APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1998, p. 3520–3524 Vol. 64, No. 9
0099-2240/98/$04.00+0

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

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Received 14 July 1997/Accepted 30 June 1998

A collection of rhizobial isolates from nodules of wild beans, *Phaseolus vulgaris* var. aborigineus, found growing in virgin lands in 17 geographically separate sites in northwest Argentina was characterized on the basis of host range, growth, hybridization to a *nifH* probe, analysis of genes coding for 16S rRNA (16S rDNA), DNA fingerprinting, and plasmid profiles. Nodules in field-collected wild bean plants were largely dominated by rhizobia carrying the 16S rDNA allele of *Rhizobium etli*. A similar prevalence of the *R. etli* allele was observed among rhizobia trapped from nearby soil. Intragroup diversity of wild bean isolates with either *R. etli*-like or *Rhizobium leguminosarum* bv. phaseoli-like alleles was generally found across northwest Argentina. The predominance of the *R. etli* allele suggests that in this center of origin of *P. vulgaris* the coevolution of *Rhizobium* spp. and primitive beans has resulted in this preferential symbiotic association.

It is generally accepted that *Phaseolus vulgaris* L. (the common bean) is native to the Americas. Domestication of wild beans took place independently in the Mesoamerican center of origin (Mexico, Central America, and Colombia) and in the Andean center in South America (Ecuador, Bolivia, Peru, and Argentina) (10). Soil bacteria of the genus *Rhizobium* induce nitrogen-fixing nodules on the roots of bean plants. The rhizobial isolates from bean nodules from various regions in Mexico and South America are known to be a very heterogeneous group. Two main types, known as types I and II, had been identified among American rhizobial isolates that share the ability to induce nodules on beans (18). Type I strains have a narrow host range restricted to *Phaseolus* spp., their DNA possesses multiple copies of the nitrogenase structural gene *nifH*, and their DNA hybridizes to the *psi* (polysaccharide inhibition) gene. Type II strains nodulate *Leucaena* spp. in addition to beans and have single copies of *nifH* (22–24). After further taxonomic characterization of these isolates by methods based on analysis of multilocus enzyme electrophoresis (MLEE), genes coding for 16S rRNA (16S rDNA) and DNA:DNA reassociation, two novel species, namely *Rhizobium etli* (type I) and *Rhizobium tropici* (type II), have been proposed in addition to *Rhizobium leguminosarum* bv. phaseoli (19, 25). This latter species also encompasses biovars viciae and trifolii. Two subspecies, A and B, with distinctive phenotypic features have been found in *R. tropici*. However, some other isolates, all of which are able to nodulate common beans with different degrees of effectiveness, appear to represent still other distinct phylogenetic lineages (9, 11–13, 15, 20, 22). Eardly et al. characterized a bean rhizobium collection by applying MLEE and analysis of 16S rDNA and found limitations in assignment of species as some *R. etli* strains have the allele corresponding to the *R. leguminosarum* 16S rRNA genes (8). Most of these data resulted from the study of a collection of rhizobia originating in Mexico and in tropical areas of South America, in Colombia and Brazil. However, the rhizobial population associated with the wild bean *P. vulgaris* var. aborigineus Burk. (Baudet), considered to be the ancestors of cultivated bean varieties and found in the southernmost region of domestication in the Southern Andes (6, 10), has not been examined yet. In this region there exist areas of virgin land that have been undisturbed by humans and that support growth of wild beans. Since the region is inhabited by other wild legumes, such as *Desmodium* spp., *Phaseolus augusti*, *Erythrina* spp., *Mimosa* spp., *Acacia* spp., and *Vigna* spp. (6), that could promote microsymbiont diversity, it is possible that the symbiotic interaction between the aboriginal, wild bean variety and naturally existing rhizobia has developed specificity in this region, thereby restricting this particular host-rhizobium association (16).

In this study our objective was to characterize the rhizobial populations naturally associated with wild beans in various areas in the Southern Andes, in northwest Argentina (NWA).

A collection of rhizobial isolates from wild beans growing in virgin lands. All rhizobia were isolates from wild beans or were retrieved in the laboratory from field soils. From each plant sampled in the field, one to three nodules were randomly excised and surface sterilized with ethanol and hydrogen peroxide. Rhizobia were isolated axenically on YEM-Congo Red agar medium as described by Vincent (30). The nitrogen fixation potential of each bacterial isolate was confirmed by detecting the presence of the *nifD* gene. This was done by testing

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TABLE 1. Rhizobial isolates from nodules of wild beans and their geographical origins

Site	No. of isolates	Location (Argentina)	Coordinates	Altitude (m)[a]
A1	2	Tilcara, Jujuy	23°35′S, 65°23′W[b]	2,460
A2	3	Yala, Jujuy	24°07′S, 65°29′W[b]	1,445
B1	3	Los Toldos, Salta	22°22′S, 64°43′W[b]	1,560
B2	1	Santa Victoria, Salta	22°15′S, 64°57′W	1,700
B3	2	Embarcación, Salta	23°16′S, 63°59′W	770
B4	9	Valle Grande, Salta	23°28′S, 64°56′W[b]	1,590
B5	2	Pichanal, