Symbiosis, 19 (1995) 197–211
Balaban, Philadelphia/Rehovot

Siderophore-Mediated Iron Acquisition Mutants in *Rhizobium meliloti* 242 and its Effect on the Nodulation Kinetic of Alfalfa Nodules

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Received July 7, 1995; Accepted October 28, 1995

Abstract

The aim of the present work was to characterize the iron acquisition system present in *Rhizobium meliloti* 242. Tn5-*mob* induced mutagenesis of Rm 242 was done using standard methods. Mutants obtained were classed in three general groups: i) those that produced no detectable or functional siderophore, ii) those that overproduce a functional siderophore, but were unable to grow on iron-starved conditions and, iii) those that overproduce a functional siderophore in both iron-limiting and iron-sufficient conditions and were able to grow on iron-starved media. According to the properties of the Tn5-*mob* induced mutants, this system appears to be complex, involving different components. The results obtained in plant assays suggest that the high-affinity iron acquisition system expressed by the free-living form is not essential for nitrogen-fixation, although it can affect the early events of nodulation. Siderophore mediated competition is a widespread phenomenon among soil microorganisms. The mutants obtained in this work will be a useful tool to study the role of iron uptake in the competition between rhizobia and other soil microorganisms.

Keywords: *Rhizobium*, siderophore, nodulation, iron, alfalfa

Presented at the 10th International Congress on Nitrogen Fixation, May 28 – June 3, 1995, St. Petersburg, Russia

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1. Introduction

The most common high-affinity iron transport system found in Gram-negative bacteria involves two major components (Neilands, 1981; Critchon, 1991). One component is the production of low-molecular weights virtually ferric specific ligands that solubilize environmental iron and are termed siderophores. The second component is an active transport system involving a complex of membrane proteins. IROMPs (Iron Regulated Outer Membrane Proteins) serve to specifically recognize the ferri-siderophore complex for subsequent uptake. Some IROMPs transport ferric complexes produced by the bacterium, whereas other IROMPs mediate the uptake of siderophores produced by other microorganisms. In *E. coli*, genes required for the biosynthesis and uptake of siderophores are negatively regulated by the Fur (ferric uptake regulation) protein. Under conditions of iron deprivation, the system is derepressed (Bagg and Neilands, 1987). Fur homologues are common in Gram-negative bacteria, although a similar protein (or gene) has not been yet detected in rhizobia.

The occurrence of a high-affinity iron uptake system is a widespread phenomenon among native Uruguayan *Rhizobium* strains: 90% of fast-growers tested produced siderophores on iron starved-media (Fabiano el al., 1994). Nonetheless, a low number of siderophore-producing *Bradyrhizobium* strains have been reported: one citric ac.-producing strain out of 20 strains tested (Guerinot et al., 1990), and in a separate study, 35% of slow-growers tested produced iron-binding compounds (Fabiano et al., 1994). Guerinot (1994) suggests that *Bradyrhizobium* may not possess a highly developed iron acquisition system, having evolved in the acidic soil of the tropics where iron is generally available. There is a wide variability in the chemical composition of the siderophores produced by different free-living rhizobia, suggesting that they are strain-specific (Guerinot, 1991; Reigh and O'Connell, 1993). Limited information is available regarding siderophore-mediated iron acquisition pathways in rhizobia.

It was shown that iron requirement is higher for plants in the symbiotic state (O'Hara et al., 1988). This would be expected since the nitrogen-fixation process is highly iron dependent, as this metal forms the prosthetic group of several essential proteins (e.g. leghemoglobin, nitrogenase, cytochromes, hydrogenase, ferredoxin). The mechanism used by the bacteroid to acquire iron *in planta* is not well understood. Recently it has been published that iron citrate can be transported into the symbiosomes of soybean nodules (Moreau et al., 1995), but nothing is known about the physiological form to acquire iron by the bacteroids. Previous studies on Medicago symbiosis showed that a high-affinity iron transport system seems to be required for an efficient nitrogen

fixation (Nadler et al., 1990; Gill et al., 1991). The aim of this work is to characterize the iron transport system present in an Uruguayan native *Rhizobium meliloti* strain, to obtain a collection of iron acquisition mutants and to evaluate the contribution of this system to the nitrogen-fixation process.

