ELSEVIER

FEMS Microbiology Ecology 30 (1999) 87–97

FEMS MICROBIOLOGY ECOLOGY

At least five rhizobial species nodulate *Phaseolus vulgaris* in a Spanish soil

José A. Herrera-Cervera [a], Jesús Caballero-Mellado [b], Gisèle Laguerre [c], Hans-Volker Tichy [d], Natalia Requena [a], Noëlle Amarger [c], Esperanza Martínez-Romero [b], José Olivares [a], Juan Sanjuan [a,*]

[a] *Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, E-18008 Granada, Spain*

[b] *Programa de Ecologia Molecular e Microbiana, Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Cuernavaca, AP 565-A Morelos, Mexico*

[c] *Laboratoire de Microbiologie des Sols, Centre de Microbiologie du Sol et de l'Environnement, INRA, 21034 Dijon, Cedex, France*

[d] *TÜV Energie und Systemtechnik GmbH, Biologische Sicherheit, Robert-Bunsen Strasse 1, D-79108 Freiburg, Germany*

Received 22 February 1999; revised 22 May 1999; accepted 22 May 1999

Abstract

The genetic relationships among bacteria nodulating *Phaseolus vulgaris* in a soil of Granada, Spain, were investigated by multilocus enzyme electrophoresis, restriction fragment length polymorphism and partial sequencing of the 16S rRNA genes and restriction fragment length polymorphisms of symbiotic genes. Multilocus enzyme electrophoresis analysis of 39 isolates determined 11 different electrophoretic types, clustered into three main genetic groups. Genetic distances between groups were above 0.8. Five different 16S rRNA gene alleles were identified in this population, corresponding to previously described rhizobial species, *Rhizobium etli, Sinorhizobium fredii, Rhizobium gallicum, Rhizobium giardinii* and *Rhizobium leguminosarum*. Using *R. etli* strain CFN42 *nifH* and *nodB* genes as hybridization probes, identical restriction fragment length polymorphism profiles were found among isolates belonging to four different 16S rRNA gene species, indicative of interspecific gene transfer. Most of the bean-nodulating strains carried three copies of *nifH* and strongly hybridized to the *nodB* gene of *R. etli* CFN42, suggesting that their symbiotic plasmid genes are organized similarly. Combined data from multilocus enzyme electrophoresis and 16S allele characterization indicate that more than five bacterial species compose this rhizobial population, as almost identical 16S rDNA alleles were identified in isolates belonging to deeply divergent multilocus enzyme electrophoresis lineages.

Keywords: Symbiosis; Common bean; *Rhizobium*; *Sinorhizobium*

1. Introduction

Bacteria which are able to nodulate on the roots of *Phaseolus vulgaris* L. (common bean) are genetically diverse. Based on the levels of 16S ribosomal DNA gene similarity, DNA-DNA relatedness, symbiotic gene organization and host range, five species have clearly been established [1–4]. Two of these species, *Rhizobium leguminosarum* biovar (bv.) *phaseoli* and

* Corresponding author. Tel.: +34 (958) 121011; Fax: +34 (958) 129600; E-mail: jsanjuan@eez.csic.es

0168-6496/99/$20.00
PII: S0168-6496(99)00044-6

Rhizobium tropici have been found in European and American soils, whereas isolates belonging to a third species, *Rhizobium etli*, are predominant in soils of Mexico, other Mesoamerican countries [3,5–7] and in Argentinian soils [8]. French soils contain two other bean-nodulating *Rhizobium* species, referred to as *Rhizobium giardinii* and *Rhizobium gallicum* [1,9,10]. Recently, Sessitsch et al. [11,12] have also reported the presence of *R. etli* and *R. gallicum* in an Austrian soil. In addition, a number of isolates assigned to different rhizobial species, as well as strains of an uncertain taxonomic position, have been shown to have the capacity to nodulate certain *P. vulgaris* genotypes [4,13–17].

Except for *R. tropici*, a bv. named phaseoli has been described for the other four species, mainly based on the presence of multiple copies of the nitrogenase reductase gene *nifH* in the symbiotic plasmid. Thus, bv. phaseoli strains of *R. etli, R. leguminosarum, R. gallicum* and *R. giardinii* usually show similar *nifH* restriction fragment length polymorphism (RFLP) profiles, which strongly supports the idea of interspecific sym plasmid exchange among these species. Although bv. phaseoli strains were reported to have a narrow host range, almost limited to *P. vulgaris* hosts [1,18], it seems that at least some of these strains possess the ability to interact with a larger set of legume species [15].

In this work, we have characterized a bacterial population nodulating *P. vulgaris* in a soil of Granada, Spain. Both multilocus enzyme electrophoresis (MLEE) and 16S rRNA gene characterization showed a great genetic diversity, although phylogenies deduced from both types of analysis were conflicting in some cases.

2. Materials and methods

2.1. Bacterial isolation

P. vulgaris cv. Contender seeds were surface-sterilized by treatment with a commercial sodium hypochlorite solution for 7 min, washed with sterile water and germinated on Petri dishes containing wet filter paper for 48 h at 28°C in the dark. Seedlings were planted in pots containing a loamy soil from the fields at the Estación Experimental del Zaidin in Granada, Spain (43% sand, 41% lime, 16% clay, pH 7.8) and cultivated in a greenhouse. After 4 weeks, about 1000 nodules were collected from the roots of 15 plants, surface-sterilized for 5 min in 0.25% $HgCl_2$ and thoroughly washed with sterile water. 100 randomly chosen nodules were individually crushed and streaked on TY agar plates (Tryptone 5 g l^{-1}, yeast extract 3 g l^{-1}, $CaCl_2$ 0.9 g l^{-1}). Bacterial growth was evident after 3–4 days incubation at 28°C. Out of 40 randomly chosen isolates, 39 were confirmed for nodulation of common beans and used for further studies.

