Identification of a Novel *Rhizobium meliloti* Nodulation Efficiency *nfe* Gene Homolog of *Agrobacterium* Ornithine Cyclodeaminase

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The *nfe* genes located on the large plasmid pRmeGR4b are involved in the nodulation efficiency and competitiveness of *Rhizobium meliloti* GR4 on alfalfa roots. One hundred twenty-eight base-pairs downstream of *nfe2* gene we found an open reading frame designated ORFC, 970 bp long and potentially coding for a 320 amino acid long protein. The amino acid sequence of the putatively encoded ORFC product shows similarity with ornithine cyclodeaminase (OCD) of *Agrobacterium tumefaciens* an unusual enzyme that converts ornithine into proline. The gene product of ORFC was identified as a 37-kDa protein by *in vitro*-coupled transcription-translation and *in vivo* by the T7 RNA polymerase/promoter system. DNA hybridization studies showed that strain GR4 carries a single copy of the *ocd*-like gene. No homologous sequences to GR4 ORFC DNA were found in other *R. meliloti* strains or *Rhizobium* spp. assayed. Furthermore, a GR4 derivative mutant obtained by plasmid disruption of ORFC showed an impaired nodulation efficiency as compared to that of the wild-type strain GR4. Thus, the former locus should be considered a novel *nfe* gene. We propose to rename the *nfe* genes, *nfe1*, *2* and ORFC as *nfeA*, *B*, and *D*, respectively.

Additional keywords: competitiveness, DNA sequence.

Rhizobia native populations present in the soil usually dominate legume-hosts nodulation in agricultural fields in detriment of inoculant strains, a phenomenon known as the nodulation competition problem. Different studies have shown that many factors can influence competitiveness: genetic determinants of nodulation, nitrogen fixation, and polysaccharides or environmental conditions such as temperature, soil pH, and nitrate. (Boonkerd *et al.* 1978; Beattie *et al.* 1989; Kamicker and Brill 1986; Noel and Brill 1980). This multiplicity of factors makes any genetic approach to solving the competitiveness problem a very difficult task. Previous reports from our laboratory showed that a DNA region named *nfe* (nodule formation efficiency) located on the large plasmid pRmeGR4b contains genes involved in nodulation efficiency and competitiveness of *Rhizobium meliloti* GR4 on alfalfa roots (Toro and Olivares 1986; Sanjuan and Olivares 1989). Chun and Stacey (1994) have recently reported in the soybean root nodule bacterium, *Bradyrhizobium japonicum,* a *nfe* gene (*nfeC*) essential for nodulation competitiveness, whose expression is differentially regulated from two promoters. The expression of the *R. meliloti* GR4 *nfe* genes was found to be dependent on the NifA-NtrA regulatory system (Sanjuan and Olivares 1989, 1991). Recently, we reported the nucleotide sequence of a 3,345-bp DNA of the *nfe* region showing that it contains four ORFs, two of them, termed *nfe1* and *nfe2*, are controlled at the transcriptional level by the NifA-NtrA regulatory system (Soto *et al.* 1993). However, *nfe1* gene expression could also occur on a NifA-independent manner, which suggests that the competitive ability of strain GR4 may be triggered before the onset of nitrogen fixation. Two other ORFs of unknown function, called ORFA and ORFB, also were identified upstream of *nfe1* and *nfe2* but transcribed in the opposite orientation (Soto *et al.* 1993). In this report we present the nucleotide sequence and characterization of a 1,216-bp DNA region located between *nfe2* and the left inverted repeat of insertion sequence IS*Rm3* (Soto *et al.* 1992). This region contains a single large open reading frame designated ORFC that potentially codes for a 320 amino acid long protein which shows homology with the *ocd* gene product (ornithine cyclodeaminase) of *Agrobacterium tumefaciens* (Sans *et al.* 1987, 1988; Schindler *et al.* 1989). In addition, it is shown that a GR4 ORFC-derivative mutant obtained by plasmid integration exhibited a pronounced reuction in its nodulation efficiency on alfalfa roots. Thus, the ORFC locus should be considered as a novel *nfe* gene.

This manuscript is dedicated to A. Zorzano who died in a car accident on May 28, 1991.

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RESULTS AND DISCUSSION

DNA sequence of the *R. meliloti* GR4 *ocd*-like gene.

