MPMI Vol. 8, No. 4, 1995, pp. 492–498.

Characterization of a *Rhizobium meliloti* Proline Dehydrogenase Mutant Altered in Nodulation Efficiency and Competitiveness on Alfalfa Roots

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Received 13 December 1994. Accepted 6 March 1995.

***Rhizobium meliloti* strain GRM8 is able to transform ornithine into proline by means of an ornithine cyclodeaminase and, therefore, has the ability to use either of these amino acids as its sole carbon and nitrogen source. By Tn*5* insertion mutagenesis we obtained a GRM8 mutant derivative strain (LM1) unable to catabolize either ornithine or proline. DNA hybridization studies showed that the LM1 mutant carries a single Tn*5* insertion within a chromosomally located gene that, as deduced from a partial nucleotide sequence, encodes a proline dehydrogenase (ProDH). Enzymatic assays confirmed the lack of ProDH activity in cell extracts of strain LM1 and revealed that production of this enzyme is inducible in the parental strain by proline and ornithine. Ultrastructural nodule microscopy analysis, acetylene reduction assays, and dry-weight determinations of nodulated alfalfa plants showed no obvious defect in the nitrogen fixation process of the ProDH$^-$ mutant LM1. However, nodulation tests and competition assays demonstrated that in *R. meliloti* ProDH is required for nodulation efficiency and competitiveness on alfalfa roots.**

Additional keyword: symbiosis.

In the *Rhizobium*-legume symbiosis the C_4-dicarboxylic acids have generally been considered the major carbon source exported from plant cells to the bacteroids that support the nitrogen fixation process (Bolton *et al.* 1986; Finan *et al.* 1983; Ronson *et al.* 1981). However, it has not been established to be the exclusive or even the most efficient energy source under different environmental conditions, and little is known about the carbon sources used by the microsymbiont during nodule formation and invasion. It has been suggested that oxidation of amino acids imported by the bacteroids from the cytosol of infected cells may supply additional energy needed to support nitrogen fixation in legume root nodules (Kohl *et al.* 1988), and attention has focused mainly on the possible role of glutamate and proline. However, the impermeability of the plant and peribacteroid membranes to glutamate suggests that this amino acid may not be made available to the bacteroids in significant quantities (Day and Copeland 1991; Udvardi *et al.* 1990). Proline is usually catabolized in prokaryotic cells via pyrroline-5-carboxylate (P5C) by means of proline dehydrogenase (EC 1.5.99.8) (ProDH), yielding NADPH, or by proline oxidase (EC 1.4.3.2). It has been reported that ProDH is associated with bacteroids and not with plastids or mitochondria in soybean root nodules (Kohl *et al.* 1988), but, in general, this enzyme activity is higher in bacteroids from determinate than from indeterminate nodules (Kohl *et al.* 1990). It has also been observed that exogenously applied proline stimulates the nitrogen fixation rate, as much as exogenous succinate or glutamate does, and increases ProDH activity (Zhu *et al.* 1992). Moreover, the accumulation of proline and ProDH activity are high in bacteroids from nodules induced in soybean plants subject to drought (Kohl *et al.* 1991). Both the compartmentation of ProDH within soybean nodules and its potential ability to contribute to the energy requirements of the bacteroid suggest a role for this enzyme in nitrogen fixation.

Recently, we demonstrated the presence of ornithine cyclodeaminase (OCD) activity in *R. meliloti* (Soto *et al.* 1994), which showed enzymatic properties similar to those of *Agrobacterium tumefaciens* (Sans *et al.* 1988; Schindler *et al.* 1989), suggesting that the ornithine catabolic pathway in this bacterium proceeds via proline formation. In this work we describe the isolation and characterization of a *R. meliloti* mutant affected in ornithine and proline utilization and lacking ProDH activity. We also investigated the nodulation properties of this mutant and its possible effect on nodule development, plant vigor, and nitrogen fixation in nodulated alfalfa plants. We discuss the importance of proline catabolism not only in bacteroids but also in the infectivity, competitiveness, and nutrition of free-living rhizobia.

RESULTS

Isolation and genetic characterization of a *R. meliloti* strain unable to utilize ornithine and proline.

