·39 gene copies (Fig. 3). The correlation coefficients R^2 were very high both for *rpo*E1 gene (0·984) and for *nod*C gene (0·992). Slope was −3·618 for *rpo*E1 and −3·385 for *nod*C. Calculated PCR efficiencies were 88·8% and 97·4% for *rpo*E1 and *nod*C, respectively.

The applicability of the assay to quantify *rpo*E1 and *nod*C gene copies in environmental samples was evaluated by spiking known titres of *S. meliloti* Rm1021 in soil aliquots (soil 3 from Grezzano, Italy) and testing the protocol on two soils (soil 1 and soil 2 from Soliman, Tunisia) in which the number of nodulating *S. meliloti* cells was previously estimated by MPN. Obtained qPCR results (Table 2) were well in agreement with known bacterial titres spiked in soil 3, down to around 10^3 cells g^{-1}. Spiking of low number of cells (10^1 and 10^2) did not produce positive amplification signals. Moreover, qPCR estimates were also in agreement with the number of CFUs present in nodulated root tissues of *M. truncatula*. Concerning the Soliman soil 1 and soil 2 in Tunisia, comparing *rpo*E1 and *nod*C qPCR estimates, these were similar for soil 1 and one-order magnitude different for soil 2. Interestingly, for both soils, qPCR estimates were higher than MPN estimates.

Table 2 qPCR estimates of *S. meliloti* titres in soil and in *M. truncatula* root tissues*

Sample	pH	Organic matter (%)	Total carbon (%)	Total nitrogen (%)	Electrical conductivity (EC) (mmho cm^{-1})	Moisture (%)	Texture	Number of *rpo*E1 gene copies†	Number of *nod*C gene copies†	Titres‡
Soil 1 (Soliman, Tunisia)	8·9	1·13	0·7	1·8	4·6	54	Clay	$1{\cdot}75 \pm 0{\cdot}16 \times 10^5$	$1{\cdot}26 \pm 0{\cdot}21 \times 10^5$	$3{\cdot}3 \times 10^4$
Soil 2 (Soliman, Tunisia)	8·5	0·9	0·4	1·4	0·7	32	Sandy	$4{\cdot}18 \pm 0{\cdot}67 \times 10^5$	$1{\cdot}50 \pm 0{\cdot}42 \times 10^4$	$3{\cdot}3 \times 10^3$
Soil 3 (Grezzano, Italy) + Rm1021	Nd	Nd	Nd	Nd	Nd	Nd	Clay	$1{\cdot}64 \pm 0{\cdot}87 \times 10^6$	$1{\cdot}89 \pm 0{\cdot}32 \times 10^6$	$3{\cdot}4 \times 10^6$
Soil 3 (Grezzano, Italy) + Rm1021	Nd	Nd	Nd	Nd	Nd	Nd	Clay	$3{\cdot}58 \pm 0{\cdot}46 \times 10^5$	$2{\cdot}21 \pm 0{\cdot}34 \times 10^5$	$3{\cdot}4 \times 10^5$
Soil 3 (Grezzano, Italy) + Rm1021	Nd	Nd	Nd	Nd	Nd	Nd	Clay	$2{\cdot}58 \pm 0{\cdot}20 \times 10^4$	$1{\cdot}89 \pm 0{\cdot}42 \times 10^4$	$3{\cdot}4 \times 10^4$
Soil 3 (Grezzano, Italy) + Rm1021	Nd	Nd	Nd	Nd	Nd	Nd	Clay	$5{\cdot}68 \pm 3{\cdot}21 \times 10^2$	$7{\cdot}43 \pm 0{\cdot}25 \times 10^2$	$3{\cdot}4 \times 10^3$
M. truncatula root								$4{\cdot}92 \pm 0{\cdot}89 \times 10^6$	$1{\cdot}44 \pm 0{\cdot}22 \times 10^6$	$3{\cdot}6 \times 10^6$

*The estimated number of *S. meliloti rpo*E1 and *nod*C gene copies (copies g^{-1} of soil) in three soils with different physico-chemical characteristics; and in *M. truncatula* nodulated root tissues. Soil 3 from Grezzano was spiked with serial dilutions known quantities of *S. meliloti* cells (see column 'Titres').

Nd, not determined.

†(±)SD from triplicate reactions of two independent experiments (six total reactions).

‡Titres are: most probable number estimates of *S. meliloti* cells g^{-1} in soil 1 and soil 2, spiked Rm1021 cells (CFU g^{-1}) in soil 3, CFU g^{-1} fresh weight in *M. truncatula* roots.

Discussion

In this paper, we reported the development of qPCR assays for the detection and quantification of *S. meliloti* cells in soil and plant samples. Primer pairs designed to amplify portions of the chromosomal gene *rpo*E1 and of the pSymA gene *nod*C of *S. meliloti* gave successful results in terms of specificity on both pure cultures and on DNA extracted from soil and plant tissues. qPCR assays based on both *rpo*E1 and *nod*C gave reliable results down to $3{\cdot}4 \times 10^3$ cells g^{-1} of soil, which is quite lower than for other qPCR protocols in other rhizobial species (Duodu *et al.* 2005). In plant roots nodulated with *S. meliloti* Rm1021, both assays were well in agreement with viable titres after surface sterilization. In native soil from Soliman, estimated qPCR titres were one–two orders of magnitude higher than MPN estimates. As reported also for other rhizobial species (Duodu *et al.* 2005), this higher qPCR estimates could suggest that qPCR approach could be more effective than nodulation-based protocols, allowing the estimation of the whole *S. meliloti* population which includes both strains nodulating the plants and free-living in soil (Duodu *et al.* 2005). It is interesting to note here that the titre of Soliman soil 2 estimated with *nod*C was significantly lower than that obtained with *rpo*E1, better approximating the MPN-estimated titre. This evidence could suggest the possibility that the chromosomal gene *rpo*E1 target a wider fraction of *S meliloti* populations which could not harbour the symbiotic-required gene *nod*C. Actually, earlier reports on *Rh. leguminosarum* indicated that only a small fraction of the rhizobial population contains genes required for symbiosis with host plant (Soberòn-Chavez and Nàjera 1989; Segovia *et al.* 1991). However, further investigations are necessary to elucidate the biological significance of the observed differences.

In conclusion, the qPCR protocols presented here, targeting *rpo*E1 and *nod*C genes of *S. meliloti* can be used as a fast and complementary tool for the estimation of the number of *S. meliloti*, DNA and cells in soil samples and plant tissues.

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Chapter V

In/Out nodules. Pattern of diversity at community and population level in plant-associated bacteria in *Medicago sativa* L. (*Fabaceae*)

Plants host a plethora of bacteria from commensal, mutualist, symbionts to pathogens. It is know that certain bacterial taxa more specifically associate with plants. However, it is not clear if specificity is restricted to higher taxonomic ranks or may be present at the species level also. *Medicago sativa* L. is one of the most investigated models for biological nitrogen fixation and is host, as all plants, by a bacterial community. Moreoveor, *M. sativa* associates with a specific symbiont, the nitrogen fixing bacterium *Sinorhizobium meliloti*. These features resulted in a good system for investigating the taxonomic and genetic specificity of plant-bacterial association from the bacterial community to the single species level. A comprehensive set of cultivation-independent molecular tools, from Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis, quantitative PCR, to sequencing of 16S rRNA gene libraries was applied to DNA extracted from nodules, stems and leaf tissues of 12 individual plants and from surrounding soil.

Results indicated a high taxonomic diversity as well as a high presence of members of the class *Alpha-Proteobacteria* in plant tissues, identifying a clear differential pattern of bacterial community diversity between soil and plant tissues at class level. Interestingly, within *Alpha-Proteobacteria* the same differential pattern was observed at the family

level (with high prevalence of members from *Sphingomonadaceae* and *Methylobacteriaceae* inside plant tissues) and also at the single species level. In particular, in *S. meliloti* population, a relatively low sharing of haplotypes (30-40%) between root nodules and soil was detected, suggesting that different ability of environmental (plant) colonization are indeed present at single species also. This latter part is the very first population genetic analysis of a bacterial species without cultivation, opening new scenarios on the extent and genetic diversity within bacterial species. As for bacterial community analysis, the development of the so-called "cultivation-independent tools", opened the way to the discovery of the large biodiversity, with entire new phyla being present as "uncultivated", now the possibility to use similar tools for population studies, could be rich of future discoveries on the ecology of single bacterial species, as rhizobia.

As a biological conclusion from our study, we have shown that environmental adaptation in plant-associated bacteria is tightly linked to a wide range of taxonomic ranks down to the species level.

V.1 Introduction

Similarly to the intensively studied animal gut, plants harbor a wide range of diverse bacteria forming a complex biological community, which includes, view from the plant side, pathogens, mutualists, and commensals (*1, 2*). Depending on the colonized compartment, these bacteria are rhizospheric (root colonizers), endophytic (colonizing the endosphere, the bulk of internal tissues) and phyllospheric (leaves surface). Moreover, bacteria can be classified as 'obligate' or 'facultative' endophytes in accordance with their life strategies; obligate endophytes strictly depend on the host plant for their growth and survival, and transmission to other plants occur only by seeds or via animal vectors, while facultative endophytes could also grow outside host plants (*3*). In the recent years endophytic bacteria have been widely studied, mainly as promising tools for biotechnological applications (see for instance (*3-7*), but studies also have been carried out in order to investigate the ecological perspectives and relationships of the endophytic bacterial communities (see for instances (*8-11*). In particular, a very small fraction of the endophytic bacterial diversity can be considered associated to all plants of a given species, most bacterial taxa being found only in single individual plants (*8*). However, few bacterial taxa, have been found in the endosphere of all individual plants, notably members of *Alpha-Proteobacteria* division (see for instance (*2, 7, 12*). Consequently, the idea generally accepted is that the ability to colonize a plant is not a general, widespread, feature in a soil bacterial community, but preferentially reside in specific taxa, which may be considered more ecologically versatile. However, all investigation performed so far have focused on the whole community level only and no studies have been performed comparing the pattern of endophytic colonization at the different taxonomic ranks, i.e. class, family and species.

Leguminous plants (*Fabaceae*) are the specific partners of the symbiotic association with nitrogen-fixing rhizobia forming root nodule (*13*). *Medicago sativa* L. (alfalfa) and the diploid relative *M. truncatula* Gaertn. (barrel medic) are among the most studied species for the molecular issues related to their symbiotic bacterial partner, the nitrogen fixing bacterium *Sinorhizobium* (syn. *Ensifer*) *meliloti* (*14-16*). *S. meliloti* is present in most temperate soils, and, when conditions are suitable, it forms specialized structures, called nodules, in roots of host plants where it differentiates in bacteroids (*14*). It is assumed that a fraction of the bacterial cells is released from dehiscent nodules to soil, giving rise to new free living rhizobial clones (*17*). While the endophytic bacterial flora of *M. sativa* has never been investigated, *S. meliloti* population genetics has been extensively studied in the past years (*18-23*); however, due to the lack of efficient selective culture media, most of the population genetics studies on *S. meliloti* have been performed only on bacteria isolated from nodules with a few early studies done on bacteria directly recovered from soil (*24, 25*); in other words there are no reports of direct analysis from nodules (or even other plant tissues) without cultivation. These earlier studies suggested that the composition, in genetic terms, of *S. meliloti* population sampled in root nodules by traditional cultivation techniques may not be representative of the actual population in

soil, allowing to hypothesize the co-existence of ecologically (and genetically) different populations (i.e. residing in soil or nodulating), due to well known problems of bacterial unculturable state (*26*). *M. sativa* then may constitute a suitable and unexplored model system for investigating the pattern of colonization by endophytic bacteria from the whole community to the single species (*S. meliloti*) level.

In this work we aimed to shed more light on the specificity of plant colonization patterns by bacteria. Bacterial taxonomic composition patterns of *M. sativa* plants and of their surrounding soil from the community level (class) to the single species level (*S. meliloti*) were investigated by using cultivation-independent techniques, which allow to sample a much wider diversity than bacterial isolation procedure. In particular T-RFLP profiling and 16S rRNA library screening ((*27*) and references therein) were used to have the taxonomic profiling of bacterial communities from single samples and an overall description of taxa composition, respectively. Moreover, to analyze the taxonomic pattern at specie level (i.e. haplotypic), a new marker system targeting the 16S-23S rDNA intergenic gene spacer (IGS) sequence was used to specifically detect *S. meliloti* DNA in environmental sample (*28*), allowing to explore for the very first time the population genetics of a single bacterial species without its cultivation.

V.2 Materials and Methods

IV.2.1 Experimental design and sampling procedure

Three experimental pots called 46, 79 and 189 (60 cm diameter 150 cm depth) filled with sandy loamy soil and planted with *Medicago sativa*. Each pot contained from 6 to 8 single plants. Plants were allowed to grow on pot for two years with a cutting after one year in a greenhouse at the CRA-FLC, Lodi, Italy. In October 2009 plants were harvested and pots were opened to allow the sampling of the whole eye-detectable nodules present and of soil. In Figure S1 a schematic representation of the experimental setting and sampling is presented.

Stems, leaves (three pools of around 10 leaves per plant) and nodules were washed with water and with $MgSO_4$ 10mM two times and slightly sterilized with 1% HClO for 1' to allow most of soil and dust particles to be removed and the elimination of bacteria loosely adhering to the surface, without disturbing the bacteria present under the plant epidermis. Samples were then stored at -80°C from 1-2 weeks before DNA extraction.

V.2.2 DNA extraction Real-Time PCR and T-RFLP profiling

DNA was extracted from soil by using a commercial kit (Fast DNA Spin kit for soil, QBiogene) following manufacturer's instruction. DNA extraction from plant tissues was performed by a 2X CTAB protocol as previously described (*29*). Real-Time PCR for quantization of *S. meliloti* DNA was carried on *rpo*E1 and *nod*C loci as previously reported (*30*). 16S rRNA gene pool of total bacterial community was amplified from the extracted DNA with primer pairs 799f (labeled with HEX) and pHr which allow the amplification of most bacterial groups without targeting chloroplast DNA (*31*). PCR conditions and Terminal-Restriction Fragment Length Polymorphism (T-RFLP) profiling were as previously reported (*8*). For sinorhizobial populations, T-RFLP was carried out on 16S-23S ribosomal intergenic spacer amplified from total DNA (IGS-T-RFLP) with *S. meliloti* specific primers as already reported (*28*).

V.2.3 Library construction and sequencing

Amplified (with 799f and pHr primer pair) 16S rRNA genes from DNA extracted from soil, nodules, pooled stems and leaves of a 1:1:1 mix of all posts were inserted into pGemT vector (Promega) and cloned in *E. coli* JM109 cells. Positive clones were initially screened by white/blue colouring and the inserted amplified 16SrRNA genes sequenced. Plasmid purification and sequencing reactions were performed by Macrogen Europe Inc. (Amsterdam, The Netherlands).

V.2.4 Data processing and statistical analyses

Differences in mean values of diversity were statistically evaluated by nonparametric tests (Kruskal-Wallis) with Bonferroni error protection. For qPCR data, 1-way ANOVA

with Tukey post hoc test was employed. Analyse-it 2.0 software (Analyse-It, Ldt.) was used for both tests. For T-RFLP, chromatogram files from automated sequencer sizing were imported into GeneMarker ver. 1.71 software (SoftGenetics LLC, State College, PA, USA) by filtering with the default options of the module for AFLP analysis. Peaks above 100 fluorescence units and whose size ranged from 35 to 500 nt were considered for profile analysis. Only presence/absence of peaks were considered as informative data from the chromatograms. Statistical analyses were performed on a binary matrix obtained as previously reported (*8*) and ribotype richness reported as number of Terminal-Restriction Fragments (T-RFs). Past 2.02 (*32*) software package was used to compute Non-Metric Multidimensional Scaling (N-MDS) and UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering based on Jaccard distance. To test the distribution of the variance of T-RFLP profiles within plant tissues and among pots, Analysis of Molecular Variance (AMOVA(*33*)) was applied using Arlequin 3.5.1.2 software (http://cmpg.unibe.ch/software/arlequin3/). Although being developed for population genetic analysis, the general procedure implemented by AMOVA is enough flexible to allow to estimate the statistical significance of groupings of bacterial communities as reported previously (*34-36*). Pairwise F_{ST} distances (*37*) between T-RFLP profiles of plant tissues and soils, were used to infer a Neighbor-Joining dendrogram with the software MEGA4 (*38*).