2. Materials and Methods

Bacterial strains, plasmid and media

R. meliloti 242, a spontaneous streptomycin-resistant derivative of the field-isolate Rm259 (Fabiano et al., 1994) was used in this work. The plasmid pSUP5011 was developed by Simon (1984). This plasmid carries Tn5-*mob* and is unable to replicate in *Rhizobium* spp. *Rhizobium* strains were cultured in either tryptone-yeast extract medium (TY) (Beringer, 1974) or in Vincent minimal medium (Vincent, 1970) without added iron and enriched with 0.03% (w/v) yeast extract (MVY medium). The water used was from a Milli-Q system. Iron-limiting media were made by supplementation with 75 μM 2,2'-dipyridyl (DP) in liquid media or 100 μM ethylendiamine di-(o-hydroxyphenylacetic) acid (EDDHA) in solid media. EDDHA is a strong iron chelator virtually Fe^{3+} specific. Iron-sufficient media were supplemented with 37 μM $FeCl_3$ from a filter sterilized stock solution of 3.7 mM $FeCl_3$ in 0.1 N HCl. Luria broth (LB) (Miller, 1972) was used for *E. coli* growth. Kanamycin (50 μg/ml), neomycin (100 μg/ml) and streptomycin (100 μg/ml) were added to the media when appropriate. Siderophore production was analyzed by using the CAS plate-assay (Schwyn and Neilands, 1987).

Isolation of iron-assimilation mutants

Tn5-*mob* was introduced into Rm242 by conjugation with *E. coli* S17-1 containing the suicide vector pSUP5011 (Simon, 1984). Mutant screening was performed according to Gill and Neilands (1989). Transconjugants that produced either no halo or very large halos on CAS-plates were selected. The mutants were tested for a functional iron-assimilation system on EDDHA-plates. Other chemical reactions to identify the mutants were not performed as the chemical structure of the Rm242 siderophore is not yet known. Catechol and hydroxamate groups could not been detected by using the Arnow (1937) and the periodate oxidation methods (Emery and Neilands, 1962) respectively.

Tn5-hybridization assays

Genomic DNA of Rm242 and of each mutant was digested with *Eco*R1. DNA/DNA hybridization analysis was performed essentially by the method of Southern (Maniatis, 1982). The vector pSUP5011 was labeled by random primers with biotin using the NEBlot-Phototope kit (Biorad).

Growth curves

Tubes were soaked at least 24 hours with 6 N HCl to eliminate traces of iron, and washed with MilliQ water. Ten ml of iron-sufficient media (TY) or iron-deficient media (TY supplemented with different concentration of EDDHA) were inoculated with 100 µl aliquots taken from log-phase cultures that grew on TY media. Tubes were incubated with shaking at 30°C. Growth was determined by measuring the optical density at 620 nm. At least, three replications for each treatment were performed. When indicated, 37 µM $FeCl_3$ was added at 43 hours.

Outer-membrane proteins enriched fractions (OM) and SDS-PAGE

Washed bacterial pellets (4×10^{11} cells) were suspended in 10 ml of 50 mM Tris-HCl buffer (pH 8.5), 10 mM ditiothreitol, 0.2 mg/ml DNAse, 0.2 mg/ml RNAse, 20% sucrose. Cells were disrupted with a French-press at 25,000 lb/in^2 (three times). Lysozyme (0.1 mg/ml) was added and the suspension was incubated for 30 min at room temperature. Unbroken cells were removed by centrifugation at 5,000 xg for 10 min and 2M KCl was added to a final concentration of 0.2 M. The resulting supernatant was supplemented with Sarkosyl to a final concentration of 1% (w/v). After incubation for 30 min at room temperature, the mixture was centrifuged at 60,000 xg for 2 hours. The pellet was resuspended with a glass-homogenizer in 400 µl of water and was mixed with an equal volume of sample buffer (Wright et al., 1986). The samples were placed in boiling water for 15 min and to promote solubilization of aggregated membrane proteins, the preparation was sonicated 10 s at 20,000 Hz (Bjerrum, 1981) at 4°C. The SDS-solubilized membrane material was recovered as the supernatant after centrifugation at 10,000 xg for 10 min. Samples were stored frozen at –20°C until they were analysed by SDS-PAGE. Prior the electrophoresis the samples were supplemented again with 2% (v/v) 2-mercaptoethanol and 10 mM EDTA and heated 1 min at 95°C. SDS-PAGE was performed according to Laemmli (1970). SDS-10% polyacrylamide gels were run at 30 mA. After electrophoresis, gels were stained with Coomasie brillant blue R.