R. meliloti strain GR4 is able to use ornithine as its sole carbon and nitrogen source and shows OCD activity similar

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to that of *A. tumefaciens*. This enzymatic activity does not depend on either of the two cryptic plasmids harbored by this strain (Soto *et al.* 1994). Thus, its pRmeGR4a$^-$ and pRmeGR4b$^-$ cured derivative strain, GRM8, is also able to grow with ornithine as its sole carbon and nitrogen source. To test the possible importance of the metabolism of ornithine and its related metabolites, such as glutamate or proline, in the *R. meliloti*–alfalfa symbiosis, we attempted to obtain GRM8 mutants defective in the utilization of these amino acids. By screening 5,000 random Tn*5*::*mob* insertion mutants on minimal medium (MM) agar (Robertsen *et al.* 1981) containing mannitol and glutamate and on MM with ornithine as the only carbon and nitrogen source, we isolated five mutants (LM1, LM2, LM3, M1, and M2) unable to grow in the latter medium and, therefore, potentially affected in ornithine metabolism. As discussed below, only one of these mutants, LM1, appeared to be defective in ProDH activity. This mutant was first tested for growth in MM broth with mannitol and glutamate or either proline, ornithine, or glutamate as a carbon and nitrogen source. Unlike the parental strain GRM8, which was able to grow in all media, the LM1 strain showed no growth in proline- or ornithine-MM, indicating its inability to utilize either amino acid as a carbon and nitrogen source.

The location of the Tn*5* transposon insertion in the LM1 mutant was physically analyzed by probing with plasmid pSUP5011. Total DNA from the mutant and the parent strain GRM8 was digested with *Eco*RI, for which there are no recognition sites in Tn*5* (de Bruijn and Lupski 1984). Only one fragment hybridized the pSUP5011 probe in the mutant LM1, indicating that it carries a single transposon insertion (data not shown). In addition, a Southern blot of an Eckhardt gel of LM1 lysate demonstrated that the Tn*5*::*mob* insertion was not located on any of the symbiotic plasmids, and the insertion was therefore assumed to be chromosomal (data not shown). We attempted to complement the mutant phenotype of LM1 by carrying out conjugative mating with a GR4 cosmid clone bank constructed in pLAFR1 (Soto *et al.* 1992). Three cosmids sharing common *Eco*RI restriction fragments, which we designated pJIJ101, pJIJ103, and pJIJ108, were found among transconjugants selected in ornithine-MM agar. The *Eco*RI fragment containing the Tn*5* insertion of LM1 (11 kb) was first cloned into pUC18 to yield pDIL100 and was later cloned into pSUP102 to yield plasmid pDIL106. The restriction map of that *Eco*RI fragment is shown in Figure 1A. Hybridization of plasmid pDIL100 with the complementing cosmids and with total DNA (*Eco*RI-digested) from the wild-type strain GR4, GRM8, and the mutant derivative LM1 (Fig. 1B) showed that the Tn*5*::*mob* insertion was located in an *Eco*RI fragment of about 3.7 kb and that the cosmids analyzed contained this wild-type DNA. Plasmid pDIL106 was introduced into GRM8, and the strains arising from the marker exchange exhibited the mutant phenotype of LM1. In

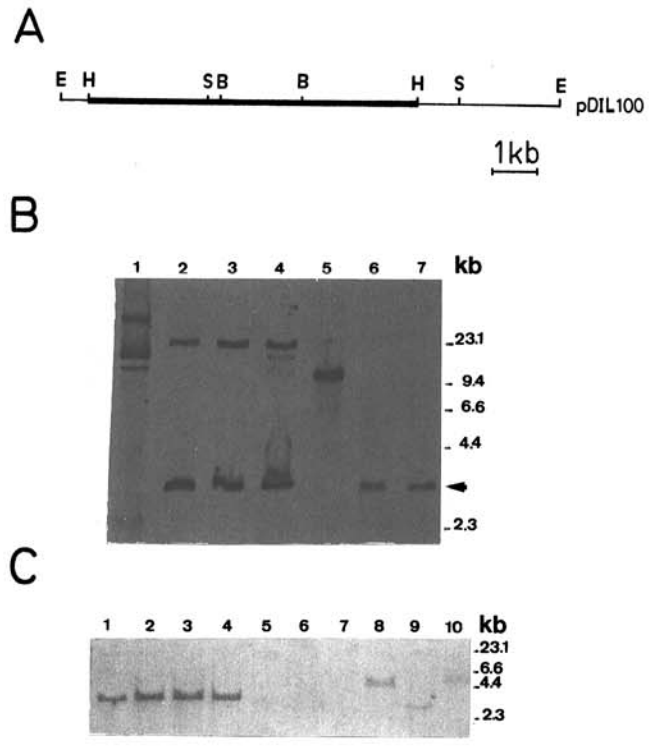

Fig. 1. A, Restriction map of the *Rhizobium meliloti* LM1 mutant *Eco*RI fragment containing the Tn*5* insertion (thick line) cloned into pUC18 (plasmid pDIL100). E, *Eco*RI; H, *Hpa*I; B, *Bam*HI; S, *Sal*I. **B,** Southern blot hybridization with pDIL100 DNA as the probe against pDIL100 (lane 1); cosmids pJIJ101, pJIJ103, and pJIJ108 digested with *Eco*RI (lanes 2, 3, and 4); and total DNA from the mutant LM1 (lane 5), the parental strain GRM8 (lane 6), and the wild-type GR4 (lane 7), also digested with *Eco*RI. The arrow indicates the wild-type 3.7-kb *Eco*RI fragment that was altered by the Tn*5* insertion in the mutant LM1. **C,** Southern blot hybridization with pDIL100 DNA as the probe against total DNA from *R. meliloti* strains GR4 (lane 1), 41 (lane 2), 2011 (lane 3), and 102F34 (lane 4); *R. l.* bv. *trifolii* RS1050 (lane 5); *R. l.* bv. *phaseoli* 8002 (lane 6); *R. l.* bv. *viciae* VF39 (lane 7); *R. fredii* HH003 (lane 8); *Bradyrhizobium japonicum* USDA110 (lane 9); and *Agrobacterium tumefaciens* A348 (lane 10), all digested with *Eco*RI.

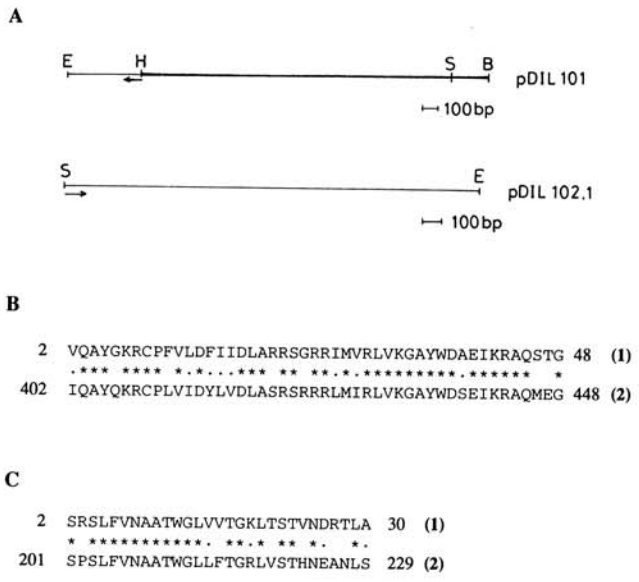

Fig. 2. Sequencing the LM1 Tn*5* insertion. **A,** Restriction maps of cloned DNA in plasmids pDIL101 and pDIL102.1. The arrows indicate the extent and direction of DNA sequenced. **B,** Protein alignment between (1) the predicted amino acid sequence encoded by the DNA region sequenced from pDIL101 and (2) the corresponding region of the PutA protein of *Salmonella typhimurium.* **C,** Protein alignment between (1) the predicted amino acid sequence encoded by the DNA region sequenced from pDIL102.1 and (2) the corresponding region of the PutA protein of *S. typhimurium.* Identical (*) and conserved (•) amino acids are marked.

addition, plasmid pDIL100 was used as a probe against total DNA from wild-type strains of different rhizobia. *R. fredii, Bradyrhizobium japonicum,* and *A. tumefaciens* strains showed homology to the pDIL100 probe, as did other wild-type strains of *R. meliloti* (Fig. 1C). However, no homology was detected with DNA from the *R. leguminosarum* strains tested.

Sequencing the LM1 Tn*5* insertion.

The 3.5-kb *Eco*RI-*Bam*HI fragment of pDIL100 (Fig. 1A) containing the kanamycin resistance gene of Tn*5* was subcloned into pUC18, to yield plasmid pDIL101 (Fig. 2A). We sequenced 144 nucleotides of the Tn*5*-DNA junction of pDIL101 by using a primer derived from the Tn*5* inverted repeat sequence (Fig. 2A). The predicted amino acid sequence encoded by this DNA region showed 70% identity (Fig. 2B) with a region extending from residue 402 to residue 448 of the PutA protein of *Salmonella typhimurium.* This protein exhibits ProDH and P5C dehydrogenase activities (Allen *et al.* 1993). In addition, a partial DNA sequence, extending 90 nucleotides, was determined from the 2.2-kb *Sal*I-*Eco*RI fragment of pDIL100 (Fig. 1A) contained in plasmid pDIL102.1 (Fig. 2A). As shown in Figure 2C, the predicted amino acid sequence encoded by this DNA region showed 65% identity with a region extending from amino acid 201 to amino acid 229 of the PutA protein of *S. typhimurium.* These results indicate that the gene altered by the Tn*5* insertion in the mutant LM1 may encode a ProDH.