Nucleotide sequence present in this work is deposited in the EMBL/GenBank/DDBJ data libraries with the accession number X64613. Sequence analysis of the 1,216-bp DNA region between *nfe2* and the left inverted repeat of insertion sequence IS*Rm3* revealed a single large open reading frame

(position 130–1089) designated ORFC (Fig. 1). ORFC is preceded by a putative ribosome binding site with strong homology to the consensus binding site of *E. coli* (Shine and Dalgarno 1974). This ORF is 970 bp long and potentially codes for a 320 amino acid long protein. ORFC was located 128 bp downstream of the competitiveness *nfe2* gene and 128 bp upstream of the unique copy of insertion sequence IS*Rm3* (Fig. 1) harbored by strain GR4 (Soto *et al.* 1992). Sequence analysis of the 5′-region reveals a potential promoter; the –35 and –10 sequences, with an optimal 17-bp spacing, showing the 4/6 bp (TTCAGA/TTGACA) and the 3/6 bp (TTAAAC/TATAAT) match, respectively, with the promoter consensus sequences of *E. coli*. Comparison of the DNA sequence of ORFC to sequences in the GenBank and EMBL databases did not show any significant similarities. On the other hand, when we searched the Swissprot and PIR protein databases for homology to the putative ORFC encoded product, we found homology with the *ocd* gene product (ornithine cyclodeaminase) of *A. tumefaciens*, a 26% identity in 265 amino acid-overlap (Fig. 2). It also shows homology with mammalian μ-crystallin (24% identity), a homolog of *A. tumefaciens* OCDs (Kim *et al.* 1992).

ocd-related sequences in *Rhizobium*.

We first investigated whether *R. meliloti* GR4 carries more than one copy of the *ocd*-like gene. Southern blot hybridization of *Eco*RI-digested total DNA from wild-type strain GR4 and derivative strains cured of plasmid pRmeGR4a, pRmeGR4b, or both: GRO13, GRM6, and GRM8, respectively (Sanjuan and Olivares 1989; Mercado-Blanco *et al.* 1993), were hybridized with a 452-bp *Pst*I-*Sma*I restriction fragment internal to the GR4 *ocd*-like gene. Only one hybridization signal was detected in all those strains harboring plasmid pRmeGR4b (GR4 and GRO13), which suggests that the *ocd* -like gene was present as a single copy in the GR4 genome. Similar studies performed with total DNA from different *R. meliloti* strains (L5-30, 102F34, 41, 2011); *R. leguminosarum* bv. *trifolii* strain RS1051, bv. *phaseoli* strain 8002, bv. *viciae* strain VF39; *Rhizobium* sp. *Acacia* strain GRH2, and *A. tumefaciens* strain A348 did not show homologous sequences to the *ocd* -like gene of strain GR4 (data not shown).

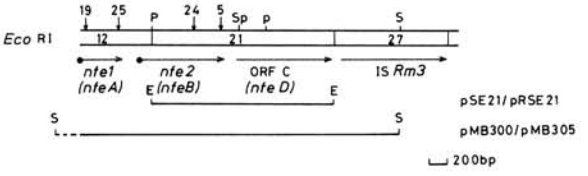

Fig 1. Physical map of the *nfe* region. The vertical arrows indicate the position of NFE⁻ Tn*3*HoHo1 insertions (Sanjuan and Olivares 1989; Soto et al. 1993). Location of insertion 5 is according to this work. ORFs identified in the *nfe* region (Soto *et al.* 1993) are indicated below the physical map. ORFC and the putative transposase of IS*Rm3* (Soto *et al.* 1992) are also shown. Closed circles correspond to *nfe nif*-like promoters. Cloned fragments used as primary sources for DNA sequencing and protein synthesis are shown below the coding regions. *Pst*I (P), *Spe*I (Sp), and *Sal*I (S).

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GR4     2 IAPVRWLSRKDVALVNLPFASALNIIEVTLRDHGN......GAFENPPKI 45
Ach5    7 LNIVPFISVEN..MMDLAVSTGIENFLVQLAGYIEEDFRRWESFDKIPRI 54

GR4    46 GIHPRHDALIHAMPGWLPTQRRAGLKWIATYSSNRSVGLPSITGLLVLND 95
Ach5   55 ASHSR.DGVIELMPTSDGTL..YGFKYVNGHPKNTKSGRQTVTAFGVLSD 101

GR4    96 PDTGLPVCVMDAAYLTAVRTAAASAVTSKYLSPSHVRKIAVIGAGIQGLY 145
Ach5  102 VDSGYPLLLSEMTILTALRTAATSAIAAKYLARKDSRTMALIGNGAQSEF 151

GR4   146 HVEMLSLVHPAAEFHIVDIDDDA.VRLLAQMVRSKARIVPVKEAEIAIRT 194
Ach5  152 QALAFKALIGVDRIRLYDIDPEATARCSRNLQRFGFQIEACTSAEQAVEG 201

GR4   195 ADVVVTATSQLEEVAFQFSWVKEGSLVLPVHPRGWSEDITTASEVLLADD 244
Ach5  202 ADIITTATADEHNATI....LSDNMIGPGVHINGVGGDCPGKTE..MHRD 245

GR4   245 VAQFASYIMALGSPYRDISRV........LGSVSDVITGQVTGRANDSDR 286
Ach5  246 ILLRSDIFVEFPPQTRIEGEIQQLAPDHPVTELWRVMTGQDVGRKSDKQI 295

GR4   287 IAVFNLGIAVHDVAIGSAIFDIAEQLGLGTIVSY 320
Ach5  296 TIFDSVGFAIEDFSALRYVRDRVEGSSHSSPLDL 329
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Fig. 2. Amino acid sequence comparison of *Rhizobium meliloti* GR4 ORFC-encoded product and OCD from *Agrobacterium tumefaciens* pTiAch5.

Identification of GR4 ORFC-coded product and OCD activity.

The ORFC-encoded product was identified by coupled *in vitro* transcription-translation of either plasmid pSE21/pRSE21 (Fig. 1) or derived *Pvu*II linear fragments that release the insert from the *lacZ* promoter. SDS-PAGE of the *in vitro* synthesized products revealed an insert-specific polypeptide of 37 kDa (Fig. 3A). The 37-kDa protein appeared with either orientation of the insert relative to the *lacZ* promoter, suggesting that ORFC carries its own functional promoter. In *E. coli* the existence of the ORFC encoded product was also demonstrated by *in vivo* synthesis with the coupled T7 polymerase/promoter system. The 37-kDa protein was detected with plasmid clone pMB305 which contains the *nfe* and ORFC DNA sequences (Fig. 3B).

Strain GR4 shows an OCD activity which is not dependent on the ORFC gene product (Soto *et al.* 1994). Therefore, ornithine cyclodeaminase enzyme assays were performed on extracts of *E. coli* cells expressing the ORFC-coded product.

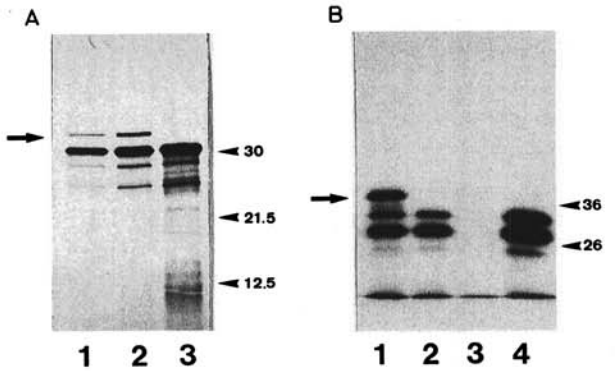

Fig. 3. SDS-polyacrylamide gel of *Rhizobium meliloti* ORFC-coded product synthesized *in vitro* and *in vivo*. **A,** *In vitro* synthesis with an *E. coli* S-30 linear system (Promega Biotech) using *Pvu*II restriction fragments of plasmids pSE21 (lane 1); pRSE21 (lane 2), and pUC18 (lane 3). **B,** *In vivo* synthesis using the coupled T7 RNA polymerase/T7 promoter system performed as described by Tabor and Richardson (1985). *E. coli* K38 cells containing plasmids pGp1-2/pMB305 (lane 1), pGp1-2/pMB300 (lane 2), pGP1-2 (lane 3), and pGp1-2/pT7-3 (lane 4). Relative molecular masses (kDa) of protein standard are shown at the right of the panels. Insert-specific polypeptides are indicated by arrows. Protein samples obtained by either *in vitro* or *in vivo* synthesis were electrophoresed on 12% polyacrylamide gels according to Laemmli (1975). Gels were fluorographs with Amplify (Amersham), dried, and exposed to autoradiography.