A semi-micro procedure was performed for the study of the OM proteins of the *Tn5*-mutants. Briefly, 50 ml bacterial culture were harvested (ca 3×10^{10} cells) and washed with 1 M NaCl. The pellet was resuspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.5), 0.25 mM EDTA. Lysozyme (0.1 mg/ml), DNAse (0.2 mg/ml) and RNAse (0.2 mg/ml) were added, and the cell preparation incubated at 37°C for 15 min. Unbroken cells were removed by centrifugation and the resulting supernatant was supplemented with Sarkosyl to a final concentration of 1% (w/v). After incubation for 1 hour at room temperature the mixture was centrifuged at 60,000 xg for 2 hours. The pellet was resuspended in 40 µl of water and was mixed with an equal volume of sample buffer. Sample preparation was performed as described in this section.

Plant assays

Alfalfa creola plants were grown aseptically on 15 ml of N-free Jensen medium (Vincent, 1970) without added iron or with 60 µM $FeCl_3$. Plant tubes were maintained at 21°C ± 2 in a light-controlled room with a photoperiod of 12 h. Relative humidity was not lower than 60%. Uninoculated and N-control plants (0.05% KNO_3) were used as references.

R. meliloti 242 and the iron-assimilation mutants were grown to early stationary phase (1×10^9 cells/ml) on TY media, washed and resuspended in 0.15M NaCl. Five-days-old plants were inoculated with 1×10^6 bacteria per plant. At least twelve tubes (two plants per tube) were used for each inoculation. After inoculation, the number of nodulated plants and the number of nodules per plant were recorded daily (Sanjuan and Olivares, 1989). The complete experiment was performed twice. Nitrogen fixation efficiencies were estimated by determining plant dry weight 60 days after planting. Mutant phenotype of bacteria recovered from the nodules was tested on CAS plates. This assay was performed on two nodules from one plant of each inoculation condition.

3. Results

Isolation of iron-assimilation mutants

To identify mutants defective in the production, transport and regulation of a siderophore system, 6000 Tn5-*mob* transconjugants obtained from 8 different mating reactions were screened on CAS-plates and further on EDDHA-plates. Mutants were analyzed by Southern hybridization to select those containing a single Tn5 insertion.

Two classes of mutants were obtained using the CAS assay, those producing no halo and those overproducing CAS-reactive material. The enlarged halo could be diffuse or with defined borders. The two classes (with- or without halos) could be further subdivided based on their ability to grow on an iron-limiting medium. Mutants that produced no halo and could not grow in the presence of EDDHA (8 mutants) were provisionally classed as siderophore production mutants. They may be defective in the biosynthesis of the siderophore or in the production of a regulatory protein required for activation. We can not discard also the possibility of mutations in some siderophore secretion functions. Using the CAS and EDDHA assays it was not possible to further differentiate this class of mutants.

Mutants that overproduced CAS-halos, but which could not grow in the presence of EDDHA were provisionally classed as uptake mutants (11 mutants). It is presumed that the siderophore is still produced, but that there is some defect at the membrane level preventing uptake of the corresponding ferric complex. Once again, we cannot discard the possibility of production of a defective siderophore which still binds iron but can not be recognized by the receptor. This class could be characterized based on the ability to produce CAS reactive material even when the medium was supplemented with additional iron, at a one hundred fold excess of that normally required to repress the system in the wild type strain. Six mutants were obtained that could produce CAS-reactive material on 200 μM $FeCl_3$-CAS plates.

One mutant was also identified which overproduced CAS reactive material, but was still able to grow on a EDDHA-containing medium. In addition, production of CAS-halo could be observed even when the CAS medium was supplemented with 200 μM $FeCl_3$. It could have some mutation in the operator sequence of a biosynthetic gene or it could be defective in some Fur-like protein, not yet detected in rhizobia. No *fur*-like gene could be detected in Rm 242 genome by low stringency hybridization analysis using *E. coli fur* gene as a probe (data not shown). Table 1 shown some properties of the mutants obtained.