2.2. MLEE

Isolates were grown on an orbital shaker (220 rpm) for 24 h at 29°C in 50 ml PY liquid medium (peptone 5 g l^{-1}, yeast extract 3 g l^{-1}, $CaCl_2$ 0.1 g l^{-1}). Cells were harvested by centrifugation at $12\,300 \times g$ for 10 min at 4°C, suspended in 0.4 ml of 10 mM $MgSO_4 \cdot 7H_2O$ containing 300 g lysozyme and incubated for 10 min at room temperature. Each suspension was frozen at −70°C for 15 min and thawed. After this process was repeated, lysates were stored at −70°C. The procedures used for starch gel electrophoresis and selective staining of enzymes have been described previously [19]. The following nine metabolic enzymes were assayed: malate dehydrogenase (MDH), malic enzyme (ME), isocitrate dehydrogenase (IDH), phosphoglucose isomerase (PGI), glucose-6-phosphate dehydrogenase (G6P), hexokinase (HEX), phosphoglucomutase (PGM), indophenol oxidase (IPO) and aconitase (ACO). The buffer system used was Tris-citrate, pH 8.0. The electrophoretic mobility of each enzyme was determined and distinctive combinations of alleles for the nine enzyme loci (multilocus genotypes) were designated different electrophoretic types (ETs). The level of genetic diversity for each enzyme locus was calculated as described previously [19].

2.3. Analysis of 16S rRNA genes

Restriction fragment analysis of PCR-amplified 16S rDNA was performed basically as described previously by Laguerre et al. [20]. The following enzymes were used: *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Msp*I, *Nde*II and *Rsa*I. Restriction patterns were

compared with those of known *Rhizobium* species. For sequencing of PCR-amplified fragments, bacteria were suspended in 20 μl lysis buffer (0.05 M NaOH, 0.25% SDS) and boiled for 15 min. The lysate was diluted in 200 μl distilled sterile water and spun in a microcentrifuge for 5 min. 2 μl of the supernatant was used directly for PCR amplification using 10 pmol of the primers 41f (5′-GCTCAGATTGAACGCTGGCG-3′) and 1488r (5′-CGGTTACCTTGTTACGACTTCACC-3′), 200 μM dNTPs and 2 U *Taq* polymerase (Boehringer) in a total reaction volume of 50 μl. Primers were designed according to the corresponding sequence positions in the *Escherichia coli* 16S rDNA. Amplifications were carried out in a Perkin-Elmer thermocycler model 2400 with the following temperature profile: 2 min at 94°C, 10 cycles of denaturation (40 s at 94°C), annealing (1 min at 60°C, lowering 1°C each cycle) and extension (2 min at 72°C), 25 cycles of denaturation (40 s at 94°C), annealing (1 min at 50°C) and extension (2 min at 72°C) and a final extension for 3 min at 72°C (H.-V. Tichy, unpublished). PCR fragments were purified from the excess of primers by using MicroSpin™ S-300 HR columns (Pharmacia), according to the manufacturer's instructions, and sequenced with a Perkin-Elmer ABI Prism 373 automated sequencer using the 41f primer. In some cases, primer 358f (5′-AGACTCCTACGGGAGGCAGCAGT-3′) was used to sequence the central portion of the amplified 16S rRNA genes. The sequences described in this work are available from EMBL Nucleotide Sequence Database under accession numbers AJ238925 through AJ238930.

2.4. RFLP analysis of symbiotic genes

Genomic DNA of individual isolates was digested with restriction enzymes (*Eco*RI or *Bam*HI) and subjected to agarose gel electrophoresis. DNA was transferred to positively charged nylon membranes by the method of Southern [21]. The probes used were plasmid p15b, which contains a 0.3-kb *Sal*I internal fragment of the *nifH* gene of *R. etli* CFN42 cloned into pBR329, and plasmid p42d-7 containing a 4.1-kb *Bam*HI fragment with the *nodB* gene of strain CFN42 [9]. DNA hybridization with non-radioactive probes labelled with digoxigenin-dUTP and detection were performed according to the manufacturer's instructions (Boehringer, Mannheim, Germany). Hybridizations and membrane washes were carried out under high stringency conditions. Membranes were prepared for chemiluminescent detection (Boehringer, Mannheim, Germany) and exposed to Kodak X-Omat film.

3. Results

3.1. MLEE and genetic diversity

We examined 39 isolates nodulating common bean and all nine enzyme loci assayed were found to be polymorphic. The mean number of alleles was 4.56, ranging from three to six alleles per locus (Table 1). A total of 11 distinctive ETs were identified (data not shown, allele profiles will be provided upon request), among which the mean genetic diversity per locus (H) was 0.780 (Table 1). Almost half of the isolates (19 of 39, 49%) showed an identical multilocus genotype, represented by ET1. Other ETs were represented by four, five or one or two isolates (Table 2). Cluster analysis placed the 11 ETs into three main genetic groups (Fig. 1). Genetic distances between these groups were greater than 0.80. Group 1 (ETs 1, 2, 7, 11) included two subgroups, with ET2 diverging from the other three ETs at a distance of 0.58. Group 2 included the closely related ETs 3, 5 and 6. Group 3 included the single-strain ETs 8 and 4, ET10 represented by two isolates and ET12

Table 1
Genetic diversity at nine enzyme loci among electrophoretic types of 39 Spanish isolates nodulating common bean

Enzyme locus[a]	Number of alleles	Genetic diversity (H)[b]
MDH	4	0.800
EM	5	0.836
IDH	5	0.782
PGI	5	0.836
G6P	6	0.891
HEX	4	0.782
PGM	6	0.836
IPO	3	0.691
ACO	3	0.564
Mean	4.56	$H = 0.780$

[a]The enzymes assayed are listed in Section 2.
[b]$H = (1-\Sigma x_i^2)(n/(n-1))$, where x_i is the frequency of the ith allele and n is the number of ETs.

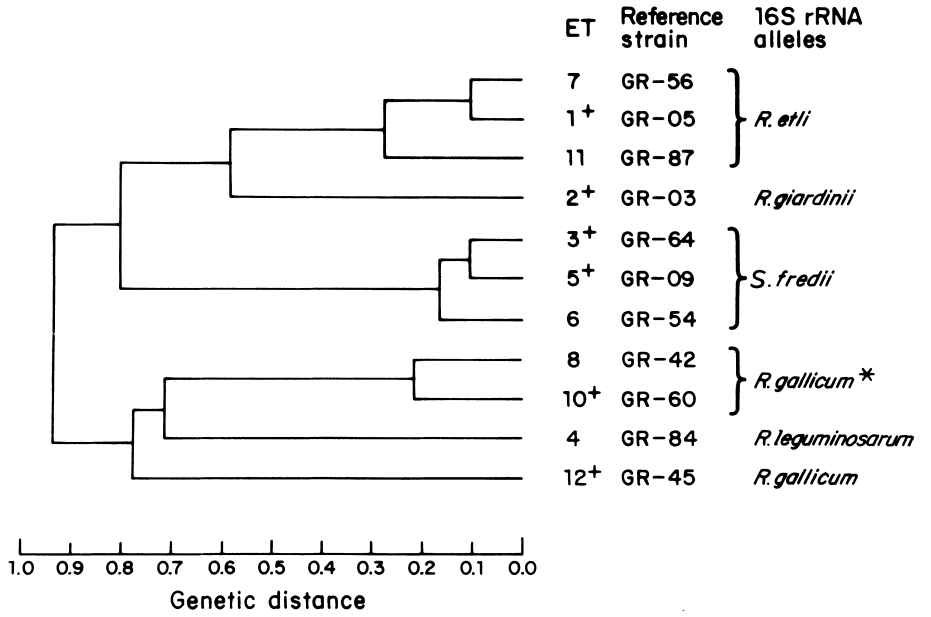

Fig. 1. Genetic relationships of ETs identified among bean-nodulating rhizobia strains in a soil of Granada. A + after the ET number indicates that the ET represents two or more isolates. Only reference strains for each ET are indicated (see Table 2). The 16S rDNA species assignment is indicated for clarity purposes. *R. gallicum** indicates a 16S rDNA allele differing from *R. gallicum* by one *Msp*I site at position 951.

including five isolates. Whereas the genetic distance between ET8 and ET10 was only 0.2, indicating that these strains were closely related, distances between these two ETs and ET4 and ET12 were 0.72 and 0.78, respectively. Thus, MLEE analysis indicated a great diversity amongst the strains isolated from bean nodules.

To determine the relationships between the Spanish bean symbionts and other strains world-wide, a comparative MLEE study was performed between these isolates and 23 bacterial strains, of which some were previously characterized by Piñero et al. (strains CFN42, F-6, Bra-5, Viking-1, CFN1, FL27, Nitragin 8251 [22]) and others were Mexican isolates of the collection of the CIFN (Cuernavaca, Mexico), isolated from different bean cultivars [23]. As shown in Fig. 2, most Spanish isolates formed unrelated clusters with little or no relationship to the non-Spanish strains. However, the isolates from ET1, ET7 and ET11, representing the larger subgroup of Spanish isolates, clustered along with strain CFN1 and the Mexican isolate FNJ11. The most distantly related ET from group 1 (ET2) was also clustered with these strains in this new analysis. In contrast, ET12 grouped together, albeit at a distance of 0.55, with the Mexican strain FL27, which has been classified as *R. gallicum* [12]. The other representatives of the Spanish population (ETs 8 and 10 and ETs 3,

Table 2
List of strains included within each electrophoretic type identified among isolates from common bean nodules

Electrophoretype[a]	Isolates (GR)[b]
1	**05**, 10, 12, 14, 20, 24, 29, 35,38, 57, 62, 69, 73, 75, 81, 82, 90, 91, 92
2	**03**, 93
3	**64**, 99
4	**84**
5	06, **09**, 21, 28
6	**54**
7	**56**
8	**42**
10	18, **60**
11	**87**
12	15, **45**, 46, 55, 89

GR stands for Granada, the location of the sample soil.
[a]Electrophoretic types shown in Fig. 1.
[b]Bold face numbers indicate isolates representing each ET in Fig. 1.