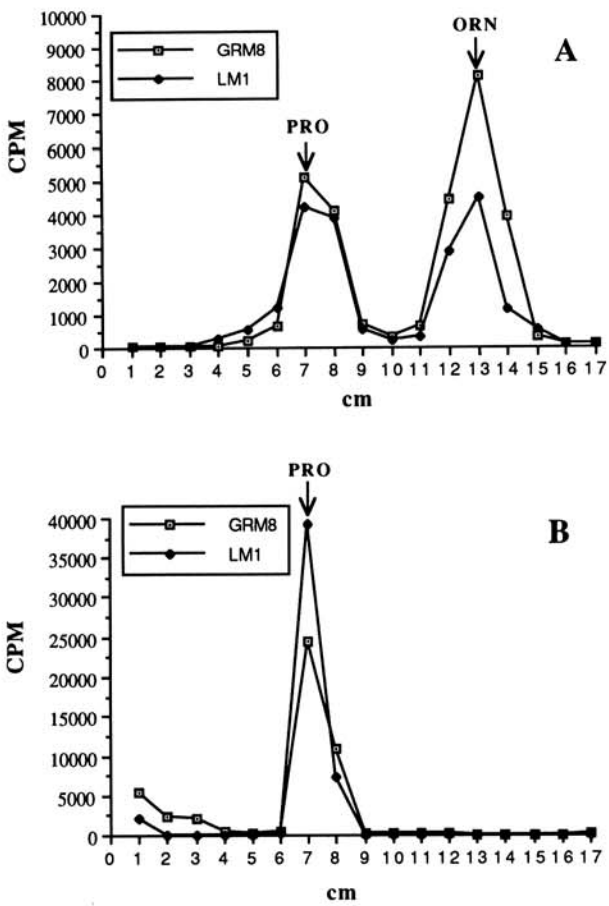

Fig. 3. *In vivo* uptake and catabolism of ornithine (**A**) and proline (**B**) in *Rhizobium meliloti* GRM8 and its insertion mutant derivative LM1. Cells were grown in minimal medium with mannitol and glutamate and incubated with L-[1-^{14}C]ornithine or L-[U-^{14}C]-proline for 20 min.

Ornithine and proline uptake and catabolism.

To determine the ability of GRM8 and LM1 strains to take up and transform ornithine and proline, bacteria were incubated with these radiolabeled amino acids, and cell extracts were analyzed by high-voltage paper electrophoresis.

Extracts from the parental strain GRM8, previously grown in MM with mannitol and glutamate and exposed to L-[^{14}C]-ornithine, yielded two radiolabeled compounds that behaved like proline and ornithine under the electrophoretic conditions described (Fig. 3A), indicating its ability to take up and transform ornithine into proline. Similar results were obtained when cell extracts from the mutant LM1 were analyzed (Fig. 3A). Data obtained when cells were exposed to L-[^{14}C]-proline (Fig. 3B) also demonstrated that the mutant LM1 was able to incorporate L-proline into the cell.

In prokaryotic cells, L-ornithine can be converted directly into P5C by an ornithine transaminase, ornithine 5-aminotransferase (OAT), or converted into proline with the release of ammonia by an OCD enzyme. In this case, conversion of proline into P5C depends on a ProDH enzyme. These enzyme activities were evaluated in cell extracts of GRM8, mutant LM1, and the complemented strain LM1 (pJIJ108). Cells were grown in complete MM (containing mannitol and glutamate) and MM supplemented with ornithine or proline as potential inductors of the enzyme activities. As shown in Table 1, OCD activity was not detected when cells were grown in complete MM but could be measured in cell extracts of the three strains tested when ornithine was added to the medium. Similar rates of proline formation and ammonia release were found for all strains. However, when we added pyridoxal phosphate and 2-oxoglutarate to the reaction mixtures, formation of P5C was not detected in any case (Table 1), indicating

Table 1. Enzyme activities associated with the ornithine catabolic pathway in *Rhizobium meliloti*

Strain	Supplement to minimal medium[a]	OAT[b]	OCD[c]	ProDH[c]
GRM8	None	...	...	...
	Ornithine	...	7.1	0.45
	Proline	ND	ND	0.58
LM1	None	...	...	...
	Ornithine	...	5.8	...
	Proline	ND	ND	...
LM1(pJIJ108)	None	...	...	...
	Ornithine	...	6.1	4.3
	Proline	ND	ND	5.2

[a] Carbon and nitrogen sources in minimal medium were mannitol and glutamate, respectively. The amino acids were added to the medium at a concentration of 0.2% (w/v).

[b] OAT = ornithine 5-aminotransferase; OCD = ornithine cyclodeaminase; ProDH = proline dehydrogenase; ND = not determined; ... = not detected.