No activity was observed under optimal conditions for *Agrobacterium* OCDs. Similarly, Kim *et al.* (1992) described that although mammalian μ-crystallin shows a 31–33% identity with *Agrobacterium* OCDs, no enzymatic activity could be detected. Both proteins lack the C-terminal extension of the *Agrobacterium* OCDs which may have an effect on activity or specificity. Another possibility is they require different conditions for activity. For example, OCD from *Clostridium sporogenes*, an anaerobic bacterium, is highly sensitive to O_2 (Muth and Costillow 1974), while the Ti plasmid-coded enzymes are not. Nevertheless, we cannot rule out that the ORFC coded product is an OCD-related but funcionally different enzyme.

R. meliloti GR4 ocd-like gene is required for efficient nodulation.

The rightward reported mutation (transposon insertion 5) affecting the nodulation efficiency of GR4 (Sanjuan and Olivares 1989) is located at the left site of the unique *Spe*I site (internal to ORFC) within the *Eco*RI fragment 21 (Fig. 1). By using the 24-mer oligonucleotide, 5′-AGGCAGAA-AACGTTGCTTAACGTG-3′ specific for the Tn*3*HoHo1 transposon (Stachel *et al.* 1985), we found that insertion 5 was outside of the coding region of ORFC at co-ordinates position 3127 and 3128 (Soto *et al.* 1993) in the coding region of *nfe2* gene (Fig. 1). Therefore, to test whether the GR4 *ocd*-like gene was in fact a novel *nfe* gene, ORFC was mutated by plasmid integration with pSUP202 containing the ORFC internal 452-bp *Pst*I-*Sma*I fragment (Fig. 4A). The expected gene disruption achieved in the mutant, named GR2034C (Fig. 4A), was confirmed by DNA hybridization using the pSUP202 vector and the former ORFC internal restriction fragment as probes. When GR2034C mutant strain was tested on alfalfa plants, it exhibited a less efficient nodulation phenotype (delay nodulation and lower amount of nodules) to that of the wild-type strain GR4 (Fig. 4B and C), and similar to the GR4 cured derivative strain GRM8 (lacking plasmids pRmeGR4a and pRmeGR4b) and to the previously reported Nfe⁻ mutants (Sanjuan and Olivares 1989). By introduction of plasmid clones containing ORFC (pFGR100 and 101) into mutant GR2034C the wild-type nodulation efficiency was recovered, demonstrating that this phenotype was specific to the ORFC mutation. Our data indicate that the ORFC locus is involved in the nodulation efficiency of strain GR4 and therefore it should be considered as the third *nfe* gene indentified so far. We propose to rename the *nfe* genes, *nfe1*, *2* and ORFC as *nfeA*, *B* and *D*, respectively.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The strains and plasmids used and constructed in this work are listed in Tables 1 and 2, respectively. *E. coli* strains were