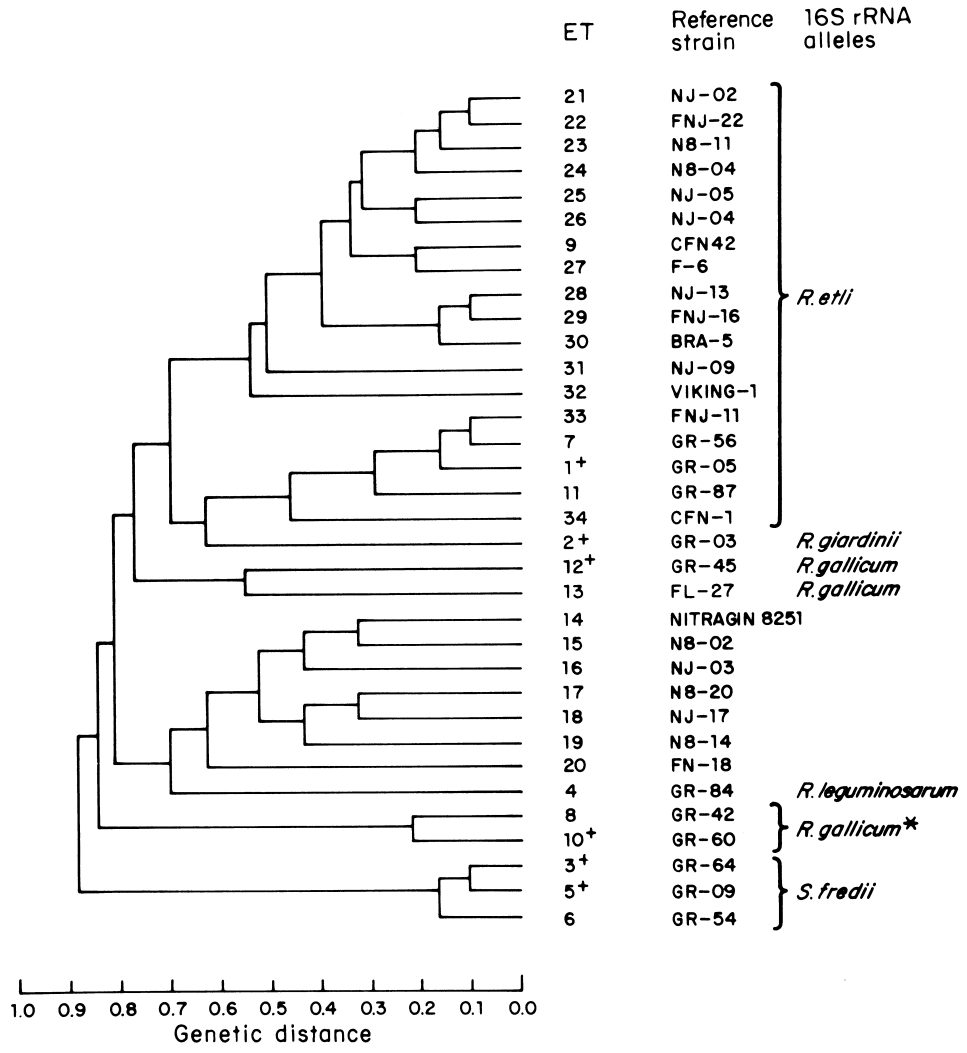

Fig. 2. Genetic relationships of ETs identified among bean-nodulating *Rhizobia* from soils of Spain and Mexico. ET reference strains labelled GR- correspond to Spanish isolates. Strains CFN42, F-6, Bra-5, Viking-1, CFN-1, FL-27 and Nitragin 8251 were previously characterized ([22]). The remaining strains are isolates from different bean cultivars ([23]). When known, the 16S rDNA species names are indicated. *R. gallicum** refers to a 16S rDNA differing from *R. gallicum* by one *Msp*I site at position 951.

5 and 6) were clustered at a large distance from each other and from any other group of strains.

3.2. Analysis of amplified 16S rRNA genes

A preliminary restriction fragment analysis of amplified 16S rDNA with enzymes *Dde*I, *Hin*fI and *Hae*III showed that isolates from ETs 1, 7, 8, 10, 11 and 12 were related to *R. etli*/*R. gallicum*, those from ETs 3, 5 and 6 were related to *Sinorhizobium meliloti*/*S. fredii* and isolates from ETs 2 and 4 were related to *R. leguminosarum* or *R. giardinii*. A detailed RFLP analysis of 16S rDNA with five more restriction enzymes was carried out with one or two isolates from each electrophoretic type, followed by partial sequencing of the 5′-end of the amplified 16S rDNA. A sequence segment of at least 400 nucleotides (nt) was obtained for each isolate and compared with the corresponding 16S rDNA sequences available in the databases. The results, shown in Ta-