Growth curves

To demonstrate that the provisionally assigned biosynthetic mutants were specifically starved in iron nutrition, some mutants were grown under conditions of iron starvation and the ability to grow was restored by the addition of iron to the medium. The growth curves of a siderophore production mutant are shown on Fig. 1.

Mutants classed as transport mutants could be further subdivided based on the growth characteristics under iron limiting conditions. Mutants A, M, 2.1 and

Table 1. Properties of Tb5-*mob* iron assimilation mutants

Strain	CAS[a]	Halo[b]	Tn5-hybridization group[c]	CAS + 200 μM $FeCl_3$	EDDHA-plates[d]
242 (w.t)	+	N	–	–	+
1	–		1		–
2	–		1		–
1.3	–		1		–
2.5	–		2		–
3.2	–		2		–
3.8	–		2		–
7.4	–		2		–
7.6	–		2		–
2.1	+++	P	3	+	–
M	+++	P	3	+	–
7.8	+++	P	n.d.	+	–
A	+++	P	n.d.	+	–
3.7	+++	P	4	–	+/–
5.3	+++	P	4	–	–
5.6	+++	P	5	–	–
2.4	+++	D	6	–	–
L	+++	D	6	–	–
D	+++	D	6	–	+/–
N	++	P	7	+/–	+/–
7.2	+++	P	8	–	+/–

[a]Production of halo on CAS plates: – absence of halo; + wild type-like halo; ++ large halos; and +++ very large halos.
[b]P: Halo with defined borders; D: halo with a diffuse border.
[c]Groups based on different sizes of genomic DNA *Eco* R1-fragment hybridized with a Tn5 probe. n.d.: not determined.
[d]MVY plates supplemented with 100 mM EDDHA: + wild type-like growth; +/– limited growth; – no growth.

7.8 were more sensitive to iron starvation than mutants D, L and 3.7. The growth curves of some representative mutants are shown in Fig. 2.

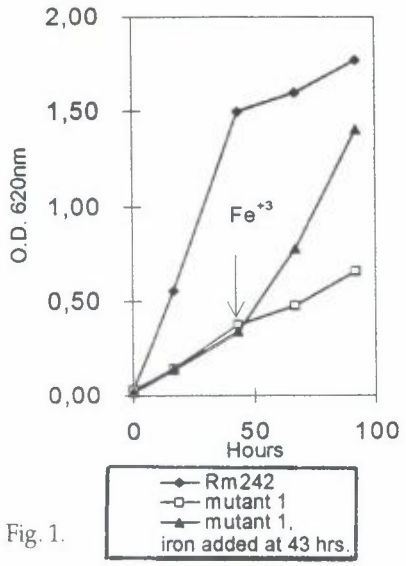

Fig. 1.

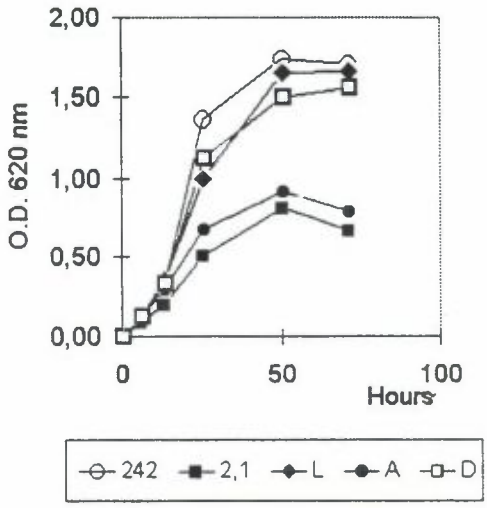

Fig. 2.

Figure 1. Effect of iron-starvation on the growth of a mutant defective in siderophore production. TY medium was iron-starved by the addition of 15 μM EDDHA; 37 μM $FeCl_3$ was added at 43 hours.

Figure 2. Growth curves of mutants that overproduce a functional siderophore but were unable to grow on 100 μM EDDHA-plates. Cells were grown on TY medium supplemented with 25 μM EDDHA.