Partial 16SrRNA sequences were manually inspected for quality, then aligned and clustered with furthest neighbor algorithm with the module present in Mothur v.1.12.3 (*39*). Diversity indices (Shannon H' and Chao-1) were calculated with the same software. Library coverage was estimated with the formula $C=1-(n/N)$ (*40*), where n is the number of singletons (defined at 97% sequence identity in Mothur) that are encountered only once in the library and N is the total number of sequenced clones. Taxonomic assignment was performed with the Classifier module present in Ribosomal Database Project 10 website (*41*) (http://rdp.cme.msu.edu/) at 80% confidence threshold. Sequences with 97% similarity were treated as a single OTU. Sequences (one for each OTU) were aligned with the 16S rRNA gene sequences of the closest match retrieved from NCBI databases, using MUSCLE (*42*) and a Neighbor-Joining dendrogram was constructed using MEGA4 (*38*). Phylogenetic inference and evolutionary distance calculations were generated using the Maximum Composite Likelihood; 1000 bootstrap replicates were used to obtain confidence estimates for the phylogenetic trees.

V.3 Result

V.3.1 Bacterial community composition and diversity

Genetic diversity of bacterial communities present in soil and associated to plant tissues (nodules, stems and leaves) was investigated by T-RFLP analysis and 16S rRNA gene library sequencing. A total of 43 samples was analyzed (Figure S1) and allowed to score 253 Terminal-Restriction Fragments (T-RFs) or ribotypes (after the restriction digest with two restriction enzymes, *Hinf*I and *Taq*I). In Table 1 the number of ribotypes detected by T-RFLP in the different sampling environment is reported. Comparing the genetic diversity values (as TRFs or number of ribotypes) of T-RFs between soil, nodules, stem and leaves, in general soil and nodules had higher (and similar to each other) values than stem and leaves.

Table V.1. Titres and genetic diversity of total bacteria in soil and plant endosphere.*

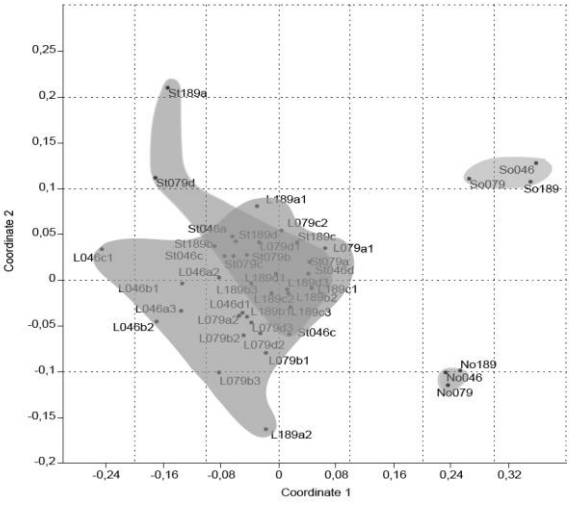
Pot 46	
Soil	41b
Nodules	58b
Stems	28.2±5.6a
Leaves	22.8±5.9a
Pot 79	
Soil	50a
Nodules	50a
Stems	23.5±13.6a
Leaves	33.3±6.7a
Pot 189	
Soil	44b
Nodules	46b
Stems	19.2±5.4a
Leaves	30.6±4.9b
Mean soils	45.0±4.6b
Mean nodules	51.3±6.1b
Mean stems	23.6±8.8a
Mean leaves	30.0±6.8a

* Bacterial titres have been estimated by qPCR on 16S rRNA gene. Diversity is the number of TRFs. ± standard deviation. Different letters indicate significant ($p<0.05$) differences after Kruskal-Wallis test

V.3.2 Bacterial community variation

Figure V.1 shows the pattern of similarity among T-RFLP profiles from total communities as Non-metric Multidimensional Scaling (N-MDS) (Figure V.1a) and cluster analysis (UPGMA) (Figure V.1b). Soil and nodule bacterial communities were strongly differentiated from stem and leaf communities, forming relatively tight clusters.

a



b

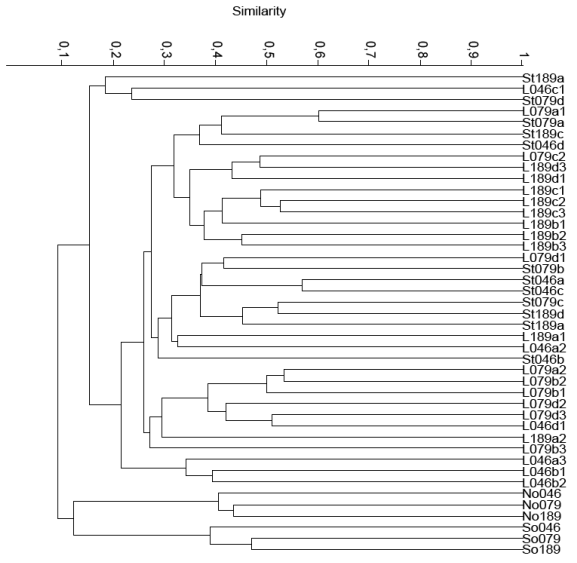


Figure V.1. Pattern of similarities of individual T-RFLP profiles from total community analysis. Nonmetric MDS (N-MDS) plot (a) UPGMA dendrogram (b) based on Jaccard similarity matrix are shown. Scale bar represents Jaccard similarity coefficient. Stress of N-MDS=0.1896.

A large heterogeneity was detected in leaves and stems communities, with only partial clustering at the pot and the plant organ level (leaves and stems). To better evaluate the statistical significance of differentiation of communities we employed AMOVA. Most part of the variation (71.75%) was due to intra-environment differences (Table V.2).

Table V.2. Hierarchical analysis of differentiation between bacterial communities.*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-value
Among environments	3	198.346	6.13749	28.25	<0.0001
Within environments	39	607.933	15.58803	71.75	
Total	42	806.279	21.72552		

* AMOVA was performed with T-RFLP profiles from samples of the four different environments (soil, nodules, stems and leaves). Data show the degrees of freedom (d.f.), the sum of squared deviation, the variance component estimate, the percentage of total variance contributed by each component, and the probability (*P*) of obtaining a more extreme component estimate by chance alone estimated computing 10000 permutations.

However, significant differences between environments were found, in particular between a soil-nodule group and a stem-leaves group. Interestingly, stem and leaf communities showed a significant, tough little, separation (Figure V.2a). A dendrogram of similarities between communities (Figure V.2b) showed three main branches: one comprising leaf and stem communities and the other two with nodule and soil communities, respectively.

a)

	Soil	Leaves	Stem	Nodules
Soil	0			
Leaves	0.42329	0		
Stem	0.49207	0.05492	0	
Nodules	0.46885 n.s.	0.43966	0.50114	0

b)

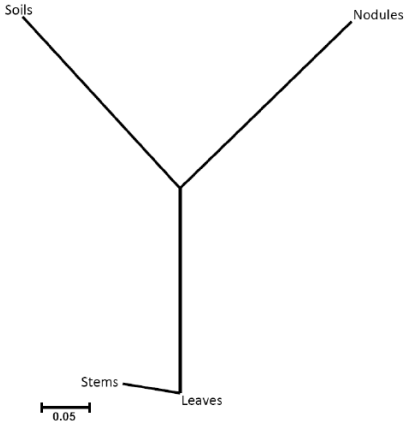


Figure V.2. Overall similarity of bacterial communities in plant and soil. a) Matrix of pairwise F_{ST} values; Statistical significance ($p<0.05$) has been computed after 1000 random permutation; n.s., not significant. Only below diagonal values are reported. b) Neighbor-Joining dendrogram from the pairwise F_{ST} distances between T-RFLP profiles. Scale bar indicates pairwise F_{ST} distance.

V.3.3 Taxonomic composition of bacterial communities in soil, nodules and plant aerial part

In order to elucidate the taxonomic distribution of the bacterial communities present in soil, nodules and plant aerial part, three libraries were constructed using 16S rRNA amplicons obtained with 799f/PH primer pair using as template the DNA extracted from soil, nodules and stems + leaves (pooled together as representative of plant aerial part, due to high similarities shown by T-RFLP). From 81 to 116 clones per library were sequenced. Stem+leaves and soil communities were the most diverse, while nodules-associated community was less different using both Shannon H' and Chao1 estimators (Table V.3).

Table V.3. Statistical analysis of 16SrRNA gene clone libraries.

	Statistics			*Diversity indices*	
	No. of sequences	No. of OTUs*	Library coverage (%)**	Chao1	Shannon (*H'*) (Diversity)
Soil	86	58	32.6	104 (78-164)	4.48
Stems	116	38	67.2	117 (115-295)	4.56
Nodules	81	20	75.3	34 (23-76)	2.19

*OTUs were arbitrarily defined at 97% sequence identity based on Mothur clustering. Confidence intervals at 95% are given in parentheses.
** Coverage is defined $C = [1 - (n/N)] \times 100$, were n is the number of unique clones, and N is the total number of clones examined

As a consequence, the library from nodules was more complete in terms of coverage than that of stems+leaves and soil. A representation of the diversity in terms of prokaryotic classes was produced (Figure V.3). Seven classes were represented only in both soil and

stem+leaves communities (11 an 9 classes in total, respectively), and 5 in nodules. The most represented class was the *Alpha-Proteobacteria* for nodules (as expected due to the presence of the alpha-proteobacterium *S. meliloti*) and stem+leaves.

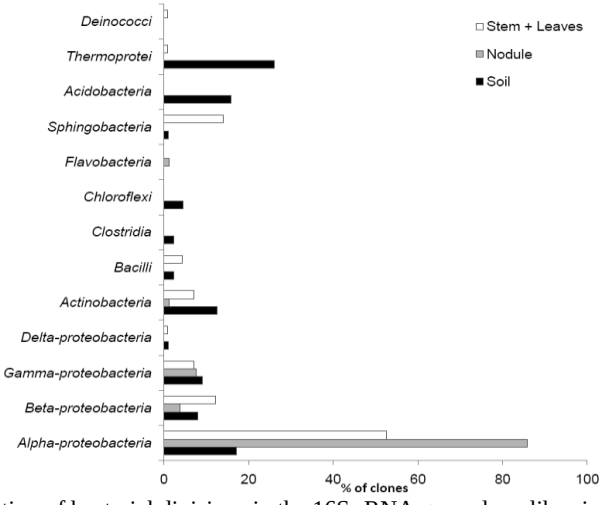


Figure V.3. Representation of bacterial divisions in the 16S rRNA gene clone libraries. The number of clones accounting for each division with respect to its origin (nodule, stem+leaves, soil) is reported.

In soil we found a prevalence of *Alpha-Proteobacteria*, *Acidobacteria* and of *Crenarchaeota* (class of *Thermoprotei*). *Flavobacteria* were found only in nodules. *Beta-* and *Gamma-Proteobacteria* and *Actinobacteria* were found in all three libraries. Concerning *Alpha-Proteobacteria*, in nodules were only found members of the family *Rhizobiaceae* with all sequences assigned as expected to the genus *Sinorhizobium/Ensifer* (Figure V.4). *Alpha-Proteobacteria*, present in soil, belonged to the families of *Rhizobiaceae*, *Bradyrhizobiaceae*, *Methylocystaceae*, *Hypomicrobiaceae and Caulobacteraceae.* Stem+leaves tissues harbored *Rhizobiaceae*, *Aurantimonadaceae* and *Methylobacteriaceae* for the order *Rhizobiales* and members of the order *Sphingomonadales*

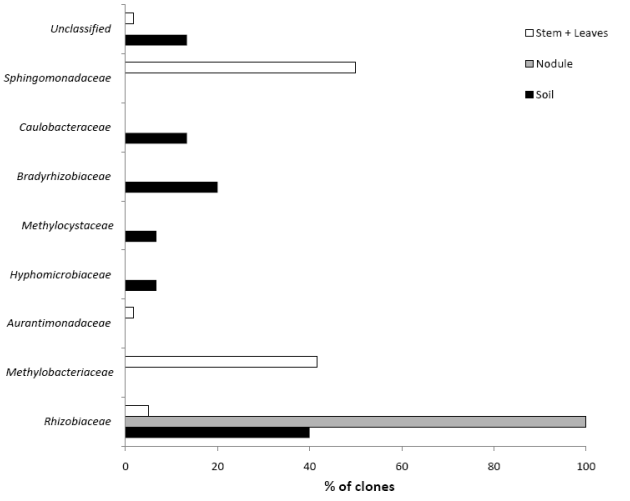


Figure V.4. Distribution of families in *Alpha-Proteobacteria* with respect to their origin (nodule, stem+leaves, soil)

V.3.4 Detection and diversity of *Sinorhizobium meliloti* in soil and plant tissues

The presence of *S. meliloti* DNA was analyzed by qPCR using two species-specific primer pairs as illustrated in the Materials and Methods section, which amplify respectively chromosomal (*rpo*E1) and megaplasmidic loci (*nod*C on pSymA). Obtained results are reported in the first column of Table V.4. Relatively high titres of *S. meliloti* DNA were detected in root nodules. Lower values were obtained in soils, leaves and in stems. Interestingly, in nodules titres of *S. meliloti* DNA detected by rpoE marker were higher than those estimated by nodC marker (roughly one order of magnitude). The viable titres of *S. meliloti* cells from crushed nodules after in-vitro infection of *M. sativa* plants ranged from $2.1x10^8$ to $5.0x10^8$cells/g of fresh tissue) suggesting the titres from nodC-marker as a better proxy to the number of bacteria involved in the symbiotic nitrogen fixing process.

TableV. 4. Titres of *S. meliloti* in soil and plant tissues.[‡]

Sample	***Titres***	
	***rpoE1*-based**	***nodC*-based**
Pot 46		
Soil	$4.92\pm2.82 \times 10^4$	$2.78\pm 0.63 \times 10^4$
Nodules	$3.07\pm0.67 \times 10^9$	$4.25 \pm1.24 \times 10^8$ **
Stems	$2.73\pm1.21 \times 10^4$	$3.22 \pm2.4 \times 10^3$ *
Leaves	$8.65\pm4.04 \times 10^3$	$4.28\pm 1.23 \times 10^3$
Pot 79		
Soil	$1.16\pm0.33 \times 10^4$	$2.88\pm 1.09 \times 10^4$
Nodules	$1.20\pm0.50 \times 10^{10}$	$1.01\pm0.10 \times 10^9$ **
Stems	$2.37\pm0.49 \times 10^3$	$1.13\pm 0.15 \times 10^3$
Leaves	$9.74\pm5.08 \times 10^2$	$2.34 \pm0.78 \times 10^2$
Pot 189		
Soil	$2.70\pm0.41 \times 10^5$	$7.42 \pm0.93 \times 10^4$ *
Nodules	$6.02\pm1.45 \times 10^9$	$2.02\pm 3.22 \times 10^7$ **
Stems	$4.91\pm0.95 \times 10^5$	$1.07\pm 3.74 \times 10^5$
Leaves	$5.54\pm2.83 \times 10^3$	$5.21\pm 3.01 \times 10^3$

[‡] Titres have been estimated by qPCR as reported in Material and Methods with *rpoE1* and *nodC* markers and are expressed as n° of gene copies/g of tissue or soil; ±, standard deviation from triplicate experiments. Asterisks indicate significant differences between estimates based on *rpoE1* and *nodC* markers (*, P<0.05; **, P<0.01).