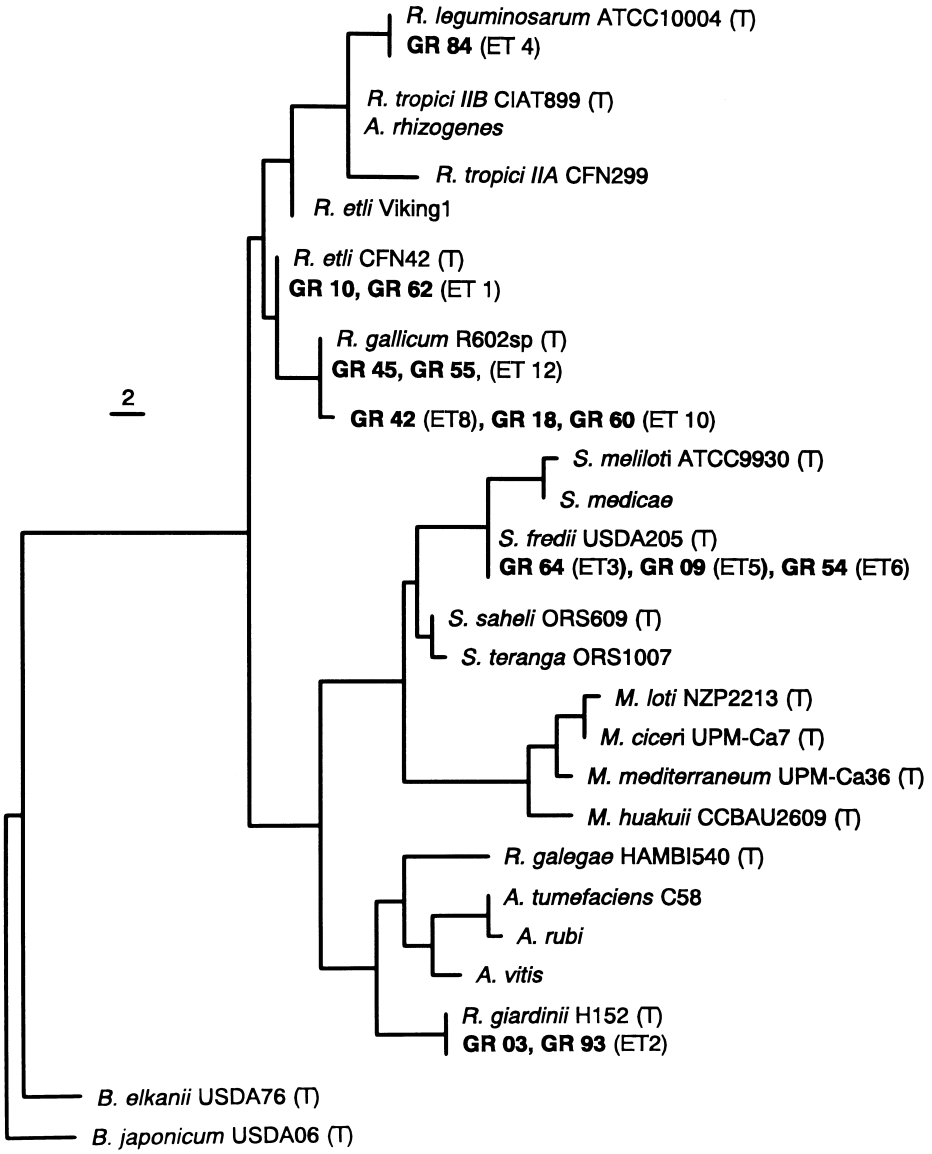

Fig. 3. Phylogenetic tree constructed by Dollo parsimony analysis (PAUP computer programme) from a databank of mapped restriction sites in 16S rDNA genes within the *Rhizobiaceae* ([24]). The horizontal branches are drawn proportionally to the number of restriction site changes. Bold face numbers indicate isolates representing the different electrophoretic types of Spanish isolates (ET numbers between parentheses). (T) indicates the species type strain.

ble 3, determined the existence of five 16S alleles in this population, corresponding to previously recognized rhizobial species. Isolates from ETs 1, 7 and 11 had 16S rDNA RFLPs identical to the *R. etli* type strain CFN42. A segment of more than 600 nt from isolates GR-10 and GR-62 was sequenced and aligned with the sequence of the 16S rRNA gene of strain CFN42 (GenBank accession number U28916). Whereas both isolates' 16S rDNA sequences were identical, a mismatch was found with respect to the *R. etli* type strain, exactly at position 322 of the CFN42 sequence (C to G change), which would actually represent a very small 16S heterogeneity.

Isolates from ET2 (strains GR-03 and GR-93)

Table 3
16S rDNA alleles identified and presence of three copies of the *nifH* gene amongst Spanish isolates nodulating common beans

Allele 16S rDNA	ETs[a]	Number strains[b]	Three copies of *nifH*[c]
R. etli	1, 7, 11	21	+(21)
S. fredii	3, 5, 6	7	–
R. gallicum	12	5	–
R. gallicum[d]	8, 10	3	+(3)
R. giardinii	2	2	+(1)
R. leguminosarum	4	1	+(1)

[a]Presence of the corresponding 16S rDNA allele among MLEE electrophoretic types.
[b]Total number of isolates carrying a 16S rDNA allele.
[c]Numbers in parentheses indicate strains carrying three copies of *nifH* out of the total number harboring the corresponding 16S rDNA allele.
[d]Refers to strains carrying a 16S rDNA allele divergent from *R. gallicum* (see text for details).

showed RFLPs identical to *R. giardinii* strain H152 and, over a segment of 583 nt, both strains showed an identical sequence to the 16S rDNA of *R. giardinii* H152 (GenBank accession U86344, nt positions 43–625).

Isolate GR-84, representing ET4, had a 16S rDNA RFLP identical to that of *R. leguminosarum*. Over two non-overlapping sequence stretches, totalling 422 nt, this strain showed an identical sequence to *R. leguminosarum* bv. trifolii (GenBank accession X67227) and one and two mismatches with *R. leguminosarum* bv. viciae (U29386) and *R. leguminosarum* bv. phaseoli (U29388) 16S rRNA genes, respectively.

Isolates from ET12 showed 16S rDNA RFLPs matching *R. gallicum* R602sp, whereas RFLPs of isolates from ET8 and ET10 were slightly divergent from *R. gallicum* (Table 3, Fig. 3). These isolates (strains GR-18, GR-42 and GR-60) presented an additional *Msp*I site at position 951, defined by Laguerre et al. [24], as compared to *R. gallicum* strain R602sp, which would represent a 0.2% divergence from the *R. gallicum* 16S rDNA allele. In spite of this difference, the 5′-portion (505 nt) of the 16S gene of two of these isolates showed an identical nucleotide sequence, when we could only find a gap with respect to the *R. gallicum* 16S rDNA sequence (GenBank accession U86343, nt positions 71–576). C-344 of the *R. gallicum* sequence was missing in strains GR-45 and GR-42.

Strains from ETs 3, 5 and 6 had 16S rDNA RFLP patterns matching *S. fredii*. The amplified 16S rDNA from strain GR-06 was partially sequenced and compared to the available sequence of the *S. fredii* USDA205 strain 16S rDNA (GenBank accession X67231). No difference between these two strains was observed over a segment of 536 nt (from nt 71 to 606 of the USDA205 sequence), in agreement with the results from RFLP analysis. Since this group of isolates represented a significant portion of the total population analyzed (seven out of 39, 18%), we tested their ability to nodulate soybeans, the primary host for *S. fredii*. All seven strains were inoculated on axenically germinated seedlings of soybean cultivars Williams and Peking. Nodulation was recorded 4 weeks after inoculation, but no nodules were observed in either case. Thus, these *S. fredii*-like strains represent an unusual set of bacteria able to form nodules on common beans, but are unable to nodulate cv. Williams or cv. Peking of *Glycine max*.