[c] Specific activity of OCD is expressed as μmol of ammonia released per minute per milligram of protein. Specific activity of ProDH is expressed as nmol of pyrroline-5-carboxylate produced per minute per milligram of protein. Values reported in both cases are means of duplicate assays of samples derived from equivalent numbers of cells.

the absence of significant OAT activity in *R. meliloti* GRM8, which confirms previous results (Soto *et al.* 1994) that demonstrated the lack of OAT in the wild-type strain GR4. ProDH activity was detected only in crude extracts of strain GRM8 grown in the presence of ornithine or proline, which indicates that these amino acids induce enzyme activity. Similar values of specific activity were determined when cells were grown in the presence of either proline or ornithine (Table 1). As expected, ProDH activity was not detected in cell extracts of mutant LM1 grown under the same conditions. When cosmid pJIJ108 was introduced into LM1, the ProDH activity was recovered, and it was determined to have a 10-fold higher specific activity than in cell extracts of GRM8 (Table 1), which may be due to the higher number of copies of the ProDH gene present in the complemented strain.

These results corroborate the conclusions that 1) catabolism of ornithine in *R. meliloti* proceeds via proline formation and depends on an OCD enzyme; 2) mutant LM1 is not affected in the uptake of ornithine and proline or in the catabolism of ornithine; and 3) LM1 is a ProDH$^-$ mutant.

Symbiotic characteristics of the ProDH$^-$ mutant LM1.

Nodulation and competition tests, ultrastructural nodule microscopy analysis, and acetylene reduction assays were performed to study the symbiotic characteristics of mutant LM1. As shown in Figure 4 and Table 2, this mutant strain nodulated 100% of the plants 1 day later and induced fewer nodules per plant than the parental strain. Introduction of cosmid pJIJ108 into mutant LM1 restored the parental nodulation ability (Fig. 4 and Table 2). Differences between the mutant and GRM8 were more evident in competition assays (Table 3). In 1:1 coinoculation mixtures, GRM8 appeared to be more competitive than LM1, as deduced from their respective percentages of nodule occupancy (only 6% of the nodules analyzed were found to be occupied by the mutant strain). Surprisingly, when we performed competition assays between GRM8 and the complemented strain, we found that 73% of the nodules were occupied by the latter strain. This indicates that cosmid pJIJ108 not only restores the competition ability of the mutant LM1 but also improves the competitiveness of the parent strain GRM8.

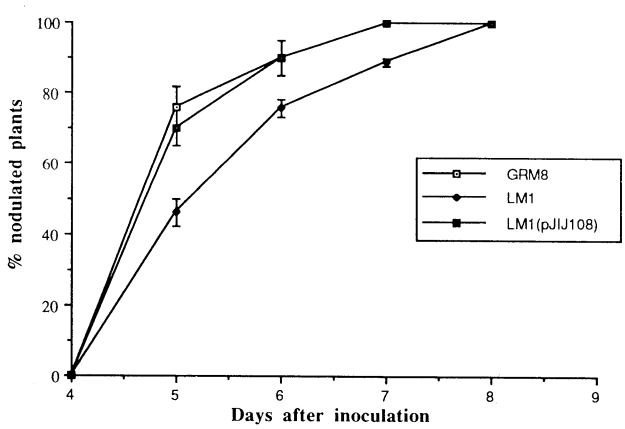

Fig. 4. Nodule formation kinetics of *Rhizobium meliloti* GRM8, its insertion mutant derivative LM1, and the complemented strain LM1(pJIJ108). Each point represents the mean ± standard error (bars) of three independent experiments.

In spite of that symbiotic defect, mutant LM1 was able to form nodules that fix nitrogen at the same rate as the parent strain, as determined by acetylene reduction assay (data not shown). Moreover, no significant differences were found between the dry weight of plants nodulated by the mutant and that of plants infected by the parental strain (data not shown). In addition, structural analysis of nodules did not reveal obvious differences between those formed by GRM8 and those induced by its derivative mutant LM1 (data not shown).

DISCUSSION

In this paper we describe the isolation and characterization of a ProDH$^-$ mutant of *R. meliloti,* obtained by Tn*5* mutagenesis, that is impaired in nodulation efficiency and competitiveness on alfalfa roots. Our results indicate that the disability of mutant LM1 to use ornithine or proline as its sole carbon and nitrogen source resulted from the Tn*5* transposon insertion within a chromosomally located gene that encodes a ProDH enzyme. Because the mutant exhibited no other growth defect and its nodulation ability is recovered to the parental level when the ProDH activity is supplied by genetic complementation, we conclude that its symbiotic defects are a direct result of the loss of this enzyme activity. To our knowledge, this is the first report describing a *R. meliloti* ProDH enzyme mutant.