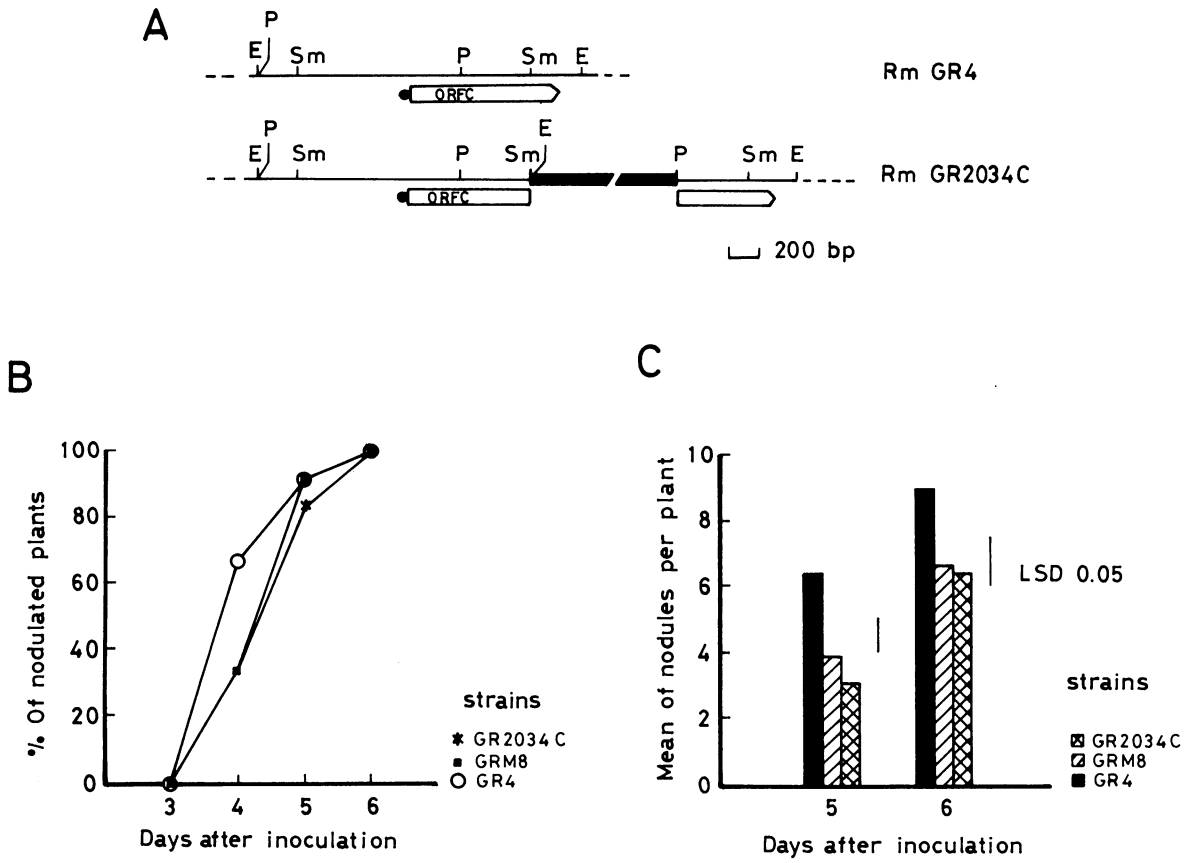

Fig. 4. Effect of ORFC in the nodulation efficiency of strain GR4. **A,** Plasmid integration into the GR4 ORFC DNA. Coding region of ORFC is indicated by arrows. pSUP202 vector sequences are shown by a solid bar. *Eco*RI (E), *Pst*I (P), and *Sma*I (Sm). Kinetics of nodule formation of wild type *Rhizobium meliloti* GR4 and its derivatives mutated in ORFC (GR2034C), as well as a derivative cured of plasmids pRmeGR4a and pRmeGR4b (GRM8). **B,** percentage of nodulated plants. **C,** mean of nodules per plant.

grown at 37° C in LB medium (Miller 1972). *Rhizobium* strains were cultured at 30º C in TY medium (Beringer 1974). All plasmids for sequencing were propagated in *Escherichia coli* DH5α (Bethesda Research Laboratories). Expression of ORFC using the coupled T7 RNA polymerase/T7 promoter was conducted in *E. coli* K38 (Russel and Model 1988).

DNA manipulation.

All restriction enzymes and DNA-modifying enzymes were from commercial sources and used according to the manufacturer's instructions. Standard procedures such as isolation and purification of plasmids, DNA fragments, electrophoresis on agarose gels, transformation of plasmid DNA, and Southern hybridization were carried out as described (Maniatis *et al.* 1982).

DNA sequencing.

The nucleotide sequence presented in this work was determined for both strands by the chain termination method (Sanger *et al.* 1977), using Sequenase version 2.0 T7 DNA polymerase (United States Biochemical, Cleveland, OH) according to the manufacturer's indications. For labeling, 2 μg of denatured double-stranded plasmid DNA and 5 μCi of [α-^{35}S] dATP (Amersham) were used. Templates were generated by progressive unidirectional deletions of restriction fragments (Fig.1) cloned in pUC18 using the Erase-a-Base system (Promega Biotech.). Plasmid DNA for sequencing was isolated and purified using the Magic Minipreps kit (Promega Biotech.). Sequencing reactions were run on a 6% sequencing gels.

Sequence analysis.