3.3. Comparison between 16S rDNA phylogenies and MLEE lineages

16S rDNA analysis demonstrated that the bean-nodulating population in this soil is represented by bacteria belonging to at least five different 16S rDNA species, all of them related to previously characterized rhizobial species. These results were in agreement with the broad genetic diversity indicated by the MLEE studies. Nevertheless, the phylogenetic positions of the various 16S rDNA alleles represented in this population did not completely fit with the relative genetic distances indicated by MLEE. Thus, whereas isolates representing ET8, ET10 and ET12 had a similar 16S rDNA allele (*R. gallicum*), only diverging at one *Msp*I site, MLEE results indicate that they are rather distantly related,

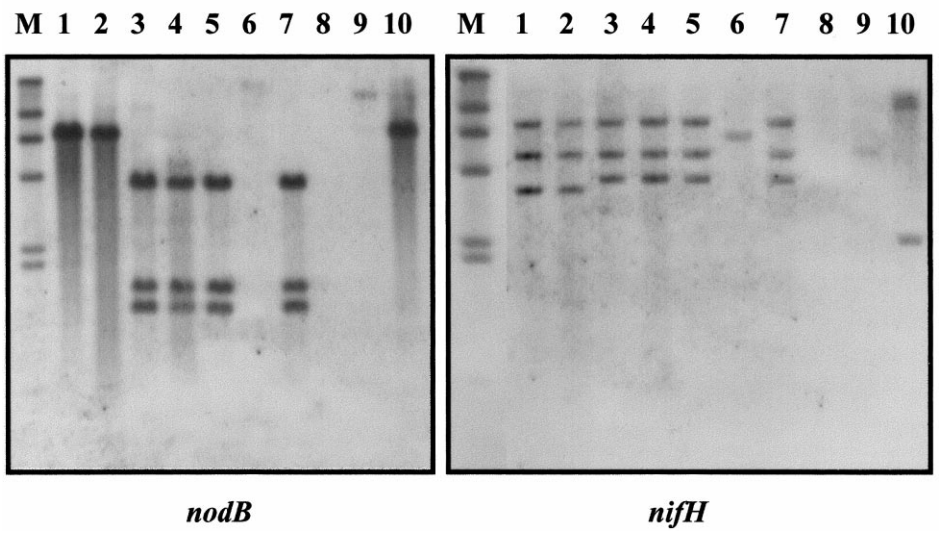

Fig. 4. Symbiotic gene organization in beans nodule isolates. Genomic DNAs digested with *Eco*RI (left blot) or *Bam*HI (right) were blotted and hybridized against a *R. etli nodB* or *nifH* gene probe, respectively. M, molecular weight marker; 1, *R. etli* CFN42; 2, isolate GR-14 (ET1); 3, isolate GR-75 (ET1); 4, isolate GR-84 (ET4); 5, isolate GR-18 (representing ET8 and ET10); 6, isolate GR-45 (representing ET12); 7, isolate GR-03 (ET2); 8, isolate GR-93 (ET2); 9, isolate GR-64 (representing ET3, ET5 and ET6); 10, *R. leguminosarum* bv. phaseoli 2616.

as ET12 diverged from ET8 and ET10 at a distance of 0.78 (Fig. 1), sufficient to consider them as different species. These two groups of bacteria yet formed unrelated clusters when non-Spanish isolates were included in the MLEE analysis (Fig. 2). However, this study was able to place strains from ET12, which carry a 16S rDNA perfectly matching *R. gallicum*, together with the Mexican strain FL27, which was recently classified as *R. gallicum* [12]. Bacterial isolates carrying a *R. etli*-like 16S rDNA clustered at a large genetic distance from the type strain and other strains classified as *R. etli*. This is not surprising, however, as *R. etli* strains are known to be genetically very diverse [6,7]. More surprising was the fact that isolates from ET2, which carry a 16S rRNA gene similar to that of *R. giardinii*, always grouped together (albeit at a distance of about 0.6) with isolates carrying a *R. etli* 16S rDNA allele (Figs. 1 and 2). 16S rDNA phylogenies, however, would place these two species as distantly related (Fig. 3).

3.4. Analysis of symbiotic genes

Hybridization of digested genomic DNAs with *R. etli nifH* or *nodB* gene probes placed bacterial isolates within two main groups, those that strongly hybridized with both probes under the high stringency conditions used and those that showed weak or no hybridization signals with either probe. Among the latter were all strains carrying a *S. fredii* 16S rDNA allele (ETs 3, 5 and 6), those carrying a 16S rRNA gene perfectly matching *R. gallicum* bv. gallicum R602sp (ET12) and one of the strains from ET2, strain GR-93, which carries a 16S rDNA similar to *R. giardinii* H152 (Fig. 4). All the remaining isolates, representing ET1, ET7 and ET11 (*R. etli*-like 16S rDNA), ET2 (strain GR-03, *R. giardinii*-like 16S rDNA), ET4 (*R. leguminosarum*-like 16S rDNA) and ET8 and ET10 (carrying a 16S rDNA slightly divergent from *R. gallicum*) appeared to contain three copies of *nifH* and showed one or more bands hybridizing to the *R. etli nodB* probe (Table 3, Fig. 4). These features are characteristic for the bv. phaseoli described for these four species [1,3]. Among strains carrying a *R. etli* 16S gene, two different *nifH*/*nodB* hybridization patterns were found, one identical to that of the CFN42 type strain and a second, the most abundant, that was also observed in isolates showing three copies of *nifH* and carrying different 16S rDNA alleles (Fig. 4). *nifH* RFLP pat-

terns identical to those described in this study have also been found among bean isolates from different locations in France [9] and in Austrian *R. etli* isolates [11]. The presence of similar hybridization patterns for symbiotic genes among isolates belonging to different 16S rDNA species indicates interspecific transfer of symbiotic genes.