To inspect the genetic diversity of *S. meliloti* population present in the different environments, the amplification of the 1.3 kbp long 16S-23S ribosomal intergenic spacer (IGS) was attempted using the extracted DNA, but only the DNA from nodules and soil gave a PCR product, probably as a result of the low bacterial titres and of high content in inhibitors present in the DNA extracted from stems and leaves. Consequently, nodule tissue of taken as representative of plant environment and was compared with soil. On the six DNA samples (three from soil, three from nodules), after IGS amplification and T-RFLP profiling, a total number of 121 different IGS-T-RFs (16S-23S ribosomal intergenic spacer Terminal-Restriction Fragments) was detected after the restriction digestion with four restriction enzymes (*Alu*I, *Msp*I, *Hinf*I, *Hha*I) (Figure V.5a). Within the 121 detected IGS-T-RF, most of them (71.9%) could be considered as "private" IGS-T-RF, being detected in one sample out of 6, only while 8 (6.6%) IGS-T-RFs only were "public", being present in all six samples (Figure V.5b). Considering the sharing of T-RFs between root nodules and soil (Figure V.5c), the presence of IGS-T-RFs present in soil and shared with nodules, or present in nodules and shared with soil was observed. In particular values ranged from 25.5 to 53.3% of IGS-T-RFs present in soil and then

detected in nodules and from 31.4 to 40.1% of IGS-T-RFs present in nodules and then detected in the respective soil sample.

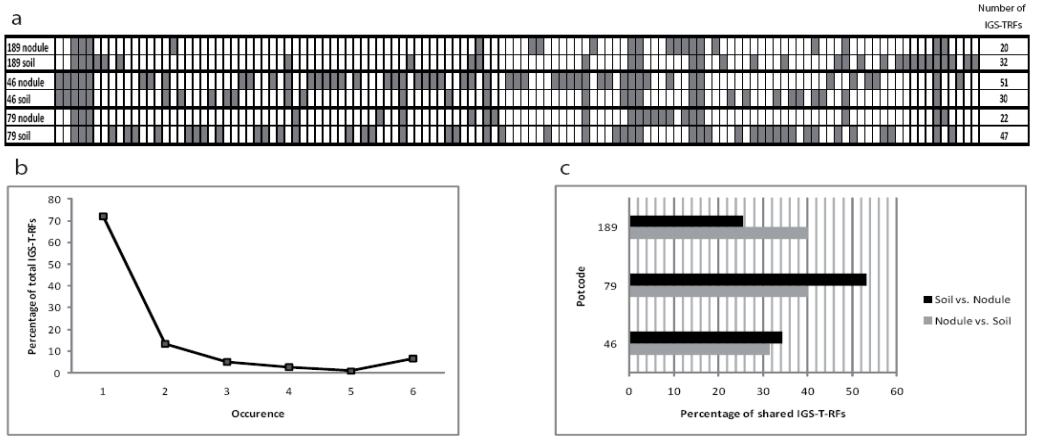


Figure V.5. *S. meliloti* IGS-T-RFLP profiling of nodule and soil samples. A), the schematic representation of the binary matrix of IGS-T-RFs presence (black) and absence (empty cell); IGS-TRFs number is reported on the right side of each row. B) The occurrence of "private" to "public" IGS-T-RFs. The percentage to the total number of scored IGS-T-RFs is reported for TRFs present from 1 to all 6 samples analyzed. C) Sharing of IGS-T-RFs between soil and nodules in the three experimental pots. The percentage of IGS-T-RFs shared between soil and nodules (soil vs nodules) or between nodules and soil (nodule vs. soil) is reported.

In Figure V.6 the similarity relationships between IGS-T-RFLP profiles are presented. Both Non-metric MDS plot of IGS-T-RFLP profiles showed a separation of nodule and soil populations. Nodule population in pot 46 was greatly different from both the soil population of the same pot and from the populations of the other pots. On the contrary, nodule populations of pots 189 and 79 were the closest ones, with soil population of pot 189 in the same cluster.

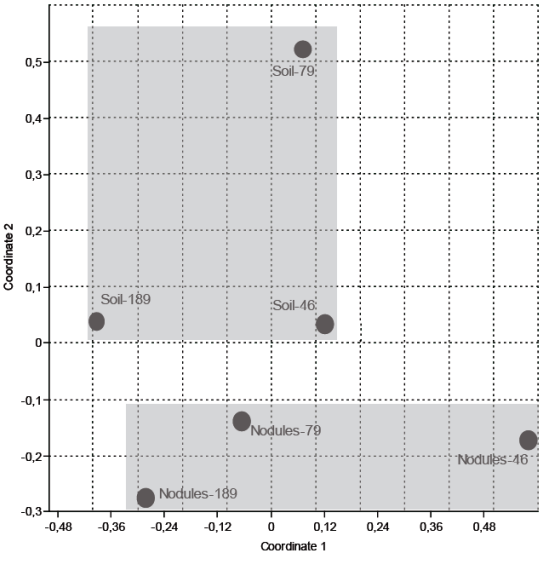


Figure V.6. Non-metric MDS plot of similarities of IGS-T-RFLP profiles from *S. meliloti* population analysis. Stress=0.0898.

IV.4 Discussion

Ecological adaptation has been viewed, depending on the scale of the analysis, as a matter of high rank taxa (classes or families) but also working at the species level were microevolution takes place. In bacteria, due to both the elevated diversity and the extent of horizontal gene transfer, the link between ecological adaptation and taxa is not obvious (*43, 44*). In the past years, the research on the adaptation of bacteria to the environment constituted by plant tissues (endophytic bacteria) has greatly increased (*45-49*). However, for the definition of the ecological significance of plants in bacterial ecology the link between bacterial taxa and the plant environment has to be investigated. We recently showed (*8*) from a field survey that plant aerial parts (leaves) harbor complex, but highly variable, bacterial communities, and only a little number of bacterial taxa are leaves-specific.

In the present work we showed that, for *Medicago sativa* plants grown on the very same soil under controlled (greenhouse) conditions, soil, nodule and aerial part of the plant harbor quite distinct bacterial communities with specific signatures at both class, family and species level. Initially, T-RFLP profiles allowed to recognize an overall statistical significant separation of bacterial communities among environments (soil, nodules, stem and leaves), with a large diversity of leaves and a separation between above ground environments (stem and leaves) to below ground (soil and nodules). The analysis of the clone libraries revealed an uneven distribution of bacterial classes, with a marked pattern highlighting the class of *Alpha-Proteobacteria* as more abundant in plant tissues than in soil (this class represents half of the clones in the library from stem+leaves). The same uneven pattern was then observed within the *Alpha-Proteobacteria* at lower taxonomic ranks with sequences of clones belonging to members of families *Methylobacteriaceae* and *Sphingomonadaceae* more abundant in stem than in soil and nodules. *Methylobacteria* and *Sphingomonadaceae* have been found as endophytes in a number of plants (*8, 12, 31, 35, 50-53*) and this group of bacteria is supposed to grow thanks to the ability to utilize the one-carbon alcohol methanol emitted by wall-associated pectin metabolism of growing plant cells.

Concerning nodule bacterial communities, mostly root nodules in legumes are classically considered as the exclusive niche of rhizobia, with the assumption that a single root nodule is colonized by a single rhizobial strain (*54*). To our best knowledge, the present report is the first attempt to characterize the bacterial community inhabiting root nodules of legumes. Data presented here shows that nodules harbor a quite large diversity in terms of bacterial taxa, the most represented taxa corresponding to the specific rhizobial host of *M. sativa*, the alpha-proteobacterium *S. meliloti*. However additional taxa have been found including members of *Actinobacteria*, *Flavobacteria*, *Gamma-* and *Beta-Proteobacteria* which may have some additional plant growth-promoting activity (see for instances (*55, 56*). Concerning soil, in agreement with many previous observations (*57*), *Acidobacteria* was one of the soil most important division (in terms of number of clones) and was present exclusively in soil clone library. Another interesting point concerning

soil clone library is the relatively high presence of *Archaea* (*Thermoprotei*). Actually, checking the 16S rRNA gene sequences present in Ribosomal Database for annealing with primers 799f/pHr, we found that PCR amplification from *Thermoprotei* was theoretically possible with this primer pair (data not shown). The presence of anaerobic *Archaea* in the soil of our experimental pots is likely due to the anoxic or nearly anoxic conditions present in the bottom of the pot.

Finally, at the lower taxonomic ranks (*S. meliloti*) an uneven pattern of distribution was again observed with haplotypes (detected as IGS-T-RFs) differentially present in soil or in nodule, suggesting the existence of trophic/symbiotic polymorphism in *S. meliloti* population. In fact, the debate about the evolutionary significance of symbiosis in rhizobia, with respect to alternative life strategies is still open (*58, 59*) and the presence of a fraction of rhizobia not participating in nodulation has previously been detected in *S. meliloti* and *Rhizobium leguminosarum* (*24, 60, 61*). *S. meliloti* has previously been show to live also as free saprophytic bacterium in soil and as endophytic colonizer of nonlegume plants as rice (*62-64*). Data obtained in the present work show the presence of *S. meliloti* in all environments analyzed (soil, nodule, stem and leaves). However, the low titres of *S. meliloti* cells in leaves and the presence of PCR inhibitors (plant DNA or phenolic compounds for instance) did not permit the amplification of 16S-23S intergenic region from plant aerial part. Consequently, we focused our investigation of *S. meliloti* population present in soil and nodule. Collectively, estimated titres were similar to those previously observed in other soil and plant tissues (*30*) and in line with soil Most Probable Number (MPN) estimates (*65*). Concerning *S. meliloti* population diversity, for the first time we shed some light on *S. meliloti* population genetics avoiding cultivation. Similar values for diversity (as number of T-RFs) were present in nodules and in soil, suggesting that both environments harbor a consistent fraction of the population's genetic diversity. Interestingly, most of the T-RFs detected were "private", that is present in one sample only, and only a very small fraction of T-RFs was shared among all samples, tough the original soil material was homogeneous and should theoretically contain the same *S. meliloti* haplotypes. A similar "single-sample effect" was previously found in an another long-term (4 years) experiment (*18*) from the analysis of ex-nodulating *S. meliloti* isolates and in *Bradyrhizobium* bacteria recovered from different *Lotus* species (*66*). A possible explanation of such findings could be linked to the relatively low titres of *S. meliloti* in soil (10^4-10^5 cell/g), which is roughly 1/10'000 of the total bacterial community of soil (estimated at ~ 10^9 16S rRNA gene copies/g of soil by qPCR, data not shown). As a consequence of this low population size, founder effect is likely to be among the main driving forces of *S. meliloti* microevolution, and could allow the fixation of sample-specific haplotypes by simple chance (*67*).

Regarding the nodule-soil relationships, our *S. meliloti* population analysis suggested the presence of both soil-specific and nodule-specific fractions suggesting that nodule, providing a niche for growth, harbor a community that do not correspond to that found in soil (where other haplotypes dominate). The differential presence of rhizobial haplotypes in nodules and soil was previously found in chickpea (*60*) and clover (*61*),

tough no simple conclusion could be drawn, because of a limited sampling. In accord to this hypothesis, we found lower titres of *S. meliloti* using nodC marker, similar to the viable titres from in-vitro single-strain infections (around 10^8 cells/g), while *rpoE* marker gave a higher titre in DNA extracted from nodules. This observation suggests the presence of a fraction in the population that do not possess nodulation genes, but which is able to colonize nodule tissues, possibly in viable but not cultivable state (*26*).

In conclusion, we have shown in the model system represented by *M. sativa* and its associated bacterial flora, that, in spite of horizontal gene transfer, patterns of ecological differentiation can be detected at different taxonomic levels from class to the single species. In particular we have shown that plant tissue harbor a diversity which mainly reside in *Alpha-Proteobacteria*. This class then show uneven presence of families between stems+leaves, nodules and soil. Again, for one of the species most abundant in nodules, *S. meliloti*, populations shows a clear pattern of differentiation between soil and nodule.

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Chapter VI

Exploring the endophytic behaviour of the nitrogen-fixing symbiont *Sinorhizobium meliloti* in the target host plant *Medicago sativa*

Rhizobia are the one of most famous examples of symbiotic association between plant and bacteria. Classically, rhizobia may live as free bacteria in soil and when conditions are suitable may form symbiotic association with leguminous plants in the root system (nodules) where nitrogen-fixation occurs. However, recently, rhizobia have been found in non-symbiotic association in non-target plant species, suggesting the presence of different life strategies other than symbiosis and free life in soil. Here we want to shed more light on the endophytic colonization of plant tissues by the nitrogen fixing symbiont *Sinorhizobium meliloti* in its natural host, the legume *Medicago sativa*.

In vitro tests of endophytic colonization were settled and the ability to colonize of the strain *S. meliloti* 1021 and of its derivative impaired in root nodule formation (*nodA*$^-$) were tested, as well as that of two natural strains for which genomic and Phenotype Microarray information are available (AK83 and BL225C).

VI.1 Introduction

Strains of the species *Sinorhizobium meliloti* are ubiquitous in soils and they specifically form symbiotic nitrogen-fixing nodules on the roots of leguminous plants such as alfalfa (*Medicago* spp.). Actually, the current model for life-style of *S. meliloti* is based on the alternation of free-life in soil and symbiosis with host plant species (1).

In recent years, scientists have found that rhizobia were capable of endophytically infecting some other plant species, such as rice, maize, barley, wheat, canola and lettuce, and colonized both the intercellular and intracellular spaces of epidermis, cortex and vascular system (2).

Rhizobia in association with certain cereal crop plants promote their growth and grain yield at harvest while reduce their dependence on chemical fertilizer inputs, independent of root nodulation and biological N_2 fixation. For instance, the beneficial growth responses of rice to rhizobia include increased seed germination, rate of radical elongation, seedling vigor, root architecture (length, branching, biovolume, surface area), shoot growth, photosynthetic activity, stomatal conductance, shoot and grain N content, harvest index, agronomic N fertilizer use efficiency, and grain yield (3).

Despite a widespread occurrence of this natural endophytic rhizobium-cereal association, much remains unknown about its infection and colonization processes (3). Important issues central to development of this plant-microbe association are the primary portals of bacterial entry into the plant tissues, the extent of their dissemination (especially ascending migration) within the plant host after primary root infection, and their population dynamics in planta (3).

Penetration of rhizobia in non-legumes such as wheat, *Brassica*, and *Arabidopsis thaliana* does not require the *Rhizobium* nodulation genes which are involved in the infection and nodulation of legume roots, however, nonlegume colonization by rhizobia is stimulated by flavonoids (4).

The first clue suggesting an ascending migration of an endophytic strain of *R. leguminosarum* bv. trifolii within rice came from early microscopic studies that found the bacteria within leaf whirls at the stem base above the roots that were inoculated and grown in gnotobiotic culture (5).

As known under N-deprived conditions, *Sinorhizobium* strain form symbiotic nitrogen-fixing nodules with alfalfa plants. Inside nodules they develop into bacteroids a differentiated forms unable to reproduce (6). However, an indeterminate nodule can contain from 10^5-10^{10} bacteria located within the zone of invasion and not yet differentiated.

The dramatic changes in bacteroids are induced by the plant (6-8), suggesting that limiting the reproduction of bacteria (by inducing the differentiation to bacteroid) could be a strategy to avoid massive bacterial colonization and then pathogen-like behaviour (6). Moreover, observing the structure of the nodule, the meristematic zone, where the undifferentiated bacteria are located, is separated from the vascular system by several cell layers; more the nodule grows, more layers of senescent cells are accumulated. The

emergence of a structure due to the infection could be related to the need to control the process of bacterial infection by plant. In this context the symbiont is seen as a "domesticated" pathogen (9).

Taken together these information, i.e. the endophytic capabilities of rhizobia and the evolution of a dedicated organ for symbiosis (to improve nitrogen fixation and control the widespread of bacteria in the plant), we could argue that rhizobia could be also endophytes of legumes.

Consequently, by growing alfalfa plants in a nitrogen rich media, to avoid nodule formation, it could be possible to analyse the behavior of rhizobia as simple endophytes, uncoupling endophytism from symbiosis.

In this work we have developed an hydroponic system to assay the endophytic capabilities of rhizobia and we have tested three different strains of *Sinorhizobium meliloti* (1021 (10)AK83-BL225C (11)) and a nodulation-defective mutant of Rm1021 in order to investigate the endophytic behavior and its connection with nodulation.

VI.2 Materials and Methods

VI.2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table VI.1. *S. meliloti* cultures were grown in TY broth (10 g of tryptone, 5 g of yeast extract, and 0.4 g of $CaCl_2$/liter) at 30°C. Antibiotics were added, as appropriate, at the following final concentrations: streptomycin (Sm), 500 µg/ml; rifampicin (Rif), 20 µg/ml; tetracycline (Tc), 10 µg/ml (AK83 exhibits an higher resistance to tetracycline and was used at 10 µg/ml final concentration). *E. coli* cultures were grown in LB broth at 37°C. Antibiotics were added, as appropriate, at the following final concentrations: tetracycline (Tc), 10 µg/ml.

Table VI.1. Strains used in this work

Species	Strain	Relevant characteristic	Resistence	Ref
S. meliloti	Rm1021	SU47 str-21	Sm	(10)
	AK83	Collected by RIAM (St. Petersburg, Russia) by trapping from soil samples of Kazakhstan, North Aral Sea region, during May 2001 using *Medicago falcata*.	-	(11)
	BM102	AK83 rifampicin resistance strain	Rif	This work
	BL225C	Isolated in Lodi, Italy	-	(11)
		Rm1021ΔnodA::tn5	Sm	D. Capela, CNRS, France
	Rm1021 (gfp)	Rm1021-GFP tagged with pHC60	Tc+Sm	(12)
	BM325	AK83-GFP tagged with pHC60	Tc+Rif	This work
	BM286	BL225C-GFP tagged with pHC60	Tc	This work
	BM270	1021ΔnodA-GFP tagged with pHC60	Tc+Sm	This work
E. coli	S17-1	*recA, pro, hsdR, RP4-2-Tc::Mu-km::Tn7*	-	(13)

VI.2.2 Electroporation of *S. meliloti* and *E.coli.*

Electro-competent cells (50µl ≈ 10^{10} cells), thawed in ice, were mixed with plasmid DNA by pipetting. The suspension was transferred to a sterile electroporation cuvette (Molecular BioProducts) with an inter-electrode distance of 0.1 cm and placed in a Gene Pulser® Apparatus connected to the Pulse controller, version 2–89 (Biorad). Immediately after pulse application (2,1KV for *S. meliloti* 1,25KV for *E. coli*), cells were resuspended with 1 ml of TY (*S. meliloti*) or SOC medium (*E. coli*), transferred to a 13 ml tube and incubated at 30 C/37°C, with shaking at 200 rpm, for 4/1 h without any antibiotic. After incubation, aliquots from serial dilutions were spread-plated on non-selective and on tetracycline-containing medium and incubated at 30 C for 3days or 37°C

for 1 day. The percentage of survival was determined as the ratio between the titer of total viable cells after electroporation and that of viable cells without treatment. (14)

VI.2.3 Conjugation.

Recipients *S. meliloti* Rm1021 was grown overnight in TY medium. Donor *E. coli* S17-1 containing the plasmid pHC60 was grown overnight in LB medium supplemented with opportune antibiotic. About 10^9 *Sinorhizobium* and 0.5×10^9 *E. coli* cells were used for each mating. Cells of both donor and recipient were separately centrifuged at 8000 rpm for 5 min and washed twice with 0.85% NaCl. Then *Sinorhizobium* and *E. coli* cells were mixed, pelleted again and resuspended in a final volume of 0.1 ml of 0.85% NaCl. Mating cells were transferred to TY plate and incubated at 30 C for 24 h. Cells were recovered from the plate with a sterile handle and resuspended in 1 ml of 0.85% NaCl. For selection of transconjugants, aliquots from serial dilutions were plated on selective (TY) medium with opportune antibiotic and streptomycin (to counter-select the donor) and non-selective (TY) medium and incubated at 30°C for three days. Efficiency of conjugation was estimated as the ratio between the number of transconjugants and the number of donor cells. (14)

VI.2.4 In-vitro tests of endophytic colonization

Medicago sativa cv 'Pomposa' seedlings were sterilized by treatment with $HgCl_2$ for 5 min, and washed three times in ddH2O. Seeds were stored in ddH_2O at RT in the dark for approximately 24 hours and then let germinated on the cover of a plate upside down in the dark at room temperature 2 days. In each glass pot were then placed 5 seeds over 15g of rockwool in 150ml of Arnon medium (15) to avoid nodule formation and nitrogen starvation and let grown in a greenhouse and allowed to grow for 7 days before bacterial inoculation. The roots were directly inoculated with *S.meliloti* colture (previously washed in Arnon medium) in order to have $3*10^7$ CFU/ml inside pot. Plants were grown in a growth chamber maintained at 26°C with a 16-h photoperiod (100 microeinstein m^{-2} s^{-1}).

After 21 days from inoculation plants were eradicated from rockwool separated in roots and aerial part and surface sterilezed 1 minute in Ethanol and then washed 2 times in ddH_2O. Then leaves stems and roots were separately grinded in a mortar and resuspended in 2 ml of physiological solution. Serial dilution of grinded tissue and washing water were then plated on appropriate plates.

VI.2.6 Microscopy and image analysis.

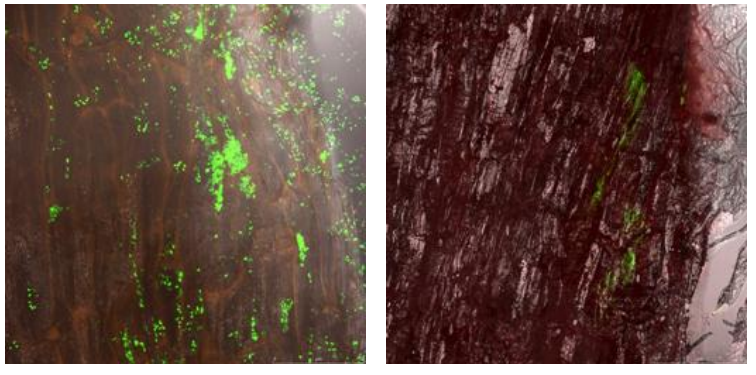
Confocal images were acquired using a Leica TCS SP5 microscope (Leica Microsystems, Mannheim, Germany) equipped with a He/Ne/Ar laser source, using a Leica Plan Apo 363/1.40 NA oil-immersion objective. A series of optical sections (102431024 pixels each; pixel size, 2003 200 nm) was taken at intervals of 0.35 µm. Confocal images were deconvolved using ImageJ 3D deconvolution software (National Institutes of Health [NIH], Bethesda, MD)

VI.3 Results

VI.3.1 Tissue localization of endophytic *S. meliloti*

Plasmid pHC60 was inserted in a Rm1021 nodulation-defective mutant (Δ*nodA*) and in two natural strains (AK83 and BL225C), allowing GFP tagging and tetracycline resistance to better retrieve bacteria from tissues.
Strains Rm1021 Δ*nod*A, and AK83 were transformed by conjugation (as described in VI.2.6) while BL225C was transformed by electroporation (as described in VI.2.2).
GFP-tagged strains of Rm1021 and of its derivative Rm1021 Δ*nodA* were inoculated in *M. sativa* seedlings. After 1 week plants were washed to remove poorly bonded bacteria and analysed by CLFM. Both Rm1021 and Rm1021 Δ*nodA* appeared to colonize the external and internal parts of roots, with no obvious difference in the pattern of colonization. Crack on lateral root emergence seems to be the point of entry for endophytic colonization. Microcolonies were found also in the vessels, suggesting the way for translocation of bacteria to above ground tissues (Figure VI.Ia,b). No bacteria were observed in leaves, probably the bacteria need more time to reach high concentration in this tissue.

a



b

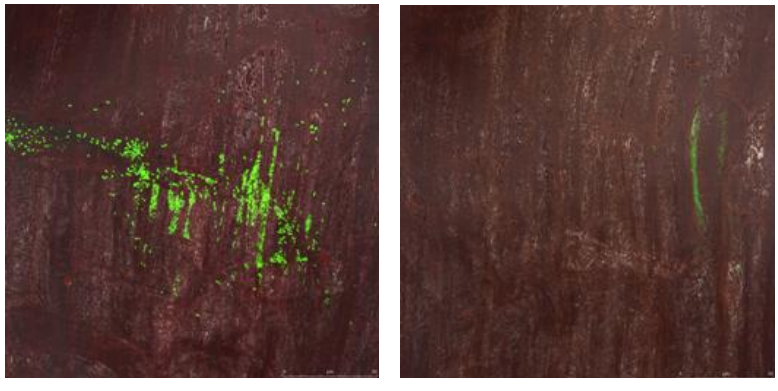


Figure VI.1 Confocal images of endophytes rhizobia inside stem a) Rm1021 b) Rm1021Δ*nodA*

VI.3.2 Endophytism test in *M. sativa*

A hydroponic system was settled up in order to optimize the condition for endophytization. Plants were grown in Arnon medium, the presence of nitrogen in the medium used to avoid the formation of nodules, so plants were not affected by difference in nodulation capability (AK83 forms less efficient nodules (16) while Rm1021Δ*nod*A do not form nodules) to better evaluate difference between strains. After bacterial inoculation ($3x10^7$ CFU/ml) plants were let grown 21 days. Then, plants were eradicated, surface sterilized grinded and plated on selective media. Results of five different experiments were summarized in Figure VI.2.

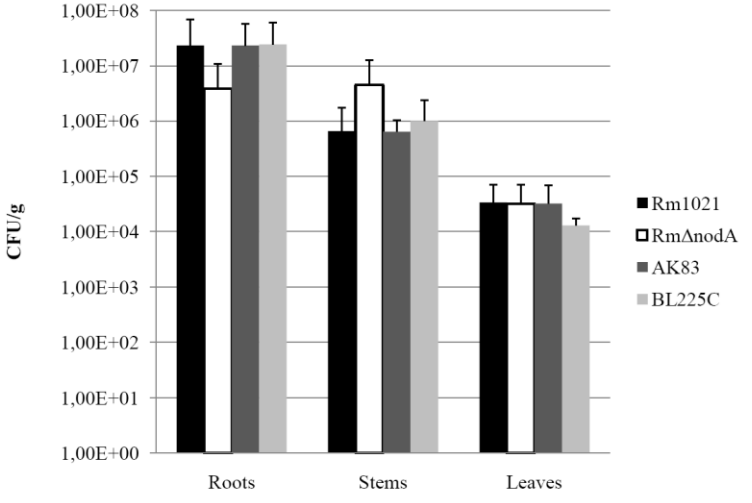


Figure VI.2. *S. meliloti* titres (Rm1021, Rm1021Δ*nod*A , AK83 and BL225C) in different tissues of *Medicago sativa* 21 day post inoculation.

All the tissue were successfully colonized by all strains. Significant difference between roots and leaves were found (one-way ANOVA, $P<0.05$), while stems show an intermediate situation even more close to roots titres suggesting an ascending migration of bacteria from roots to leaves.

Since, AK83 (which shows the less symbiotic phenotype (16)) genome has been shown to contain the gene *acd*S encoding the ACC-deaminase enzyme, which has been addressed as one of the functions related to a better plant colonization ability (17, 18), a long term experiment was settled to more in deep investigate the difference between Rm1021 and AK83 in term of plant colonization activity, plating our sample at 300 days post inoculation. Interestingly a clear difference in roots of the plants were detected, alfalfa roots of negative control and those inoculated with Rm1021 being brownish, indicating stress and consequent lignifications, while those inoculated with AK83 were still white (Figure VI.3).

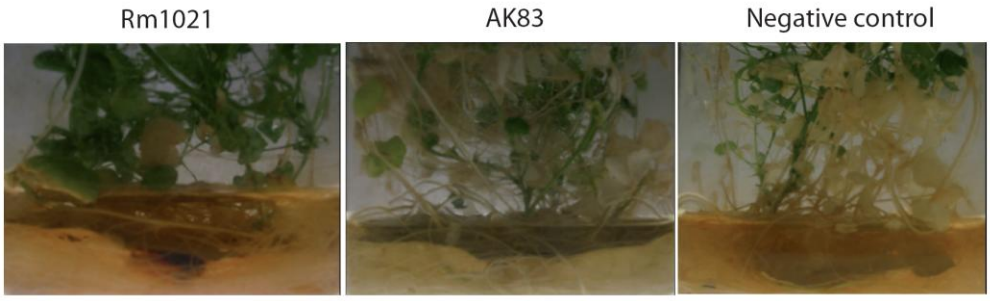


Figure VI.3. Plants 300 day post inoculation. Arnon medium looks brownish in negative control and RM1021 infected plants white in plants inoculated with AK83.

Titres obtained (Figure VI.4) show similar values in stems and leaves for both strain while in roots we found a significant difference, AK83 having higher value with respect to Rm1021 (one-way ANOVA, $P<0.05$),. Moreover, in AK83 we see the same colonization pattern observed in short term experiment, while in Rm1021 roots titres are lower than stems ones, suggesting that stress conditions of long term experiment are less perceived by plant infected with AK83, allowing a higher colonization level of roots. This could also be due to the higher biomass of roots in plants infected with AK83 (237 mg, wet weight) while in plants infected with Rm1021 roots are less grown (113 mg, wet weight) probably because of a more abundant lignifications process.

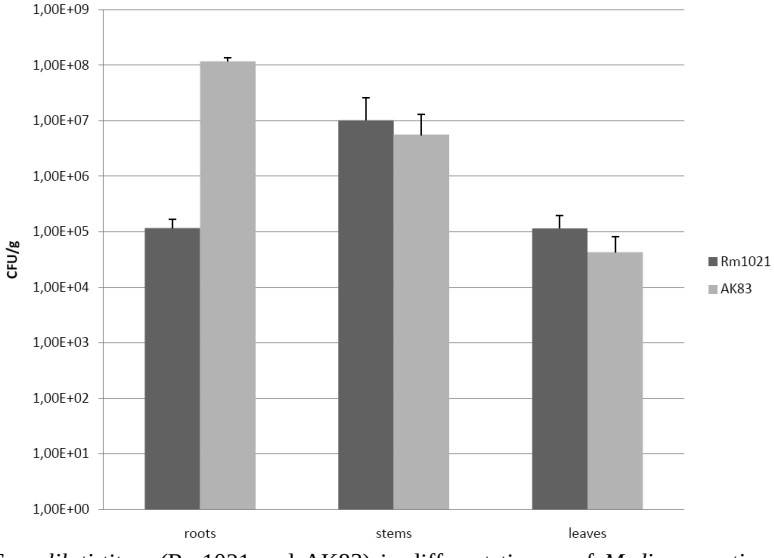


Figure VI.4. *S. meliloti* titres (Rm1021 and AK83) in different tissues of *Medicago sativa* 300 day post inoculation.

Besides, by comparing the bacteria titre in the medium at the beginning of the experiment and at the end, a decrement of one order of magnitude in Rm1021 was observed while in AK83 an increase of two orders magnitude was found, suggesting that the couple Arnon medium-plant acts in some negative or positive way towards Rm1021 and AK83 respectively.

VI.5 Conclusions

Overall, the data obtained allow to provide a preliminary response to some basic questions related to the endophytic ability of *S. meliloti*.

i) Could nodulation defective mutants endophytically colonize the plant? Data indicate that mutant strains can endophytically colonize the plant, suggesting the existence of alternatives to the symbiosis for colonizing plants. From the ecological point of view this results could explain the coexistence, in environmental strains, of multiple symbiotic phenotypes, some having also very defective phenotypes (as AK83 strain).

ii) Is endophytism a strain-variable phenotype? No significant difference in the endophytic capabilities between strains have been detected by our test system. However, it is noticed that on the long term AK83 has higher ability to colonize roots than Rm1021.

iii) Which plant compartments are colonized by *S. meliloti* endophytes? Likely sites of entry are at emergence of secondary roots and root hairs, as suggested by the high number of bacteria present at those locations. Microcolonies of bacterial endophytes were then found in the stem, particularly in the intercellular spaces and some portions of vascular tissue.

It is unclear the ecological significance of the kind of relationship that endophytic *S. meliloti* establishes on *M. sativa*. In fact, the presence of bacteria as endophytes, don't reflect an improved health of the plant, often these plants show chlorotic leaves and lower height than the negative control. Assuming that this is due to the presence of rhizobia, it could be argued that *S. meliloti* is not a simple endophytes but a bacterium that, when placed in a suitable system (e.g. a nitrogen rich soil) could become a "greedy" commensal causing a greater energy expenditure to the plant. This hypothesis, however, need to be supported by further experiments to clarify a pseudo pathogenic role in similar conditions, considering that plant-bacterium interaction under in vitro test is only a far approximation of natural condition, where the plant interacts with the whole bacterial community in which *S. meliloti* is a minority species (see data shown in chapter V).