Characterization of outer membrane proteins

Iron limited cells usually produced two OM bands of 82 and 91 Kd that are not present on OM obtained from iron sufficient cells so they are referred as Iron Regulated Outer Membrane Proteins (IROMPs) (Fig. 3). The band of ca 47 Kd is sometimes present on the iron-sufficient cell OM preparations (Fig. 4), therefore considered as not iron regulated.

The outer membrane protein profiles of all the mutants were studied. None of the mutants were defective regarding the iron regulated outer membrane proteins detected (Fig. 4).

Plant assays

Plants inoculated with all the mutants obtained formed effective nodules. There was no correlation between nodule number per plant and iron content in plant assays performed with the mutants and the wild-type strain inocula.

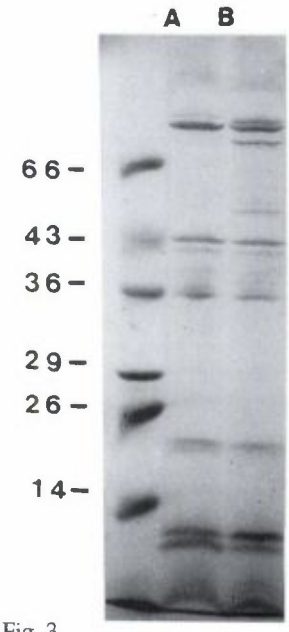

Fig. 3.

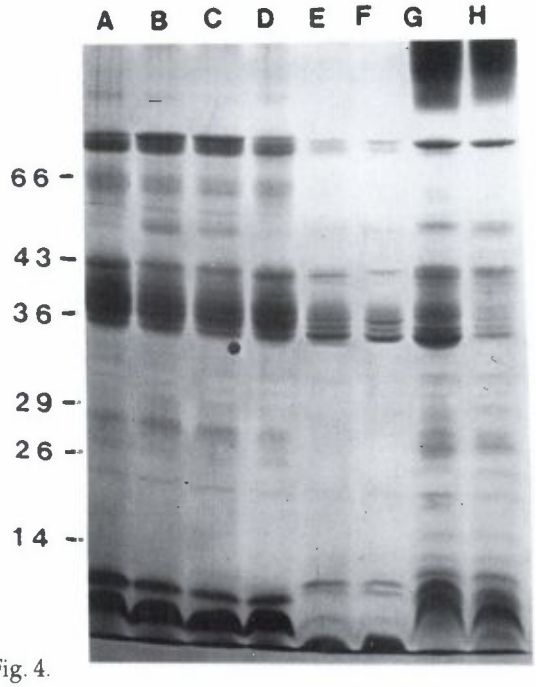

Fig. 4.

Figure 3. *R. meliloti* 242 outer membrane protein profile obtained after Sarkosyl-cytoplasmic membrane solubilization. Cells were cultured on iron-sufficient medium (lane A) or on iron-starved medium (lane B). Mass markers in kilodaltons are shown on the left. The gel was stained with Coomassie blue.

Figure 4. Outer membrane protein profile of some iron assimilation mutants. Cells were grown on iron-starved medium (lanes A to G) or on iron-sufficient medium (lane H). Media were supplemented with 75 μM DP (lane A to E and G) or with 50 μM EDDHA (lane F). Lane A: mutant 7.8; lane B: mutant 2.1; lane C: mutant 5.3; lane D: mutant 2.4; lane E: mutant 7.8; lanes F to H: Rm242.

One representative mutant of each group was selected to study the kinetic of nodule formation. It was significantly slower for mutants 1, 3.2, 7.8 and N than the one obtained with the wild type strain when the plant assay was performed on Jensen medium without added iron (Table 2 and Fig. 5). Mutants D and L having a residual growth on EDDHA-containing medium (Fig. 2) were not affected in their nodulation kinetic compared to the parental strain. Mutant 5.6 showed a lower but not statistically significant ($P<5\%$) nodulation kinetic.