4. Discussion

MLEE analysis revealed the existence of one of the highest degrees of genetic diversity encountered among *Rhizobium* isolates recovered from root nodules of a single legume in a single location [7]. In addition, the analysis showed at least three deeply diverging phylogenetic groups, indicating that several bean-nodulating *Rhizobium* species could be distinguished among the isolates analyzed. Indeed, analysis of the 16S rRNA genes demonstrated the presence of five different 16S alleles in this common bean-nodulating population, of which one, *S. fredii*, has not previously been found in European soils. The most abundant species appeared to be *R. etli*, as judged from RFLP of the amplified 16S rDNA, which was confirmed by partial sequencing. The simplest explanation of the occurrence of these strains is that they were brought into Spain from the Americas after introduction of beans about four centuries ago. Pérez-Ramírez et al. [25] have recently shown that *P. vulgaris* seeds may support the geographical spread of *R. etli* strains. Although the Spanish *R. etli*-like strains clustered at a large distance from the CFN42 type strain and other *R. etli* strains, it is interesting that two Mexican isolates, CFN1 and FNJ11, grouped closely with the Spanish isolates, which may indicate that the Spanish strains indeed derived from American ones. The fact that these strains represent the most abundant subpopulation among the bean nodule isolates (54%) may suggest that the conditions in this soil have been appropriate for persistence and development of these strains. It should be noted here that beans have not been cultivated in this soil during the past 30 years, resembling the situation of an Austrian soil where Sessitsch et al. [11] have also reported large numbers of *R. etli* nodulating common beans.

A symbiotic gene organization typical of *R. etli* bv. phaseoli, characterized by the presence of three copies of *nifH*, was found in isolates belonging to four distinct 16S rDNA species, *R. etli, R. leguminosarum, R. gallicum* and *R. giardinii*, and, according to this feature, all such strains should be classified as bv. phaseoli of the corresponding species. These results indicate that extensive interspecific symbiotic gene exchange has taken place in this site and presumably, as discussed above, *R. etli* strains could have been the original gene donors. The other three species, which have also been found at different locations in Europe, probably represent bacteria that pre-existed in European soils when beans were first introduced and have received genetic material from the introduced *R. etli*. This species has been found in other European locations, but no evidence was reported of gene transfer to other species [11,12]. In contrast, the typical *R. etli* sym gene organization has been identified in species populating other European soils, but no *R. etli* strains were found co-existing at those locations ([1,9,11]). Thus, the soil studied in this work represents a unique case where a donor and the putative DNA recipients co-exist and probably compete for the same ecological niche.

It was surprising to find strains carrying a 16S rDNA similar to that of *S. fredii* among the common bean-nodulating bacteria in this soil. These isolates do not nodulate American or Chinese soybean cultivars. Moreover, we have been unable to isolate any soybean-nodulating bacteria from this soil, which further supports the observation that these *S. fredii*-like strains are not soybean symbionts. Thus, this is not a case similar to previous reports showing that certain soybean symbiotic bacteria, classified as *S. fredii*, also had the capacity to establish in symbiosis with certain *P. vulgaris* genotypes [16,17]. Bacteria classified as *S. fredii* are the fast growing representatives of soybean microsymbionts. They have always been isolated from soybean nodules and are abundant in Chinese soils [7]. Thus, with the data available, it is not possible to speculate about the relationships between so far identified *S. fredii* bacteria and the *S. fredii*-like strains described in this work.

A puzzling situation was encountered when MLEE lineages were compared with 16S rDNA species assignments. Two sets of strains carrying 16S rDNA alleles similar to *R. gallicum* (those from

ETs 8 and 10 and ET12) were always distantly clustered by MLEE cluster analysis. As different species can share similar 16S rRNA genes, the two MLEE lineages could correspond to different species. It may be noteworthy that the three strains representing ET8 and ET10 belonged to the bv. phaseoli, whereas strains representing ET12 had characteristics of bv. gallicum. This situation recalls the difficulties in defining species limits [26] and resembles the case reported by Eardly and coworkers [6] for certain common bean-nodulating isolates. These authors showed the occurrence of strains carrying the *R. leguminosarum* or the *R. etli* 16S rDNA allele within closely related chromosomal lineages and suggested transfer and recombination of the 16S rRNA gene as a possible explanation for their observations. Recently, Ueda et al. [27] have shown that certain cases of intrastrain 16S rRNA heterogeneity can only be explained by horizontal gene transfer events, which can also explain the cases where almost identical 16S rRNA sequences are harbored by phenotypically divergent bacteria [28]. In the present study, two groups of isolates carrying almost identical 16S alleles, similar to *R. gallicum*, correspond to distantly related MLEE lineages and could therefore represent different species that may have exchanged their 16S rDNA alleles. Confirmation of this extreme will require a more detailed analysis of their genomes.

Acknowledgements

This work was supported in part by a CSIC-CONACYT cooperation grant to J. Sanjuan and E. Martínez-Romero and by Grant BIO2-CT930053 of the European Union. We acknowledge Rocio Bustillos for technical support.

References

[1] Amarger, N., Macheret, V. and Laguerre, G. (1997) *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. Int. J. Syst. Bacteriol. 47, 996–1006.

[2] Martínez-Romero, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P. and Pardo, M.A. (1991) *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* trees. Int. J. Syst. Bacteriol. 41, 417–426.

[3] Segovia, L., Young, J.P.W. and Martínez-Romero, E. (1993) Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. 43, 374–377.