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Chapter VII

The cell cycle kinase DivJ in *Sinorhizobium meliloti*

Sinorhizobium meliloti is a symbiotic soil bacterium that forms nodules in alfalfa roots where it fixes atmospheric nitrogen. Inside cells of plant nodules, bacteria become elongated, unable to duplicate, polyploid and are defined bacteroids. Those features suggest an alteration of cell cycle progression during differentiation. A model of regulation of cell cycle, applicable also for *S. meliloti*, has been proposed in *Caulobacter crescentus*: the principal regulator of the cell cycle, CtrA, is inhibited by another regulator, DivK, in a cell cycle-dependent fashion. The activation of DivK depends on the histidine kinase DivJ while PleC is its principal phosphatase. Another histidine kinase, CbrA, putatively controlling DivK phosphorylation level, was previously discovered in *S. meliloti*.

Here in this thesis, I analyzed the role of the DivJ in *S. meliloti* and its interaction with CbrA. First, the deletion of *divJ* was constructed demonstrating that *divJ* is not essential. However the deletion strain resulted in a reduced growth rate and in a dramatic cell elongation and branching. As in *Caulobacter*, Δ*divJ* is still motile and it showed an enhanced ability to bind calcofluor suggesting higher levels of exopolysaccharides on the surface of the cells. Moreover, over-expression of CtrA, which is lethal in combination with the *divJ* mutant, is able to partially rescue the *cbrA* phenotype. *S. meliloti* Δ*divJ* is able to form nodules but inefficient as the dry weight of the plant infected by the mutant resembled the non-inoculated one. This suggest that factors involved in cell cycle regulation are involved in the differentiation process that takes place in nodules.

VII.1 Introduction

Alpha proteobacteria group is a very heterogeneous group of bacteria and includes symbionts of plants (*Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Azorhizobium*)), pathogens for animals (*Brucella*, *Rickettsia*), pathogens for plants (*Agrobacterium*), photosynthetic bacteria (*Rhodobacter*) and also several genera metabolizing C1-compounds (*Methylobacterium*). Among symbiotic alphas, *Sinorhizobium meliloti* is one of the most studied systems, being able to form nodules in roots of alfalfa where bacterial cells undergo a differentiation process becoming polyploid and elongated cells named bacteroids (1). Other alphas are characterized by asymmetric cell division (2) including model organisms for the study of bacterial cellular biology.

Cell cycle machinery, controlling DNA replication, cell division, morphogenesis of polar structures, is the engine of each organism and it has been extensively studied in the alpha proteobacterium *Caulobacter crescentus* (3). Many factors are known to regulate cell cycle progression and most of them are members of the family of two-component signal transduction proteins, comprised of histidine kinases (HK) and their response regulator substrates. Among those proteins CtrA is the master regulator of the *Caulobacter* cell cycle, an essential response regulator whose activity as a transcription factor varies as a function of the cell cycle (4-6).

CtrA controls various functions during cell cycle progression by activating or repressing genes expression. CtrA also blocks DNA replication trough the binding of the replication origin. Among genes regulated by CtrA we can find those involved in cell division (*ftsZ*, *ftsA*, *ftsQ* and *ftsW*), the protease encoding gene *clpP*, which is essential in *Caulobacter*, the DNA methylation gene *ccrM*, flagellar biogenesis genes, stalk biogenesis regulators genes, pili biogenesis genes such as *pilA*, and chemotaxis genes (7-12).

CtrA activity and stability varies during the cell cycle; maximum peak of CtrA binding to DNA is at the predivisional stage before cell division. This oscillation of CtrA levels is achieved by different mechanisms: transcription, proteolysis and phosphorylation control as discussed in details below.

An essential regulatory control on CtrA is carried out by phosphorylation. In fact, CtrA must be phosphorylated to bind DNA and its phosphorylation depends on cell cycle progression. An essential phosphorelay, composed by the hybrid histidine kinase CckA and the histidine phosphotransferase ChpT, is responsible for CtrA phosphorylation (13, 14).

DivK, which is a response regulator, plays an essential role as positive regulator of cell cycle progression because, when phosphorylated,, inactivates CtrA and thus promotes DNA replication. Two histidine kinases are known to interact with DivK: PleC and DivJ (15-18). A null *Caulobacter pleC* mutant produces almost symmetric cells at the division and shows an abnormal polar development. The DivJ histidine kinase plays a role in controlling the length and location of the stalk and cell division. PleC and DivJ are

considered respectively the principal phosphatase and kinase of DivK and they are localized at opposite places during cell cycle progression (19).

Phosphorylation is connected with regulation of CtrA proteolysis that is in fact also controlled by the aforementioned phosphorelay. ChpT transfers the phosphate of the CckA receiver domain also to a second response regulator, named CpdR. CpdR, together with RcdA, are factors involved in CtrA proteolysis mediated by ClpP-ClpX protease. CpdR directs ClpXP localization to the cell pole and RcdA mediates proteolysis of CtrA in *Caulobacter* by the dynamically localized ClpXP protease at specific times in the cell cycle (20-22). CtrA is degraded at the stalked pole at the G1/S transition when the origin of replication needs to be cleared and also in the stalked department where initiation of DNA replication occurs immediately after cell division (23, 24).

In *Caulobacter* CtrA controls transcription of *divK*, coding a response regulator whose activity is controlled by multiple kinases, such as DivJ and PleC, through phosphorylation. In *Caulobacter* disruption of *divK* transcriptional control by CtrA leads to a severe cell cycle defect demonstrating that transcriptional feedback of CtrA on *divK* is essential for circuitry (14).

It is known that different histidine kinases could control DivK phosphorylation and several ones have been characterized using biochemical and genetic techniques in *Caulobacter*, such as DivJ, PleC and CckN and also in *Brucella* and *Sinorhizobium* by genetic analysis only, and named respectively PdhS (25) and CbrA (26, 27).

The *C. crescentus* regulatory scheme of CtrA in *Rhizobiales* shows several variations. For example, the control of CtrA on the response regulator *divK*, observed in *C. crescentus*, is shifted to the gene encoding the DivK kinase (*divJ*) and/or the phosphatase (*pleC*) in most *Rhizobiales*. This observation may suggest that feedbacks can be conserved even when connections are rewired (28).

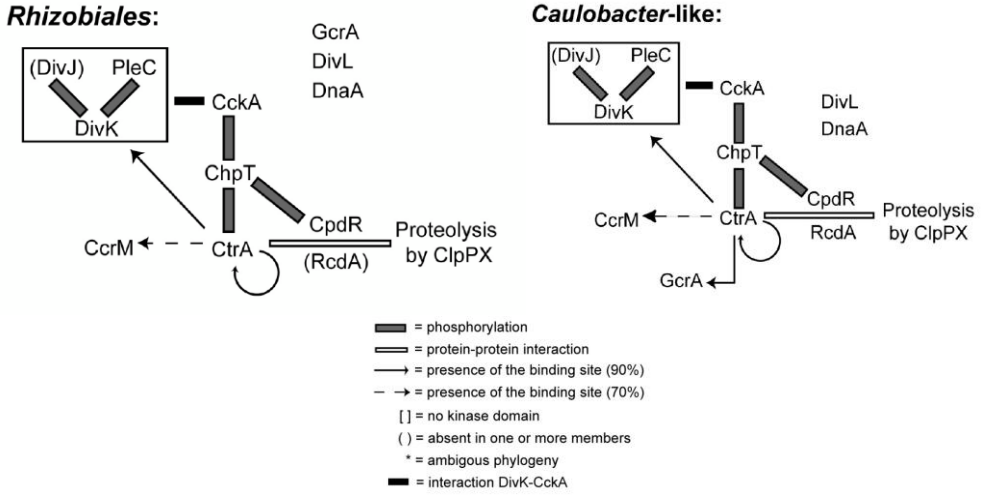

Figure VII.1. Regulatory circuits of rhizobiales and caulobacter-like. Interactions via phosphorylation, as well as proteolysis, were suggested only considering the interaction demonstrated in *Caulobacter*. The *Caulobacter*-like group corresponds to *B. japonicum*, *P. lavamentivorans* and *M. maris.* Modified from Brilli *et al.* (28)

It has been proposed that CtrA in *S. meliloti* has the same role as in Caulobacter but its function has not been revealed yet. Multiplication of chromosomes and elongation are known to be features of a *Caulobacter ctrA*-loss of function allele suggesting that, also in the bacteroids, CtrA may be involved in the differentiation process.

CtrA is essential for viability in *S. meliloti* (29); to reveal the *ctrA*-loss of function phenotype, a *S. meliloti* Δ*ctrA,* complemented with an inducible *ctrA,* was constructed (Ferri *et al.* in prep) by fusing the *ctrA* coding region with an IPTG-inducible promoter (30). Three alleles were tested, *ctrA* coding region of *S. meliloti*, *ctrA* coding region of *C. crescentus*, *ctrA401*^{ts} coding region. *S. meliloti ctrA* restored the viability of chromosomal *ctrA* deletion for *S. meliloti*, demonstrating that regulation of CtrA at the transcriptional level is not essential for cell cycle progression (Ferri *et al.* in prep). This suggests that CtrA activity can be sufficiently regulated only at the post-translational level by both phosphorylation/dephosphorylation and proteolysis as demonstrated for *C. crescentus (4).*

Conditional mutant was not viable when induction is stopped (Figure VII.2a) allowing observation of CtrA-loss of function phenotype of *S. meliloti*. Stop of CtrA expression provoked the lack of motility and strong modifications of the morphology (Ferri *et al.* in prep). As revealed by CtrA regulon bioinformatics prediction, cell motility should be regulated by CtrA in *S. meliloti (28)*. Microscope observations revealed that cells that lose CtrA appeared clearly non motile with respect to the cells where CtrA expression is induced by IPTG and the wild type Rm1021, confirming consistency of the prediction (Ferri *et al.* in prep).

Considering the overall morphology, all cells showed abnormal growth of cell volume in both dimensions (Figure VII.2b). Moreover cells develop an enlargement of the envelop that can be located in the center or in one pole and it appears bright in a phase contrast microscopy observation, suggesting a different 3-dimensional structure (Figure VII.2b).

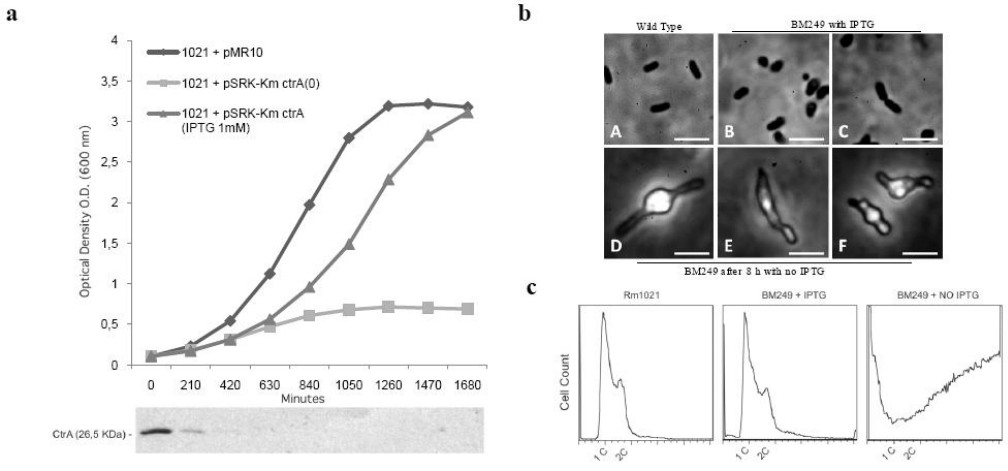

Figure VII.2. a) Growth curve of BM113 (Rm1021 + pMR10) and BM249 (Rm1021Δ*ctrA* + pSRKKm *ctrA*) with and without IPTG, and western blot of CtrA at different time in the conditional strain grown without IPTG b) morphology of Rm1021 and BM249 with IPTG and after 8h without IPTG c) FACS analysis on Rm1021 and BM249 with and without IPTG. Ferri *et al* in prep.

Cell elongation range is between 3 and 10 times the size of the strain induced with IPTG and the wild type Rm1021 (Ferri *et al*. in prep). Moreover FACS analysis (Figure VII.2c) show an increment in the DNA content of the conditional mutant when grown in absence of IPTG.

All this features are typical in bacteroids, so 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.

VII.2 Material and methods

VII.2.1 Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are described in Table VII.1. *Escherichia coli* strains were grown in liquid or solid Luria-Bertani (LB) broth (Sigma Aldritch) (39) at 37°C supplemented with opportune antibiotics: kanamycin (50 µg/ml in broth and agar), tetracycline (10µg/ml in broth and agar). *S. meliloti* strains were grown in broth or agar TY (31) supplemented with kanamycin (200 µg/ml in broth and agar), streptomycin (500 µg/ml in broth and agar), tetracycline (1 µg/ml in broth, 2 µg/ml in agar) as necessary. For mutants the counter-selection was performed in 10% sucrose added to agar plates. For calcofluor analyses of succinoglycan production, LB agar was buffered with 10 mM MES (morpholineethanesulfonic acid), pH 7.5, and calcofluor white MR2 Tinopal UNPA-GX (Sigma Aldrich) was added at a final concentration of 0.02%.

Table VII.1 Strain and plasmid used.

Organism or plasmid category	Strain or plasmid name	Description	Resistence	Source
Strain				
Sinorhizobium meliloti	Rm1021	SU47 *str-21*	Sm	(31)
	KEG2016	Transduced *cbrA*::Tn*5*	Sm, Km	(26)
	BM113	1021 + pMR10	Sm, Km	Ferri *et al.* in prep.
	BM132	1021 + pMR10-*ctrA* (S.mel)	Sm, Km	Ferri *et al.* in prep.
	BM240	1021 + pSRKKm*ctrA* (S.mel)	Sm, Km	Ferri *et al.* in prep.
	BM184	1021 + pMR10 *divJ*	Sm, Km	This work
	BM224	1021 Δ*divJ* ::tc + pMR10 *divJ*	Sm, Km, Tc	This work
	BM225	1021 Δ*divJ* ::tc pMR10	Sm, Km, Tc	This work
	BM253	1021 Δ*divJ* ::tc pMR10 (deletion transduced from BM225)	Sm, Km, Tc	This work
	BM423	1021 Δ*divJ* ::tc (deletion transduced from BM253)	Sm, Tc	This work
	BM264	1021ΔdivJ::tc+ pSRKKm*ctrA* (S.mel)	Sm, Km, Tc	This work
	BM280	1021 + pSRKKm*divK* (S.mel)	Sm, Km	This work
	BM316	1021 + pSRKKm*divJ* (S.mel)	Sm, Km	This work
Escherichia coli	DH5α	F, *supE44, lacU169, hsdR17, recA1, endA1, gyrA96, thi-1, relA1* (80*lacZ*M15)	-	(32)
	S17-1	*recA, pro, hsdR, RP4-2-Tc::Mu-km::Tn7*	-	(33)
Plasmid				
General purpose vectors	pNTPS138	Suicide vector, *oriT, sacB*	Km	D.Alley
	pMR10	Broad host-range cloning vector, low copy number	Km	(34)
	pSRKKm	pBBR1MCS-2-derived broad-host-range expression vector containing *lac* promoter and *lacI*q, *lacZ*	Km	(30)
Deletion plasmid	pΔ*divJ*		Km, Tc	This work
Overexpression plasmid	pSRKKm*ctrA*	pSRKKm containing *ctrA* nserted between NdeI and KpnI sites	Km	Ferri *et al.* in prep.
	pSRKKm*divJ*	pSRKKm containing *divJ* nserted between NdeI and XhoI sites	Km	This work
	pSRKKm*divK*	pSRKKm containing *divK* nserted between NdeI and KpnI sites	Km	This work

VII.2.2 Transductions with ΦM12

In all experiments, the medium used for phage propagation was LB containing 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$. (LB/MC). Liquid lysates were made by the addition of phage ΦM12(35) (with a ratio cells:phages of 1 : 1) to 5 ml of an actively growing culture of *S. meliloti* (10^9 cells/ml) and incubation at 30°C in a shaking incubator. Cell lysis normally occurred in 6 to 12 h. All lysates were inactivated with $CHC1_3$, centrifuged to remove cellular debris, and stored at 4°C adding a drop of $CHC1_3$. Titers were estimated mixing 100µl of *S. meliloti* fresh overnight colture grown in LB/MC and 100µl of phage stock with opportune dilutions, mix was incubated at room temperature for 30 minutes. Then 5 ml of molten (50°C) LB/MC top agar (agar 0,7%) were added, mixed and poured onto an LB/MC agar plate. Plates were incubate at 30°C over-night and PFU were calculated.
For transduction, equal volumes of phage and bacteria (in LB/MC) were mixed to give a multiplicity of infection of ca. 0.5 phage per cell. The mixture was incubated at 30°C for 30 min. To select for the transduction of an antibiotic resistance marker, cells were centrifuged, suspended in LB, and plated directly on LB agar containing the antibiotic.