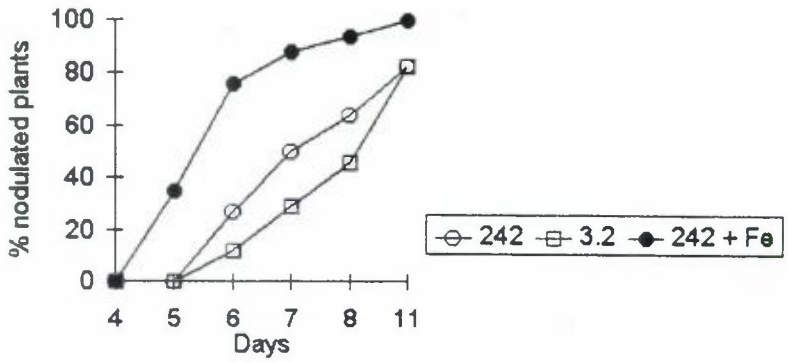

Figure 5. Iron effect on the kinetics of nodulation of *R. meliloti* 242 and its derivative mutant defective in siderophore production. Empty symbols represent data obtained from plant assays performed without added iron; bold symbols represent data obtained from plant assays performed with the addition of 60 μM $FeCl_3$ to the Jensen medium.

Table 2. Nodulation kinetics of *R. meliloti* 242 strain and iron assimilation mutants on alfalfa grown without added iron.

Strain	Days after inoculation 6	7	8	11	21
242 (w.t.)	[a]27A	50A	64A	83A	100A
1	12B	30B	50A	83A	100A
3.2	12B	29B	46B	83A	100A
7.8	10B	30B	40B	90A	100A
5.6	14A	35A	41B	77A	100A
D/L	25A	42A	50A	83A	100A
N	9B	18B	25B	77A	100A

[a]The data are the % of the nodulated plants calculated on the basis of 24 plants of one experiment. Different letters mean significant differences between values in the same column ($p<5\%$). Probabilities have been calculated using cumulative binomial distribution.

Plants were harvested 60 days after inoculation with mutants representative of each class. Plant dry weights of 15 plants per inoculant mutant were determined. All the mutants tested showed lower but not significant differences in the dry weight values compared to that of the wild type strain (data not shown). Evaluation of bacteria obtained from nodules, indicated that the corresponding phenotype of the inoculant (mutant and wild type) was retained.

4. Discussion

Only limited information is available regarding iron acquisition systems in rhizobia. In a previous work (Fabiano et al., 1994) it was shown that the highest siderophore accumulation was observed among microsymbionts of legumes growing on neutral or alkaline soils. The *Rhizobium meliloti*-alfalfa symbiosis is normally found in non-acidic soils and the expression of a classical system of siderophore production would be expected to be present in this bacteria. Because very little is known about iron transport in *Rhizobium*, the present study was undertaken to extend previous findings using a different strain of *R. meliloti*. We found that the native Uruguayan strain Rm242 presents a siderophore mediated iron acquisition system repressed on iron sufficient media. According to the properties of the Tn5-*mob* induced mutants, this system appears to be complex, involving different components. *R. meliloti* iron-assimilation mutants have been previously isolated by Gill and Neilands (1989) and Reigh and O'Connell (1993). The Tn5-induced mutants of R. meliloti 1021 have been provisionally classified as defective in the siderophore biosynthesis, transport or regulation (Gill and Neilands, 1989). Reigh et al. (1993) isolated *R. meliloti* 2011 and *R. meliloti* 220-5 Tn5-*mob* induced mutants producing no orange halo on CAS plates, indicating a defect in siderophore production. Using a similar methodology we obtained a mutant bank of Tn5-*mob* induced iron assimilation mutants of *R. meliloti* 242 which could be provisionally assigned as biosynthetic, transport and regulatory mutants. The transport mutants obtained could be further subdivided based on the CAS-halo produced (with defined borders or with diffuse borders), different ability to grow on iron-starved media, and the production of CAS-reactive material even when the medium was supplemented with additional iron. More genetic and biochemical studies are needed to determine the genotype/phenotye of each mutant.