In prokaryotic cells, catabolism of ornithine is generally dependent on an OAT enzyme that transforms ornithine directly into P5C. However, in a few bacteria, ornithine may be converted into proline with the release of ammonia. We have recently suggested (Soto *et al.* 1994) that in *R. meliloti* the main pathway of ornithine utilization proceeds via proline formation and depends on an OCD activity, which requires NAD^+ and L-arginine. The fact that the ProDH$^-$ mutant LM1 is unable to use ornithine as its sole carbon and nitrogen

Table 2. Nodulation of alfalfa by *Rhizobium meliloti* strain GRM8, mutant derivative LM1, and the complemented strain LM1(pJIJ108)

	Days after inoculation			
Strain	**5**	**6**	**7**	**8**
GRM8	2	3.8	4.4	5.2
LM1	0.77	1.9	2.7	3.5
LM1(pJIJ108)	1.7	3.7	4.6	5.2
LSD 0.05	0.81	1.1	1.2	1.45

[a] The values reported are means of the number of nodules on 24 plants in three independent experiments. On each day, the values for GRM8 and LM1(pJIJ108) were not significantly different, but the values for LM1 were significantly different from those for both of the other strains.

Table 3. Competition studies between *Rhizobium meliloti* GRM8 and derivative strains LM1 and LM1(pJIJ108)

		Percentage of nodules formed by:[a]		
Strain A	**Strain B**	**Strain A**	**Strain B**	**Double occupancy**
GRM8	LM1	92 ± 3	6 ± 2	2 ± 1
GRM8	LM1(pJIJ108)	17 ± 4	73 ± 5	10 ± 3

[a] Nodule occupancy was determined in 25 nodules. Data are means ± standard error of three independent experiments.

source and the results of the enzymatic assays presented in this work strongly support that conclusion. Interestingly, DNA hybridization studies also demonstrated that the *R. meliloti* GRM8 DNA fragment affected by the mutation is conserved not only in *R. meliloti* strains but also in other rhizobia, such as *R. fredii, B. japonicum,* and *A. tumefaciens,* suggesting that in these bacterial species catabolism of proline depends on a ProDH activity.

Different reports have suggested that, at least in soybean root nodules, proline catabolism may have particular importance in the bacteroid metabolism, and one hypothesized role for proline is as a source of reduced carbon for bacteroids, especially in environmentally stressed nodules (Kohl *et al.* 1988, 1991, 1994; Zhu *et al.* 1992). On the other hand, studies of the transport and catabolism of L-proline in free-living cells and bacteroids of cowpea *Rhizobium* NGR234 have shown that in this bacterium proline is metabolized via P5C and glutamate by means of proline oxidase and P5C dehydrogenase (Glenn *et al.* 1991). However, isolated snakebean bacteroids of NGR234 were unable to take up L-proline, and neither proline oxidase activity nor P5C dehydrogenase activity was detected in cell-free extracts of these same bacteroids (Glenn *et al.* 1991). Furthermore, Tn*5*-derived mutants of *R. leguminosarum* bv. *viciae* strain C1204b affected in proline catabolism and biosynthesis were found to be unaffected in their ability to form nodules with wild-type levels of nitrogenase activity (Chien *et al.* 1991). These findings suggest that, at least in these two symbioses, L-proline is not the energy-yielding metabolite supplied by the host to the bacteroid. Our results do not show an obvious defect in the bacteroid metabolism of *R. meliloti* ProDH$^-$ mutant LM1; in comparisons of the dry weight of plants, rates of nitrogen fixation measured by acetylene reduction, and the structure of nodules, nodulation by the mutant did not differ from nodulation by the parental strain. Nevertheless, we cannot rule out that proline may have a physiological effect on nitrogen-fixating metabolism in the *R. meliloti*–alfalfa symbiosis in the open field or under stress conditions, and additional work would be required to elucidate this question.

However, in the present report we show that ProDH activity is required for nodulation efficiency and competitiveness of *R. meliloti* on alfalfa roots. Moreover, the complementing cosmid of mutant LM1 enhances the competitiveness of the parent strain. This positive effect may be due not only to the overproduction of ProDH (Table 1) but also to other genes present in the cosmid. Further studies will be necessary to determine and characterize the exact DNA region that determines this improvement in competitiveness. It has been established that the bacteroid genetic system required for the metabolism of C_4-dicarboxylic acids is necessary for nitrogen fixation in the *Rhizobium*-legume symbiosis (Bolton *et al.* 1986; Finan *et al.* 1983; Ronson *et al.* 1981): dct$^-$ rhizobia were unable to fix nitrogen, but they were still able to form nodules that were invaded by bacteria, and therefore the dicarboxylates are not essential for nodule invasion or bacterial division within infection threads. Other carbon sources may be used by rhizobia during nodule formation. Amino acids and derivative compounds such as betaines released by plants into the soil are also thought to be nutritional mediators, stimulating the growth of certain rhizosphere bacteria carrying the genes for their catabolism. As a consequence, these metabolites may play an important role in *Rhizobium*-legume interactions, during the first steps of the infection process. It has recently been reported (Goldman *et al.* 1994) that symbiotic plasmid genes essential to the catabolism of proline betaine, or stachydrine, are also required for efficient nodulation. In this regard, ProDH would be a key enzyme in the catabolism of different metabolites, including some amino acids and derivative compounds that lead to proline formation and are available during the infection process. We should expect that blocking any step of these degradation pathways would result in an alteration of rhizobial nodulation efficiency and competitiveness.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Bacterial strains and plasmids used and constructed in this study are described in Table 4. *Escherichia coli* was routinely grown at 37° C on Luria-Bertani medium (Sambrook *et al.* 1989), and rhizobial strains at 30°C on TY (Beringer 1974) or defined MM (Robertsen *et al.* 1981). Carbon and nitrogen sources added to the latter medium are specified in each experiment. Antibiotics were added as required, at the following concentrations (in μg/ml): tetracycline (10), chloramphenicol (50), ampicillin (200), kanamycin (50 for *E. coli* and 180 for *Rhizobium* species), and streptomycin (250).