VII.2.3 Electroporation of *S. meliloti* and *E. coli.*

Electro-competent cells (50µl ≈ 10^{10} cells), thawed in ice, were mixed with plasmid DNA by pipetting. The suspension was transferred to a sterile electroporation cuvette (Molecular BioProducts) with an inter-electrode distance of 0.1 cm and placed in a Gene Pulser® Apparatus connected to the Pulse controller, version 2–89 (Biorad). Immediately after pulse application (2,1KVofor *S. meliloti* 1,25 for *E. coli*), cells were resuspended with 1 ml of TY (*S. meliloti*) or SOC medium (*E. coli*), transferred to a 13 ml tube and incubated at 30 C, with shaking at 200 rpm, for 4h (*S. meliloti*) or 1 h at 37°C at 200rpm (*E. coli*) without any antibiotic. After incubation, aliquots from serial dilutions were plated on non-selective and on antibiotic-containing medium and incubated at 30 C for 3days or 37°C for 1 day. The percentage of survival was determined as the ratio between the titer of total viable cells after electroporation and that of viable cells without treatment. (36)

VII.2.4 Conjugation.

Recipients *S. meliloti* Rm1021 was grown overnight in TY medium. Donor *E. coli* S17-1 containing the plasmid was grown overnight in LB medium supplemented with opportune antibiotic. About 10^9 *Sinorhizobium* and 0.5×10^9 *E. coli* cells were used for each mating. Cells of both donor and recipient were separately centrifuged at 8000 rpm for 5 min and washed twice with 0.85% NaCl. Then *Sinorhizobium* and *E. coli* cells were mixed, pelleted again and resuspended in a final volume of 0.1 ml of 0.85% NaCl. Mating cells were transferred to TY plate and incubated at 30 C for 24 h. Cells were recovered from the plate with a sterile handle and resuspended in 1 ml of 0.85% NaCl. For selection of transconjugants, aliquots from serial dilutions were plated on selective (TY with oppurtune antibiotic and streptomycin, to counter-select the donor) and non-selective (TY) medium and incubated at 30°C for three days. Efficiency of conjugation

was estimated as the ratio between the number of transconjugants and the number of donor cells. (36)

VII.2.5 Two-step gene deletion.

Two fragments of about 1000-bp long (UP and DN) on either side of *divJ* were amplified by PCR using oligonucleotides pSmc00059_P1 (5'-AACAGGCAATCGCGTTTCCCC), pSmc00059_P2 (5'-CGATATCAAGCTTATCGATACCGTGTCCATCTGCCAGCCAT), for upstream region; pSmc00059_P3 (5'-AACTTCGAATTCCTGCAGCCGGGGGACATGGCGCCGCG), and pSmc00059_P4 (5'-AGTGGTGCGCAACTGCTC) for downstream region. The first six and last 12 codons of each gene deleted were left intact to protect against disruption of possible regulatory signals for adjacent genes.

Regions of homology were amplified in 50µl reactions by PCR using the following conditions: 200 ng Rm1021 genomic DNA, 10 mM each dNTP, 400 nM each primer (P1 + P2 or P3 + P4), 1X AccuPrime™ *Pfx* Reaction Mix, 2 U AccuPrime™ *Pfx* DNA Polymerase (Invitrogen), 2% DMSO and 1,25M Betaine. For each reaction, 40 cycles of the following sequence were run: 95° C for 1 min, 55° C for 1 min, and 68° C for 2 min. Reactions included a pre-incubation at 95° C for 5 min, and concluded with a 10-min extension at 68° C. Products were then gel-purified (Qiagen, Valencia, California, United States) and used to amplify a tetR cassette by PCR: 50 µM each dNTP, 100 nM P1 primer, 100 nM P4 primer, 70ng of the products of the flanking homology PCRs,, 1X expand long template buffer 3, 3,75 U expand long template enzyme mix (Roche), 2% DMSO, 1,25M Betaine, and 200 ng of the KpnI-SacI fragment of pKOC3 containing the tetR cassette. Cycling comprised pre-incubation at 95° C for 5 min; followed by 40 cycles of the sequence 95° C for 1 min, 60° C for 1 min, and 68° C for 5 min; followed by 68° C for 10 min. Final PCR amplicons were gel-purified, blunted using the End-IT kit (Epicentre, Madison,Wisconsin, United States) and ligated into pNPTS138 (37).

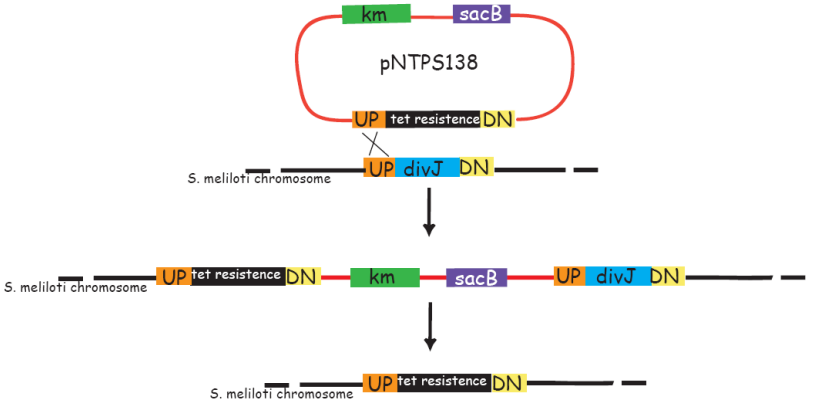

Figure VII.3. Methodology used to generate chromosomal deletion strains. For *divJ* deletion, a suicide vector was constructed, with approximately 1000-bp regions of homology upstream and downstream of the gene flanking a tetR cassette. See Materials and Methods for details of plasmid construction. In a two-step process, deletion strains are isolated by selecting first for tetracycline resistance and then by sucrose counterselection utilizing the *sacB* gene carried on the vector. Cells harboring the *sacB* gene die in the presence of sucrose. Hence, a deletion strain is identified as tetR/sucroseR. For nonessential genes, stable deletions are easily identified by screening 5–10 colonies after the two-step recombination. Modified from (37)

Ligations were transformed into DH5α and positive colonies selected by blue/white screening. Plasmids from white colonies were verified by sequencing.
Complementation plasmid were also constructed, *divJ* and its putative promoter region were amplified by PCR using 50 µM each dNTP, 100 nM P1 primer, 100 nM P4 primer, 1X expand long template buffer 3, 3,75 U expand long template enzyme mix (Roche), 2% DMSO, 1,25M Betaine, and 200 ng Rm1021 genomic DNA. Cycling comprised pre-incubation at 95° C for 5 min; followed by 40 cycles of the sequence 95° C for 1 min, 60° C for 1 min, and 68° C for 5 min; followed by 68° C for 10 min, fragment were gele purified and cloned into the low copy vector pMR10. Plasmid obtained was electroporate in Rm1021 (BM184).
Deletion plasmids were transformed into E. coli S17-1 and then transferred by conjugation into BM113 and BM184, first integrants were selected by plating on TY containing tetracycline. Colonies with the integrated deletion plasmid were inoculated into liquid TY medium with tetracycline and grown for 12–16 h. Five micro liters of each culture was then plated on TY plates containing tetracycline and sucrose. Colonies were screened for tetracycline resistance and for resistance to the activity of sacB gene (loss of the plasmid), to identify deletion strains. Proper construction of the gene deletion was verified by PCRs using primer specific to the chromosomal region up and down P1-P4 region, using pSmc00059_P1tris (5'-GCGCATCGTTATCTCACCTT) in combination with P4 and pSmc00059_P4tris (5'-CGCATGCAAAGCTGATACAC) with P1 in the same condition of P1-P4 amplification. Deletion was also confirmed by its transduction with phage ΦM12.

VII.2.6 Construction of overexpression clones.

The *divJ* and *divK* gene were amplified from genomic DNA of *S.meliloti* Rm1021 by PCR using oligonucleotides pSmc00059_P0 (5'-ggcatATGGCTGGCAGATGGACATC) and pSmc00059_P6 (5'-ctcgagCTGAAGACGACGGCAAAGAT) for *divJ* and pSmc01370_P0 (5'-ggggcatATGCCCAAACAGGTAATGATTG) with pSm01370_P6 (5'-ggtaccGCCGTAAGCACGTCGAAATA) for *divK*. Reaction were carried out in 50 µl final volume using the following conditions: 200 ng Rm1021 genomic DNA, 10 mM each dNTP, 400 nM each primer, 1X AccuPrime™ *Pfx* Reaction Mix, 2 U AccuPrime™ *Pfx* DNA Polymerase (Invitrogen) . Cycling comprised pre-incubation at 95° C for 5 min; followed by 40 cycles of the sequence 95° C for 1 min, 60° C for 1 min, and 68° C for 3 min; followed by 68° C for 10 min. Amplicons were gel purified Qiagen, Valencia, California, United States) and polyA tail were added by incubation 10' at 72°C with 10 mM dATP, 1,5mM $MgCl_2$, 1X GoTaq flexi buffer, 2 U GoTaq™ (Promega), fragments were PCR purified, ligated into pGEM-T Easy vector (Promega) and transformed into DH5α. Plasmid were then extracted using a NucleoSpin® Plasmid kit (Macherey–Nagel) following the supplier's instructions and verified by sequencing. The fragments were excised by restriction (NdeI and XhoI for *divJ*, NdeI and KpnI for *divK*; restriction site were generated as part of the primers) and gel purified, then were ligated in pSRK-Km (previously restricted with the same enzymes), generating pSRK-Km-*gene*. In this construct, the start codon of the gene is overlapping with the start codone of *lacZ*.

Ligations were transformed in DH5α prior to be transferred in *S.meliloti* by electroporation.

VII.2.7 Microscopy.

S. meliloti cells were grown to mid-log phase, fixed by addition of 0.5% paraformaldehyde in PBS, washed, and concentrated with physiological solution. Samples were deposited on microscope slides coated with 0.1% poly-L-lysine. Differential interference contrast images were obtained with a Leica DM L (Leica, Wetzlar GmbH, Germany) equipped with an N PLAN oil-immersion objective (100 X/1.25 Oel) and a Leica DFC425 C 5Mpixel CCD camera controlled by a DFCTwain7.3.0 software. Evaluation of the collected images was done by Adobe Photoshop CS2.

VII.2.8 Physiological assays.

For the efficiency-of-plating (EOP) assays, cultures were grown to exponential phase (OD_{600}, ≈0.5) in LB/MC medium and then diluted to an OD600 of 0.1 in LB. Each sample was serially diluted from 10^0 to 10^{-6} in LB, and 100 µl was spread onto LB agar containing either crystal violet (Sigma), hydrogen peroxide (Sigma) or IPTG (1mM). After 4 to 5 days of growth at 30°C, the number of CFU was determined, with the exception of the Δ*divJ* and *cbrA*::Tn*5* mutant, which required an additional 48 h of growth at 30°C for colonies to appear. The average and standard deviation for each strain were derived from two independent cultures which dilution was spread onto two plates.

VII.2.9 Nodulation assays.

Medicago sativa cv 'Pomposa' or *M. truncatula* cv.'Jemalong' seedlings were sterilized by treatment with $HgCl_2$ for 5 min, and washed three times in ddH2O. Seeds were stored in ddH_2O at RT in the dark for approximately 24 hours and then let germinated on the cover of a plate upside down in the dark at room temperature 2 days. Seedlings were transferred to buffered nodulation medium (BNM) pH 6.0 (38) supplemented with 1 µM AVG (ethylene biosynthesis inhibitor amino-ethoxy-vinyl-glycine) in 1.5% (wt/vol) noble agar (Sigma) (39), and allowed to grow for 4 to 5 days before bacterial inoculation to promote synchronous nodule development. The roots were directly inoculated with 100 µl of bacteria that had been cultured to logarithmic phase (OD_{600}, ≈0.5) in TY medium, spin down, washed in 0.5X BNM medium, and then diluted to an OD_{600} of 0.1 in 0.5X BNM medium.

Plants were grown in a near-vertical position in a growth chamber maintained at 26°C with a 16-h photoperiod (100 microeinstein m^{-2} s^{-1}). Plant photographs were taken and microscopic analysis of nodules was performed at 28 days postinoculation. Nodule development (number of nodules/plant and percent pink nodules/total number of nodules per plant) and plant height were monitored for a period of 5 weeks. Plant height was assessed by measuring the length of the epicotyl stem.

VII.3 Results and discussion

VII.3.1 Histidine kinases potentially interacting with DivK in *S. meliloti*

As illustrated in the introduction, in *Caulobacter* CtrA is negatively controlled by the two component system DivJ-DivK, while DivK is mainly dephosphoryalted by PleC, which plays indeed a positive role on CtrA. DivJ-DivK-PleC, respectively SMc00059, SMc01370 and SMc02369 have been also found in *S. meliloti*, using bioinformatic tools (2, 28).

Histidine kinases are multi-domains proteins composed by a C-terminal histidine kinase domain that is conserved among all members of this family and a non conserved N-terminal sensor domain that is presumably involved in the perception of signals and also the regulation of the auto-phosphorylation enzymatic activity. The sensor part often contains transmembrane regions allowing the localization of the protein at the membrane; usually the sensor part of histidine kinases protrudes outside the cell and perceives extracellular signals, which are then transduced inside the cell. Sensor domains of kinases are less conserved than the C-terminal part reflecting the plethora of different signals and modes of sensing.

Histidine kinases of this type can be classified on the basis of the signals they perceive: periplasmic-sensing histidine kinases detect signals (e.g. small solutes) through their extracellular input domain; histidine kinases with sensing mechanisms linked to the transmembrane regions detect stimuli from the membrane itself and cytoplasmic-sensing histidine kinases (either membrane anchored or soluble) detect cellular or diffusible signals reporting the metabolic or developmental state of the cell. For this reason, it was assumed that large extracellular domains allows a protein to sense environmental signals, while soluble histidine kinases or those with extracellular portions should sense mostly internal signals.

PleC orthologs, belonging to organisms of the alpha-proteobacteria cluster A (28), generally have a large fragment between two transmembrane regions and this membrane part is then followed by a PAS-PAC-PAS domain, the large extracellular domains may be devoted to sensing exogenous signals (Fig. S1). DivJs, show an intermediate situation, DivJs can have up to 5 transmembrane regions organized in a tight way followed by a single PAS-PAC domain before the kinase portion. In *S. meliloti*, *R. leguminosarum*, *A. tumefaciens* and *M. loti*, is characterized by short transmembrane regions. This structure is also present in *X. autotrophicus*, while in *P. lavamentivorans* the DivJ orthologs is a soluble histidine kinase, lacking transmembrane segments as predicted by SMART. Finally in the remaining alphas belonging to cluster A, the DivJs have many transmembrane regions suggesting the ability of sensing membrane signals (Fig. VII.S1).

How many histidine kinases, besides DivJ and PleC orthologs, interacting with DivK, are present in *S. meliloti*? We undertook the definition *in silico* of a family of kinases, interacting by phosphate exchange with DivK and this family was named *pleC*/*divJ* homolog family (Pdh) as previously suggested (2). Members of this family must show a

conservation of sequences involved in response regulator-histidine kinase interaction as defined by Ohta and Newton by a yeast two-hybrid experiment (Ohta *et al.*, 2003) integrated with a more recent work on specificity of histidine kinases (40). Accordingly, the fragment of the histidine kinase that defines the specific interaction with the response regulator is composed by helix 1 and helix 2 of the two helix bundle of the histidine residue surrounding region which corresponds to residues 332 to 351 (hereinafter helix1 motif) and 369 to 395 (hereinafter helix2 motif) of the DivJ sequence of *Caulobacter*. Therefore, we derived a consensus describing conservation of the specificity region in our dataset of orthologs of DivJ and PleC proteins from organisms possessing DivK (Fig. VII.4A).