Sequence analysis and homologies searches were done with the Genepro version 4.0 (Riverside Scientific Enterprises) and the GCG (University Research Park, Madison, WI) software packages. The program FASTA (Pearson and Lipman 1988) and BESTFIT from the University of Wisconsin were used.

Protein analysis.

In vitro protein synthesis was performed with an *E. coli* S-30 linear system (Promega Biotech). Reactions were routinely conducted in a 50-μl volume with 2 μg of plasmid DNA or *Pvu*II linear fragment of pUC18 clones containing ORFC. *In vivo* protein synthesis was performed using the coupled T7 polymerase/promoter system as described (Tabor and Richardson 1985) using plasmid pT7-3 as expression vector. Protein samples obtained by either *in vitro* or *in vivo* synthesis were electrophoresed on a 12% polyacrylamide gel (Laemmli 1970). Gels were fluorographed with Amplify (Amersham).

Table 1. Bacterial strains used in this work

Strain designation	Characteristics	Source
R. meliloti		
GR4	Wild-type strain	This laboratory
GR013	GR4 derivative, lacks plasmid pRmeGR4a, exhibiting similar competitive ability to that of wild type GR4	This laboratory
GRM6 and GRM8	GR4 derivative strains obtained by heat treatment; lack plasmids pRmeGR4b and pRmeGR4a/pRmeGR4b, respectively	J. Mercado
GR2034C	GRO13 derivative obtained by cointegration into ORFC of plasmid pSMT4	This study
L5.30	Wild-type strain	J. Denarié
41	Wild type strain	A. Kondorosi
102F34	Wild-type strain	G. Ditta
2011	Wild-type strain, Sm^R	J. Denarié
R. leguminosarum		
VF39	Wild-type strain bv. *viceae* Sm^R	U. Priefer
RS1050	Wild-type strain bv *trifolii*	F. Rodriguez-Quiñones
8002	Wild-type strain bv. *phaseoli*	A. W. B. Johnston
Rhizobium spp.		
GRH2	Wild-type strain isolated from *Acacia* nodules	M. A. Herrera
A. tumefaciens		
A348	A136 containing plasmid pTiA6NC	E. W. Nester

Table 2. Plasmids used and constructed in this work

Plasmids designation	Characteristics	Source
pSE21/pRSE21	pUC18 Ap *Eco*RI fragment 21 from pRmeGR4b cloned in both orientations	J. Sanjuan
pT7-3	T7 promoter-expression vector	Tabor and Richardson
pGp1-2	T7 polymerase vector	Tabor and Richardson
pMB305/300	pT7-3 clones containing the 4.9-kb *Sal*I fragment from pRmeGR4b in both orientations	Soto *et al.* 1993
pSMT4	pSUP202 containing the GR4 ORFC internal *Pst*I-*Sma*I fragment of 452 bp	This study
pJB3KmD	RK2 derivative of 6,052 bp, Km^r, Ap^r.	Janet Martha Blatny (UNIGEN)
pFGR100/pFGR101	pJB3KmD derivative containing *Eco*RI 21 fragment from pRmeGR4b cloned in both orientations	This study

OCD incubations.

Enzyme extracts were prepared as described (Sans *et al.* 1987) from *E. coli* cells harboring plasmids pSE21 or pGp1-2/pMB305. Routine assays contain in a final volume of 20 µl: 55 µg of protein, 3 mM EDTA, 0.5 mM NAD^+, 0.1 mM L-arginine, 0.1 mM unlabeled L-ornithine, 20 µM L[1-^{14}C]ornithine (2 GBq/mmol, Amersham), 20 mM potassium phosphate, pH 8.5, and were incubated at 30º C for 30 min. Reactions were stopped by mixing with 5 µl of 50% trichloroacetic acid. After removal of precipitates by centrifugation, supernatants were extracted with 50 µl of chloroform to remove lipids. The evaluation of OCD activity was performed by thin-layer chromatography and high-voltage paper electrophoresis (Dessaux *et al.* 1986, Sans *et al.* 1988).

Plant nodulation assay.

Alfalfa plants were grown on nitrogen-free medium (Rigaud and Puppo 1975) and inoculated as described previously (Olivares *et al.* 1980). Twenty-five individual plants were inoculated with each bacterial strain (the experiment was performed three times). The nodule formation on alfalfa roots was followed until 100% of the plants were nodulated.

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