Mutagenesis.

Transposon Tn*5*::*mob* random mutagenesis was performed by mating *E. coli* S17-1 (pSUP5011) with *R. meliloti* GRM8, following previously described procedures (Simon *et al.* 1983). Kanamycin-resistant transconjugants were selected on MM agar with mannitol and glutamate as carbon and nitrogen sources. To identify insertion mutants potentially affected in ornithine utilization, colonies were simultaneously screened on MM agar supplemented with mannitol and glutamate or with ornithine as the sole carbon and nitrogen source.

DNA manipulation and hybridization procedures.

Plasmid and total DNA were isolated by standard procedures (Maniatis *et al.* 1982). Restriction endonucleases and T4 DNA ligase were obtained from Boehringer (Mannheim, Germany) and used as specified by the manufacturer. For DNA-DNA hybridizations, restriction fragments, separated by agarose gel electrophoresis, were transferred to nylon filters by vacuum blotting (Pharmacia LKB) and fixed by baking the filters at 120° C for 30 min. Hybridizations were performed overnight at 42° C in the presence of 50% formamide and used digoxigenin (DIG) random primed labeled probes. Filters were washed under high-stringency conditions, and detection of hybridized DNA was performed with the DIG Luminiscent Detection Kit (Boehringer) following the specifications of the manufacturers.

DNA sequencing.

Nucleotide sequences determined in this work were obtained by the chain termination method (Sanger *et al.* 1977) using *Taq* polymerase (Promega Biotec) and by polymerase chain reaction according to the manufacturer's indications. The primers used were the IS50L-specific primer 5′-AAAGGTTCCGTTCAGGACGC-3′ for plasmid pDIL101 and the

forward sequencing primer of pUC18, 5′-GTAAAACG-ACGGCCAGT-3′, for plasmid pDIL102.1. Restriction fragments were cloned into pUC18, and plasmid DNA for sequencing was isolated and purified with the Magic Minipreps kit (Promega Biotec). Sequence analysis was done with the Genepro version 4.0 (Riverside Scientific Enterprises) GCG software packages (University Research Park, Madison, WI), and the programs FASTA (Pearson and Lipman 1988) and BLASTNCBI (Altschul *et al.* 1990) were used for homology searching.

Ornithine and proline uptake assay.

Cells were grown in MM broth with mannitol and glutamate to exponential phase, pelleted by centrifugation, washed, and finally resuspended to OD_{600} = 0.5 in MM free of carbon and nitrogen sources. L-[1-^{14}C]Ornithine (60 mCi/mmol) or L-[U-^{14}C]proline (261 mCi/mmol), both from Amersham, was added to 180 µl of the cell suspension to a final concentration of 2.5 µM. Aliquots of 50 µl were taken at intervals (5, 10, and 20 min) and centrifuged. Cells were extracted with 20 µl of 30% (w/v) acetic acid for 2 hr at 4° C. To analyze intracellular radiolabeled compounds, 5 µl of each extract was placed on a Whatman 3 MM paper and electrophoresed at 100 V/cm for 7 min in water/acetic/formic acid buffer (91:3:6). After electrophoresis, the papers were dried, and each lane was divided into 1-cm-wide stripes in which radioactivity was measured by liquid scintillation counting.

Enzyme assays.

For the preparation of the enzyme extracts, cells were grown to exponential phase in MM broth (30 ml) with glutamate and mannitol, supplemented with proline or ornithine (0.2%, w/v), as potential inductors of enzyme activities. Cultures were centrifuged at 7,000 × *g* for 10 min at 4° C, and the pellet was suspended in 3 ml of 20 mM potassium phosphate buffer (pH 7). Cells suspensions were sonicated in three cycles of 30-sec bursts at 35 W with a microprobe, and the resulting extracts (with cell debris not removed) were used as a source of enzymes. Previously described methods were used to determine OAT (E.C. 2.6.1.13) (Igeño *et al.* 1993), OCD (E.C. 4.3.1.12) (Soto *et al.* 1994), and ProDH (E.C. 1.5.99.8) (Dessaux *et al.* 1986) activities.