Notably the *S. meliloti* CbrA (26) and *B. abortus* PdhS (25), predicted to interact with DivK, were in fact found with this bioinformatic analysis. The number of those members in each organism analyzed that possess DivK are reported in figure VII.S2.

S. meliloti showed five Pdh kinases including, CbrA(26), orthologs of DivJ and PleC and two other histidine kinases belonging to the Pdh family. Moreover a binding site of *ctrA* was found upstream *cbrA* so presumably also *cbrA* is under the control of *ctrA.*

VII.3.2 Construction and characterization of *divJ* mutants in *S. meliloti*

The *S. meliloti* Rm1021 deletion mutant of the gene SMc00059, annotated as a DivK kinase encoding gene *divJ*, was created as described in the Materials and Methods section. It was viable but it showed a severe reduction of the growth rate (Fig. VII.4B). Moreover cells of *divJ* deletion were morphologically abnormal, showing longer bodies and a certain level of branching (Fig. VII.4C).

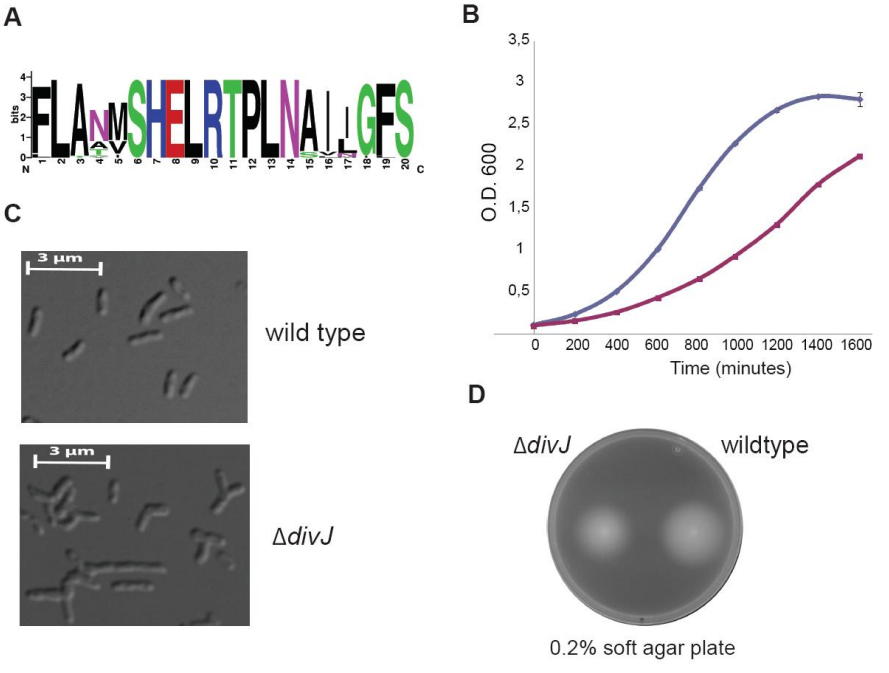

Figure VII.4. *divJ* mutant (BM253) is viable but shows a severe phenotype. A. Pdh-family specificity consensus; B. Growth curve of BM253; C. Morphology of BM253. D. Soft agar swarmer assay.

As in *Caulobacter*, the *S. meliloti* mutant Δ*divJ* was still motile (assayed by soft agar plates and directly observed) as shown in figure VII.4D. The smaller halo in the soft agar could be due to the slightly slower growth rate of the mutant and/or the branched phenotype of cells that usually retard the motility. Finally the deletion of *divJ* did not alter the DNA content since the FACS profile was not distinguishable from wild type (Data not shown).

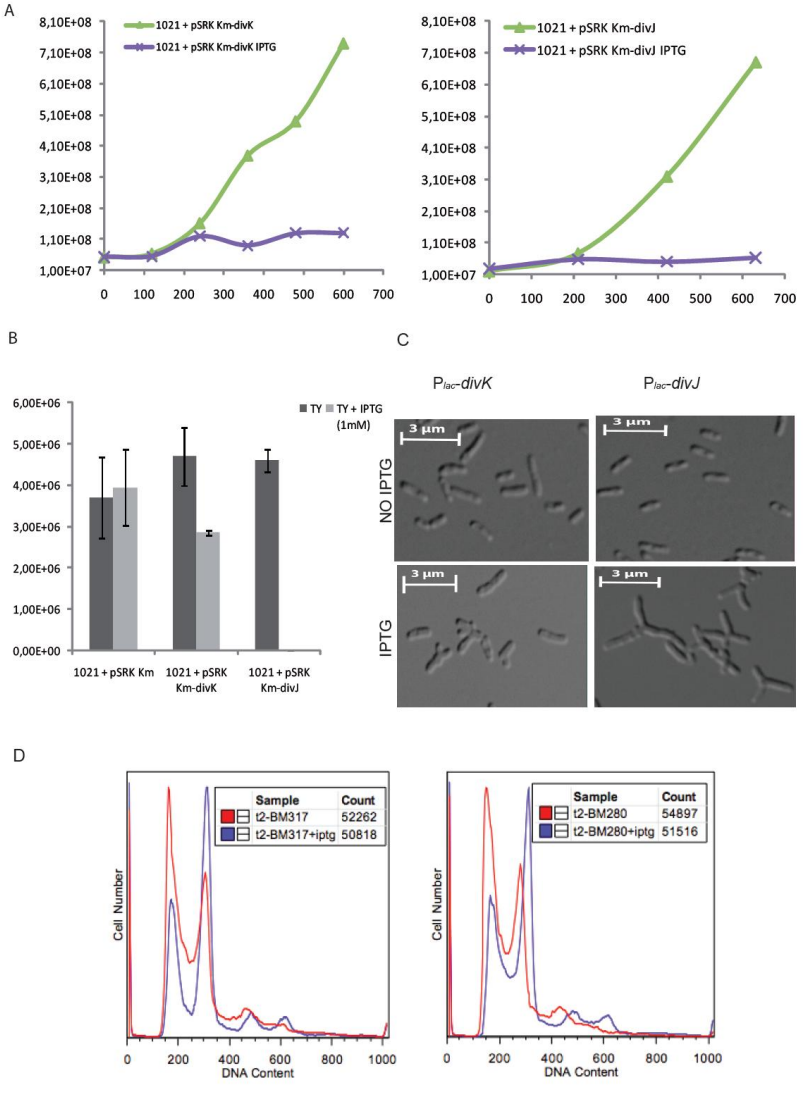

Figure VII.5. Overexpression of *S. meliloti divJ* (BM317) and *divK* (BM280). A) Growth curve of BM280 and BM317 with (purple line) and without (green line) IPTG, B) Efficiency of plating without and with IPTG of BM280 and BM317; C. Morphology of BM280 and BM317. D. FACS of BM317 and BM280.

We constructed also a strain of *S. meliloti* Rm1021 in which *divJ* was under the control of an IPTG inducible P_{lac} promoter (pSRK derivatives, (30)) (BM317). We also built an

inducible expression strain of *divK* in order to genetically confirm that DivJ and DivK are both members of the same two-component system also in *S. meliloti*, therefore sharing common phenotypes. As illustrated in Figure VII.4, both strains lead to a severe growth defect as shown by viable counts, in a strain with *divK* constitutively expressed we observe a strong reduction of the viability while with overexpression of *divJ* the reduction of growth is even higher as we can observe in a growth curve inducing the expression of the gene adding IPTG (Figure VII.5A) or by efficiency of plating on TY with and without IPTG (Figure VII.5B).
We analyzed if the over-expression lead to cell morphology defect. Both over-expression strains showed a similar elongated morphology (Figure VII.5C) suggesting together with the previous experiments that both gene are coupled in the same cascade.
Finally we checked also alteration of DNA replication by using FACS analysis (in collaboration with Prof. Graham Walker at MIT, Boston, USA). This investigation revealed that after over-expression of both *divJ* and *divK* cells accumulated two chromosomes as a G2 arrest suggesting a block of cell division (Figure VII.5D).

VII.3.3 DivJ-DivK two component system is negatively upstream CtrA

DivJ in *Caulobacter* is a negative regulator of CtrA because it phosphorylates DivK, which in turns inhibits CckA, the kinase of CtrA.
It is also reasonable to hypothesize that combining a mutation that increase the CtrA activity with the deletion of *divJ* should lead to a severe phenotype. The strain BM132 where the *ctrA* locus is cloned in a low copy vector, pMR10, has estimated a 4-5 fold higher CtrA level than wild type (Ferri *et al*. in prep).

A

Recipient strain	Number of transduced (cfu/ml)
Wild Type	130
Wild Type IPTG	48
P*lac* - *ctrA*	340
P*lac* - *ctrA* IPTG	0
pMR10	190
pMR10 - *ctrA*	0

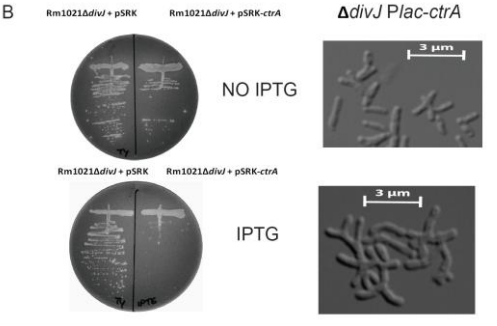

Figure VII.6. DivJ is required for down-regulation of CtrA (BM264). A. Transductions table, overexpression of CtrA in the Δ*divJ* is lethal; B. BM264 (Δ*divJ* + over-*ctrA*) agar plates and morphology with and without IPTG.

We attempted the transduction of the *divJ* deletion using a phage lysate of the strain BM253 (Δ*divJ*) and also we transduced the same lysate into the strain with *ctrA* under the inducible promoter (BM240), creating the strain BM264. Results are summarized in Figure VII.6A and strongly suggest that a strain carrying a deletion of *divJ* does not tolerate high levels of CtrA, since we did not recover colonies using BM132 as recipient strain. In order to confirm this observation we further analyzed the strain BM264 grown

without IPTG and then refreshed in medium supplemented with IPTG; the strain dies (Fig. VII.6B) and develop a phenotype highly branched and elongated (Fig. VII.6B).

VII.3.4 Comparison of Δ*divJ* with the *cbrA*:Tn5 mutant

As introduced before, CbrA is an histidine kinase, hypothesized to interact with DivK, whose mutant shows several altered phenotypes, such as abnormal EPS production and nodulation defects in alfalfa plants (26)

First we compare several phenotypes that characterized the *cbrA* mutant with the *divJ*. As mentioned above *cbrA* mutant shows abnormal EPS production. Succinoglycan plays a critical role in infection thread development and hence in nodule invasion. A reliable test to screen genes involved in succinoglycan production, is the strike of mutant strains on LB containing calcofluor, a dye that fluoresces under UV light specifically when bound to certain β-linked polysaccharides, including succinoglycan (41). Mutants with a calcofluor-bright phenotype, indicate succinoglycan overproduction or alteration in production. Both strains shows a bright phenotype the *divJ* mutant is even brighter than *cbrA* (Figure VII.7A).

Besides succinoglycan production, other two aspects of *cbrA* mutant were particularly intriguing in relation to the establishment of the symbiosis with alfalfa, the cell surface composition and the resistance to oxidative stress. In fact LPS of cell envelope plays an important role in symbiosis. Mutants with distinct LPS alterations, but a common sensitivity to detergents in the medium, are unable to establish a chronic intracellular host infection and rapidly degenerate (42-44). In contrast to the wild type strain, *cbrA* mutant is unable to form single colonies when grown on LB plates supplemented with the hydrophobic dye crystal violet (1 μg/ml), suggesting of an alteration in the cell envelope of the *cbrA* mutant (26). Moreover reactive oxygen species are produced as an early event in plant defense response against avirulent pathogens.

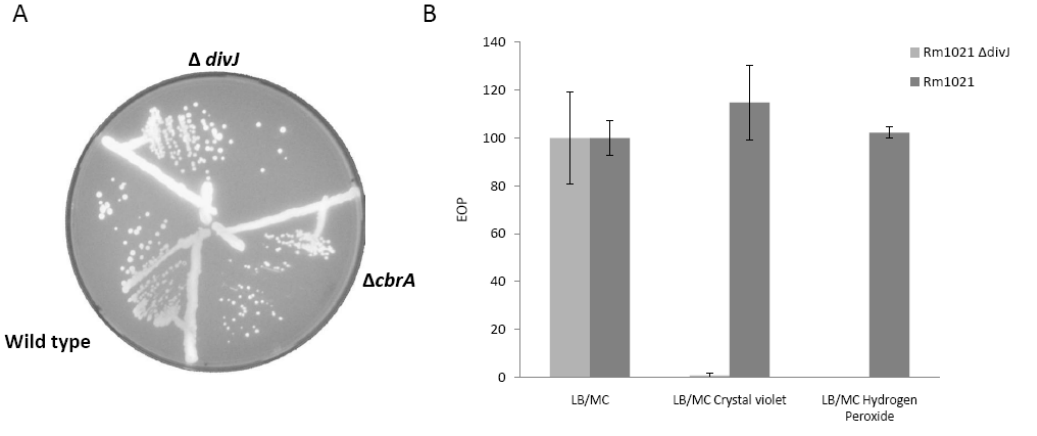

Figure VII.7. Similarities with *cbrA* mutant. A) Calcofluor LB/MC plates B) Efficiency of plating in LB/MC with crystal violet or hydrogen peroxide.

Also alfalfa during early nodule development responds to *S. meliloti* infection by production of superoxide and hydrogen peroxide, so bacterial progression through the

infection thread is concurrent with an oxidative burst derived from the plant (45), and mutants sensitive to oxidative stress were found to be symbiotically deficient (46-48). Since DivJ as CbrA contains a PAS domain that may provide a redox sensory function (49), we also investigated if this feature plays a role in bacterial resistance to oxidative stress.

In fact in contrast to the wild type, the *cbrA* mutant is unable to form single colonies when grown on LB containing the oxidative-stress agent hydrogen peroxide (1 mM) (26). So we perform an Efficient of Plating assay of *divJ* mutant (BM253) respect the complemented strain (BM224) either on LB supplemented with the hydrophobic dye crystal violet (1 µg/ml) or the oxidative-stress agent hydrogen peroxide (1 mM). Both assays shows that the Δ*divJ* strain was unable to grow in those conditions (Figure VII.7B).

VII.3.5 DivJ activity is involved in the symbiosis process

Finally we tested the *divJ* mutant (BM253) for its ability to infect plants, form nodules and differentiate in bacteroids, in nodulation experiments using *M. sativa* and *M. truncatula* (Fig. VII.8A) as described in the Methods section.

To characterize the symbiotic deficiency associated with the *divJ* mutant, alfalfa seedlings were either inoculated with 0.5 BMN medium or inoculated with several strains derived from the wild type, Rm1021.

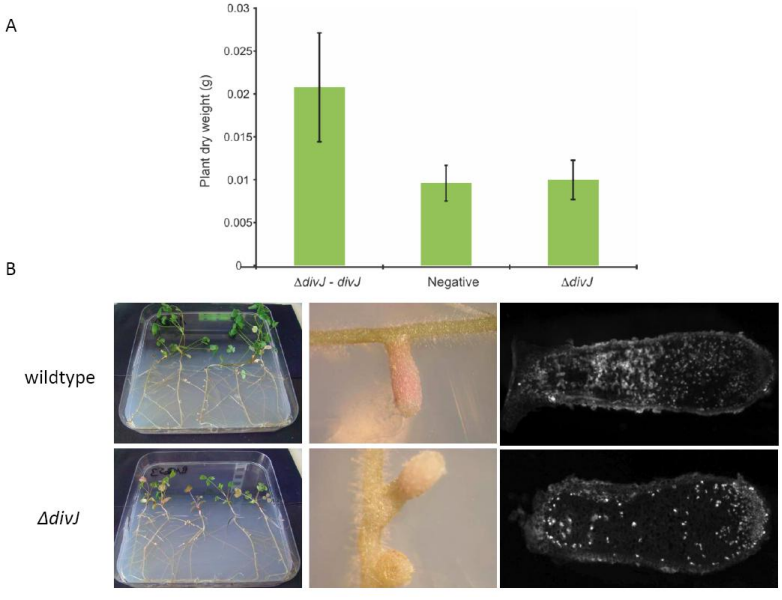

Figure VII.8. Nodulation efficiency. A. Table with plant weight; B. Pictures of plants, nodules and EM of cells inside nodules.

Alfalfa plants inoculated with the wild type grow to a weight of 21 (±6) mg after 28days of growth (Figure VII.8A), and the leaves of the plants are a healthy green color (Figure VII.8B). In contrast, plants inoculated with the *divJ* mutant are significantly shorter, with an average weight of 10 (±2) mg (Figure VII.8A), and the leaves are slightly yellowed

compared to plants inoculated with the wild type (Figure VII.8B). Plants that are not inoculated grow only 9 (±2) mg (Figure VII.8A) and display sick and yellow leaves due to nitrogen starvation on the BMN medium used to test nodulation.

As expected, nodules elicited by the wild type were elongated and pink in coloration (Fig. VII.8B). In contrast, nodules induced by the Δ*divJ* mutant were variable in both size and coloration, some have the same features of wild type ones, other show abnormal shapes and white or slightly pink colour. EM analysis (in collaboration with Prof. Anke Becker, at Albert-Ludwigs-Universität, Freiburg, Germany. Figure VII.8B) shows that meristematic part (where are localized the undifferentiated cells) of Δ*divJ* nodules are highly populated in comparison with wild type ones while the central part of the nodule where we can find the differentiated cells (bacteroids) and nitrogen is fixed is quite empty in mutant nodules and highly crowded in nodules induced by wild type strain, suggesting that differentiation in Δ*divJ* mutant is impaired. It should underlined that the *cbrA* mutant that showed similarities with *divJ* is not able to enter the nodules, suggesting a different role for DivJ.

VII.4 Conclusions

The development of nodules relies on a continuous molecular dialogue between the two symbionts and successive steps lead to the completion of nodule formation (50).
Symbiotic cells originate from progenitor cells in the meristem. During their differentiation, the cells exit the cell division cycle, which is converted into an endoreduplication cycle. These post mitotic cells are infected and gradually become filled with increasing numbers of symbiosomes. In parallel, they increase their size considerably by successive endoreduplication cycles. Mature symbiotic cells are about 80-fold larger in size and have endoploidy levels up to 64C as compared to diploid 2C cells in Zone I (50). In this process CtrA may be involved, in fact a *ctrA* depletion strain of *S. meliloti* shows similar features with bacteroids (Ferri *et al.* in prep) such as polyploidy and cell elongation. Here we analyzed the histidine kinase *divJ* and its involvement in cell cycle regulation and bacteroid differentiation. One possible trait-de-union between cell cycle and differentiations could linked by histidine kinase controlling DivK a fundamental response regulator that acts on CtrA phosphorylation and degradation.
Histidine kinase are multi-domains proteins composed by a C-terminal histidine kinase and an N-terminal sensor domain that is presumably involved in the perception of signals, on the basis of this domain it's possible to subdivided the HKs able to perceive extracellular input or citoplasmatic signals, so could be a good candidate in plant bacterium dialogue, directly or indirectly. Moreover a bioinformatic analysis on *Alphaproteobacteria* HKs potentially interacting with DivK shows an high variability on their number *per* organisms suggesting a possible involvement of HK regulation of cell cycle in response to environmental changes.
Our attention was focused on DivJ the principal kinase of DivK in *Caulobacter*. First, the deletion of *divJ* was constructed demonstrating that *divJ* is not essential in *S. meliloti*. However the deletion strain resulted in a reduced growth rate and in a dramatic cell elongation and branching. As in *Caulobacter* (19), Δ*divJ* is still motile (assayed by 0.2% soft agar plates).
Bionformatic analisys shows that in *S. meliloti* another kinase of DivK is regulated by CtrA besides DivJ and PleC (28), this kinase named CbrA and is previously been characterized by Gibson *et al.*(26, 27). Our analysis shows that *divJ* mutant share several features with this kinase such as an enhanced ability to bind calcofluor suggesting higher levels of exopolysaccharides on the surface of the cells, moreover the two mutant have in common LPS alterations and oxidative stress sensibility. *S. meliloti* Δ*divJ* is able to form nodules but inefficient as the dry weight of the plant infected by the mutant was similar to the non-inoculated one. This phenotype could be related to the reduced ability to fix nitrogen or presumably to a defect in the differentiation process as the zone III (see I.4) of the nodule is devoid of bacteroids. This feature distinguished DivJ from CbrA that is not able to enter efficiently in nodules.

In *Caulobacter*, deletion of *divJ* induces an increase of CtrA activity by reducing DivK phosphorylation level. We hypothesize that the reduced efficiency of nitrogen fixation of the *divJ* mutant is due to an enhanced activity of CtrA; so DivJ controlling CtrA phosphorylation is indirectly involved in bacteroid differentiation This may suggest that CtrA needs to be down-regulated during the differentiation process. Furthermore using transduction was demonstrated that the deletion of *divJ* combined with the over-expression (30) of *ctrA* is a lethal condition, probably stopping cell cycle progression (G1 arrest).

Overexpression mutant of *divJ* and *divK* (in *divJ* this characteristic are more pronounced) were also analysed, the two gene show similar features, branching cell morphology, bigger cell size, accumulation of two chromosome and reduced viability in particular the overexpression of the first gene is lethal while the second one shows a bacteriostatic effect. In general the phenotype of *divJ* overexpression is more severe than *divK* ones. Particularly intriguing is the bacteroid-like shape (50) in the overxpression of *divJ*, a feature which could led to hypothesize that an enhanced expression of this gene could be involved in bacteroid differentiation even if other changes are necessary to complete the development program (i.e. endoreduplication of genome).

Next step will be a detailed analysis of the other members of the Pdh family (*pleC*, *cbrA*, *pdh1* and *pdh2*), by deletion, overexpression and their phenotypic characterizations. Another aspect of great interest will be the construction of kinases double/triple mutants in order to see if the combination of more mutations of these genes will restore a wild type phenotype or it will be a lethal condition. Moreover a fascinating challenge will be to study of cell cycle kinases expression and localization during bacteroid differentiation.

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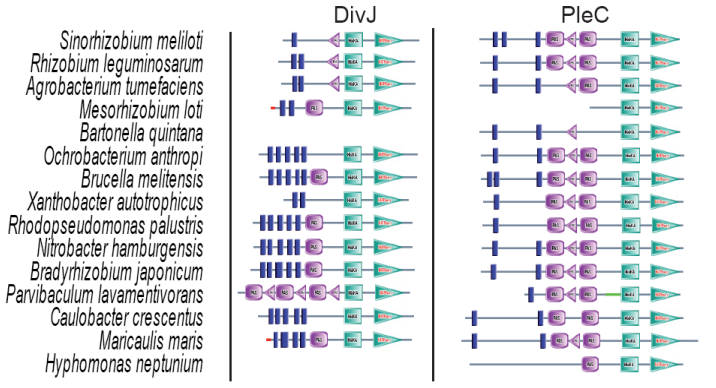
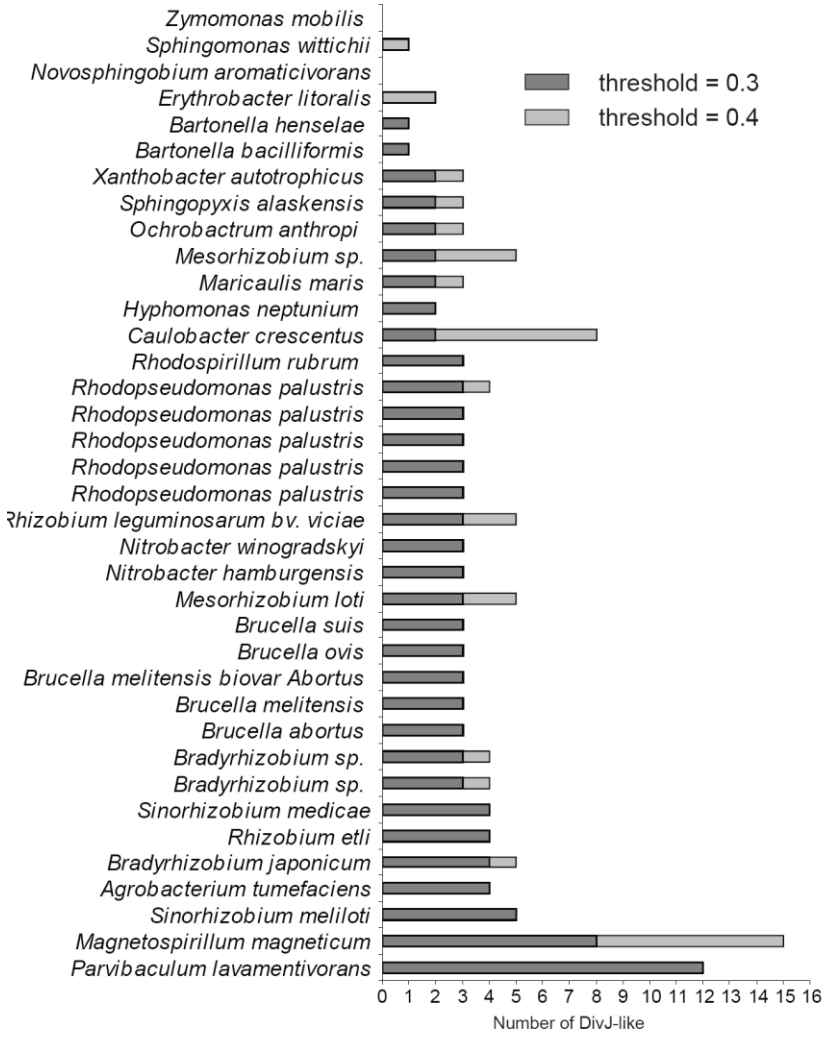


Figure VII.S1. Domains organization of the DivJ-like family. Proteins domains of Pdh family (DivJ, PleC) as predicted by SMART (Schultz et al. 1998; Letunic et al. 2002). Red line indicates a signal peptide; blue transversal bars indicate transmembrane regions; purple, pink or black shapes are domains found in sensors such as pac or pas domains; green square are H-boxes that together with the green triangles (ATPase domain) make the histidine kinase domain.



FigureVII.S2. .Histogram showing the number of Pdh in alpha proteobacterial sequenced genomes.

Chapter VIII

Conclusions and perspectives

As reported in several studies, plant-associated bacteria are promising partners which can increase plant performances and productivity. However several points are still unexplored, concerning in particular bacteria associated to above-ground plant tissues, and plant preference in bacteria recruitment. Two different plant models were analyzed in this thesis, *Alyssum bertolonii* and *Medicago sativa*. In both species a very heterogeneous distribution of leaves-associated bacteria population was found. Regarding *A. bertolonii*, plant-by-plant variability of bacterial community composition is far higher than variability due to the sampling site, suggesting that a large fraction of bacteria could be associated to the plant simply by chance and may not provide any selection by the plant, then hypothesizing no positive (or negative) relevant effect towards plant phenotypes and fitness. However, the significant level of among-plant-populations variance could lead to the hypothesis that an influence (numerically small but present) exists by different plant populations on the recruitment of some different bacterial strains (1).

In *M. sativa* a significant pattern was found that showed the class of *alpha-proteobacteria* as the more abundant in plant tissues differently than in soil. The same uneven pattern was then observed within the *alpha-proteobacteria* at lower taxonomic ranks.

In both plant species members of *Methylobacteriaceae* (strongly represented in alfalfa shoots) were found, suggesting an important role of this class in plant-bacteria association. In fact *Methylobacteria* have been previously found as endophytes in several plant taxa (2-9).

Moreover the *S. meliloti* populations were also analysed, in order to see if a differential pattern of colonization exists also at species level between nodule and soil. For this purpose two molecular tools were developed: i) a species-specific marker based on terminal-restriction fragment length polymorphism (T-RFLP) methodology, targeting

specifically the 16S-23S Ribosomal Intergenic Spacer of *S. meliloti* (10) and ii) two real-time PCR (qPCR) markers to detect and quantify the presence of *S. meliloti* in soil and plant tissues by targeting, in a species-specific fashion, the chromosomal gene *rpoE1* and the pSymA gene *nodC (11)*. Obtained data showed the presence of both soil-specific and nodule-specific fractions, suggesting a selection of the plant for the entry of *S. meliloti*. Nodules, providing a niche for growth, harbor a community that do not correspond to that one found in soil (where other haplotypes dominate).

qPCR analysis has shown the presence of *Sinorhizobium meliloti* also in stems and leaves, consequently an in-vitro experiment (setting up an hydroponic system) was performed to test the capability of *S. meliloti* to enter inside plants independently from nodulation. Plants were infected with *S. meliloti* 1021 wild type strain or with a mutant defective for nodulation (both marked with a GFP plasmid) and their entry with plants was monitored by plating and confocal microscopy.

Data suggested that the nodulation-defective mutant strain can endophytically colonize the plant, suggesting the existence of life style alternatives to the symbiosis that could explain the coexistence in the environment of strains which symbiotic characteristics are very different. Putative entry sites were detected at the emergence of secondary roots and root hairs. Microcolonies of bacterial endophytes were found in the stems, particularly in the intercellular spaces and some portions of vascular tissue. Moreover, after comparing the results from endophytic colonization by three different strains (1021, AK83 and BL225C (12)), we found that their trend of colonization is similar. However further experimental work is needed to set up a more sensitive test systems.

As we said before, *S. meliloti* is able to enter inside plants also as an endophyte. However, under nitrogen-limiting conditions, the establishment of the symbiosis and the formation of nitrogen-fixing nodules takes also place. In nodules, rhizobia undergo a terminal differentiation process driven by the plant (13-15), by which alfalfa probably control the infection (13). The typical changes of bacteroids suggest that plants act by altering bacterial cell cycle. The model of cell cycle regulation found in *Caulobacter* may work in closely related bacteria, such as those belonging to *Caulobacterales* and *Rhizobiales* (16). In *Caulobacter* the principal regulator of the cell cycle is CtrA (17) that is inhibited by another regulator called DivK in a cell cycle dependent fashion. The activation of DivK depends on the histidine kinase DivJ while PleC is its principal phosphatase. An interesting point is that in most *Rhizobiales* the control of CtrA on the response regulator-encoding gene *divK*, observed in *C. crescentus*, is shifted to the genes *divJ* and/or *pleC*. So even if the connections are rewired, feedbacks can possibly be conserved (16). Moreover cell cycle histidine kinases have different sensor domains that could modulate the cell cycle with respect to the environment; they could be theoretically used also by the plant during *S. meliloti* differentiation. We focus our attention on the histidine kinase DivJ and a deletion strain of this gene confirmed its importance in cell cycle regulation (reduced growth rate, cell elongation and branching). In fact, acting on DivK phosphorylation, DivJ indirectly control also CtrA phosphorylation and degradation, so a *divJ* mutant resulted in a strain with an enhanced CtrA activity

(combination of *divJ* deletion and *ctrA* overexpression was a lethal condition). Previous results showed that bacteroids resemble to *ctrA*-loss of function mutant (Ferri *et al*. in prep), so *divJ* deletion (enhancing CtrA activity) should interfere with the differentiation process. Our results confirmed this hypothesis in fact *S. meliloti* Δ*divJ* is able to form nodules but inefficient in nitrogen fixation (zone III of nodules are devoid of bacteroids). Furthermore overexpression of *divJ* (downregulation of CtrA) showed a strong reduction of viability (as bacteroids they were not able to duplicate) and bacteroid-like cell shape suggesting the possible involvement of this gene in the differentiation; however there was no increase in DNA content (bacteroids are polyploid), so there are probably other factors necessary to complete the development program.

Finally this picture suggests that during bacteroid differentiation cell cycle factors, such as CtrA and DivJ, are involved. This observation also suggest that the block of cell cycle in nodules could be a strategy by plants to control bacterial infection, which, in fact, can be very diffuse (endophytic) as it was showed in this thesis.

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