Reigh and O'Connell (1993) demonstrated that the utilization of a particular siderophore by a *R. meliloti* strain is correlated with the possession of specific iron-regulated outer membrane proteins. The authors presented strong evidences that the isolated cosmid pGR30 carries all the genes necessary to synthesize the *R. meliloti* 2011 siderophore and the outer membrane receptor necessary for its uptake. No iron-uptake mutants in this system have been reported and the outer membrane protein sequence is not yet available. None of our iron-uptake Tn5-induced mutants were defective in the production of the two IROMPs detected. We can not discard the possibility that some of the presumptive iron-uptake mutants could be defective in the outer membrane receptor that specifically recognize the Rm242 siderophore, but perhaps this protein is not detected with the outer membrane preparations used or it could be

masked by another IROMP. Alternative strategies must be performed to detect the ferri-siderophore outer membrane receptor. Gill and Neilands (1989) were also unable to demonstrate that iron transport mutants were defective in any of the outer membrane proteins induced by iron deprivation.

To address the question of how bacteria and bacteroids acquire iron *in planta*, mutants defective in iron acquisition functions have been analyzed. None of the mutants obtained in this work induced significant differences in plant dry weight values compared to that of the wild type strain, suggesting that this system is not essential for nitrogen fixation. Nonetheless, Nadler et al. (1990) reported that a chemically-induced *R. leguminosarum* mutant defective in iron acquisition was also defective in bacteroid maturation. In another study *R. meliloti* 1021 mutants defective in a high-affinity iron acquisition system were still capable of forming effective nodules on alfalfa plants (Gill and Neilands, 1989), but subsequent studies indicated that the siderophore mutants were less effective than the wild-type in stimulating plant growth (Gill et al., 1991), suggesting the possibility that the iron storage by rhizobia may play a role in supplying this metal to the developing bacteroid. Iron storage and efficient recycling may act in concert with iron acquisition to assure that sufficient levels are available for bacteroid development under diverse conditions. The precise mechanism used by the *Rhizobium*-bacteroid to acquire iron *in planta* remains a fertile area for subsequent research.

Although iron is required for nitrogen fixation, very little is known about the interactions of this metal with other factors controlling symbiotic development and function. Nodule development but not nodule initiation, of the groundnut-bradyrhizobia symbiosis was severely limited by iron deficiency reducing nodule mass, leghemoglobin content and nitrogen fixation activity (O'Hara et al., 1988). On the other hand, Tang et al. (1990) showed that iron deficiency depressed nodule initiation on *B. lupini-Lupinus angustifolius* symbiosis. It is very difficult to monitor iron deficiency in tube-plant experiments due to iron present in reagents and lab material: iron content could be higher than 1 μM even without external iron supplementation. Moreover many of the kinetics involved on bioavailability of iron are slow, it could take days to reach the equilibrium (Crowley et al., 1991). The soil system is very different: there are many factors that influence iron bioavailability (Crowley et al., 1991), but it is generally assumed that calcareous soils (with high pHs) have limited available iron (Leong, 1986). The plant assays performed in this work showed no chlorosis symptoms on the alfalfa plants even if no iron was added, suggesting that our system is not completely iron-stressed. Nonetheless significant differences in the kinetics of nodulation of the wild type strain could be observed when the assay was done without added iron or with the addition of 60 μM $FeCl_3$. Significant differences in the nodulation kinetic could

also be detected between the wild type strain and some representative iron assimilation mutants (mutants 1, 3.2, 7.8 and N) in plant assays performed without added iron. These results suggest that iron and the high-affinity iron acquisition system present in the free-living form can affect the early events of nodulation.

The success of legume inoculation with rhizobia depends in part on the ability of the introduced strain to persist in the soil and thus to compete with the indigenous microflora. Competition between strains in soil is usually complex and it is caused by different factors. Siderophore mediated competition is a widespread phenomenon among soil micoorganisms (O'Gara et al., 1986; Jurkevitch et al., 1992). It has been reported that siderophores in soils are present in a range of 0 to 150 $\mu g\ kg^{-1}$ soil which correspond to 0 to 2.5 μM Ferrioxamine B equivalent (Crowley et al., 1991). The mutants obtained in this work will be a useful tool to study the siderophore mediated competition between rhizobia and other soil micoorganisms and also, to asses the role of siderophores during the infection process.

Acknowledgement

We are grateful to M. Aguilar for helpful comments. This research was partially supported by SAREC, IFS and PEDECIBA.

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