Plant assays.

Alfalfa (*Medicago sativa* L.) plants were grown in a nitrogen-free medium as described by Olivares *et al.* (1980). To test the degree of infectivity of each strain, 24 individual plants were inoculated with 10^6 cells. After inoculation, the number of nodulated plants and the number of nodules per plant were recorded daily. Acetylene reduction was evaluated as indicative of the ability of the plants to fix nitrogen. After tubes containing whole plants were injected with 10% acetylene and incubated for 60 min, ethylene formation was detected with a Perkin-Elmer F33 gas chromatograph. The dry weight of 1-month-old nodulated plants was also determined.

For competition assays, 10-day-old alfalfa plants (three plants per tube) were inoculated with mixtures (10^7 cells) of GRM8 (selected for spontaneous streptomycin resistance) and its Tn*5* derivative strain LM1 (kanamycin-resistant) or the complemented strain LM1(pJIJ108) (kanamycin- and tetracycline-resistant), at a strain ratio of 1:1 in both cases. After 9 days, 25 individual nodules, corresponding to each assay,

Table 4. Strains and plasmids used in this study

	Relevant characteristics	Source or reference
Strains		
Escherichia coli		
S17-1	Modified RP4 plasmid integrated into the genome	Simon *et al.* 1983
DH5α	*recAI, endAI, ϕ80d, lacZ, dm15*	Bethesda Research Laboratory
Rhizobium meliloti		
GR4	Wild-type; Nod$^+$Fix$^+$	Casadesús and Olivares 1979
GRM8	GR4 pRmeGR4a$^-$ and pRmeGR4b$^-$ plasmids cured derivative strain	J. Mercado-Blanco
LM1	GRM8 Tn*5*::*mob* insertion mutant derivative strain	This work
41	Wild type; Nod$^+$Fix$^+$	A. Kondorosi
2011	SU47 derivative strain	J. Denarié
102F34	Wild type; Nod$^+$Fix$^+$	G. Ditta
Rhizobium leguminosarum		
bv. *trifolii* RS1050	Wild type; Nod$^+$Fix$^+$	F. Rodríguez-Quiñones
bv. *viciae* VF39	Wild type; Nod$^+$Fix$^+$; Sm^r	U. Priefer
bv. *phaseoli* 8002	Wild type; Nod$^+$Fix$^+$	A. W. B. Johnston
Rhizobium fredii HH003	Wild type; Nod$^+$Fix$^+$	Dowdle and Bohlool 1985
Agrobacterium tumefaciens A348	Wild type	E. W. Nester
Bradyrhizobium japonicum USDA110	Wild type	Maier *et al.* 1978
Plasmids		
pRK2013	Helper plasmid with replicon *ColE1*, Km^r, *tra*	Figurski and Helinski 1979
pSUP102	pACYC184::*mob*, Tc^r, Cm^r	Simon *et al.* 1986
pSUP5011	pBR325::Tn*5-mob*, Ap^r, Cm^r, Km^r	Simon *et al.* 1983
pUC18	Sequencing plasmid; Ap^r	Yanisch-Perron *et al.* 1985
pDIL100	*Eco*RI fragment of LM1 containing Tn*5* insertion cloned in pUC18	This work
pDIL101	*Bam*HI-*Eco*RI fragment of pDIL100 containing 3.5 kb of the Tn*5* insertion cloned in pUC18	This work
pDIL106	*Eco*RI fragment of pDIL100 cloned in pSUP102	This work
pDIL102.1	2.2-kb *Sal*I/*Eco*RI fragment of pDIL100 cloned in pUC18	This work
pJIJ101, pJIJ103, and pJIJ108	Cosmid clones based on pLAFRI carrying the ProDH gene of *R. meliloti* GR4	This work

were collected, surface-sterilized for 5 min in 0.25% $HgCl_2$, crushed, and sequentially plated on TY agar supplemented with the corresponding antibiotics. The plates were incubated for 3–4 days at 28° C and examined for the identity of strains to determine their percentages of nodule occupancy.

ACKNOWLEDGMENTS

This work was supported by Grant BIO93-0677 from Comisión Asesora de Investigación Científica y Técnica. We are grateful to D. Kohl for providing us with information about a *B. japonicum* ProDH mutant and proline metabolism in soybean stressed nodules prior to publication. We also thank Luis M. Ramal for technical assistance.

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