[4] van Berkum, P., Beyene, D. and Eardly, B.D. (1996) Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris*). Int. J. Syst. Bacteriol. 46, 240–244.

[5] Amarger, N., Bours, M., Revoy, F., Allard, M.R. and Laguerre, G. (1994) *Rhizobium tropici* nodulates field-grown *Phaseolus vulgaris* in France. Plant Soil 161, 147–156.

[6] Eardly, B.E., Wang, F., Whittam, T.S. and Selander, R.K. (1995) Species limits in *Rhizobium* populations that nodulate the common bean (*Phaseolus vulgaris*). Appl. Environ. Microbiol. 61, 507–512.

[7] Martínez-Romero, E. and Caballero-Mellado, J. (1996) *Rhizobium* phylogenies and bacterial genetic diversity. Crit. Rev. Plant Sci. 15, 113–140.

[8] Aguilar, O.M., Lopez, M.V., Riccillo, P.M., Gonzalez, R.A., Pagano, M., Grasso, D.H., Pühler, A. and Favelukes, G. (1998) Prevalence of the *Rhizobium etli*-like allele in genes coding for 16S rRNA among the indigenous rhizobial populations found associated with wild beans from the Southern Andes in Argentina. Appl. Environ. Microbiol. 64, 3520–3524.

[9] Geniaux, E., Laguerre, G. and Amarger, N. (1993) Comparison of geographically distant populations of *Rhizobium* isolated from root nodules of *Phaseolus vulgaris*. Mol. Ecol. 2, 295–302.

[10] Laguerre, G., Fernandez, M.P., Edel, V.E., Normand, P. and Amarger, N. (1993) Genomic heterogeneity among french *Rhizobium* strains isolated from *Phaseolus vulgaris* L. Int. J. Syst. Bacteriol. 43, 761–767.

[11] Sessitsch, A., Hardarson, G., Akkermans, A.D.L. and de Vos, W.M. (1997) Characterization of *Rhizobium etli* and other *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. in an Austrian soil. Mol. Ecol. 6, 601–608.

[12] Sessitsch, A., Ramírez-Saad, H., Hardarson, G., Akkermans, A.L. and de Vos, W.M. (1997) Classification of Austrian rhizobia and the Mexican isolate FL27 obtained from *Phaseolus vulgaris* L. as *Rhizobium gallicum*. Int. J. Syst. Bacteriol. 47, 1097–1101.

[13] Bromfield, E.S.P. and Barran, L.R. (1990) Promiscuous nodulation of *Phaseolus vulgaris, Macroptilium atropurpureum* and *Leucaena leucocephala* by indigenous *Rhizobium meliloti*. Can J. Microbiol. 36, 369–372.

[14] Eardly, B.D., Young, J.P.W. and Selander, R.K. (1992) Phylogenetic position of *Rhizobium* sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *nifH* genes. Appl. Environ. Microbiol. 58, 1809–1815.

[15] Hernandez-Lucas, I., Segovia, L., Martínez-Romero, E. and Pueppke, S. (1995) Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. Appl. Environ. Microbiol. 61, 2775–2779.

[16] Sadowsky, M.J., Cregan, P.B. and Keyser, H.H. (1988) Nodulation and nitrogen fixation efficacy of *Rhizobium fredii* with

Phaseolus vulgaris genotypes. Appl. Environ. Microbiol. 54, 1907–1910.

[17] Michiels, J., Dombrecht, B., Vermeiren, N., Xi, C., Luyten, E. and Vanderleyden, J. (1998) *Phaseolus vulgaris* is a non-selective host for nodulation. FEMS Microbiol. Ecol. 26, 193–205.

[18] Martinez, E., Pardo, M.A., Palacios, R. and Cevallos, M.A. (1985) Reiteration of nitrogen fixation gene sequences and specificity to *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris*. J. Gen. Microbiol. 131, 1779–1786.

[19] Selander, R.K., Caugant, D.A., Ochman, H., Musser, J.M., Gilmour, M.N. and Whittan, T.S. (1986) Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. 51, 873–884.

[20] Laguerre, G., Allard, M.R., Revoy, F. and Amarger, N. (1994) Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. 60, 56–63.

[21] Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.

[22] Piñero, D., Martinez, E. and Selander, R.K. (1988) Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar *phaseoli*. Appl. Environ. Microbiol. 54, 2825–2832.

[23] Caballero-Mellado, J. and Martínez-Romero, E. (1999) Soil fertilization limits the genetic diversity of *Rhizobium* in bean nodules. Symbiosis 26, 111–121.

[24] Laguerre, G., van Berkum, P., Amarger, N. and Prevost, D. (1997) Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astragalus, Oxytropis* and *Onobrychis*. Appl. Environ. Microbiol. 63, 4748–4758.

[25] Pérez-Ramírez, N.O., Rogel, M.A., Wang, E., Castellanos, J.Z. and Martínez-Romero, E. (1998) Seeds of *Phaseolus vulgaris* bean carry *Rhizobium etli*. FEMS Microbiol. Ecol. 26, 289–296.

[26] Martínez-Romero, E. (1996) Comments on *Rhizobium* systematics: Lessons from *R. tropici* and *R. etli*. In: Biology of Plant-Microbe Interactions (Stacey, G., Mullin, B. and Gresshoff, P.M., Eds.), pp. 503–508. International Society for Molecular Plant-Microbe Interactions, St. Paul, MN.

[27] Ueda, K., Seki, T., Kudo, T., Yoshida, T. and Kataoka, M. (1999) Two distinct mechanisms cause heterogeneity of 16S rRNA. J. Bacteriol. 181, 78–82.

[28] Fox, G.E., Wisotzkey, J.D. and Jurtshuk, P. (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42, 166–170.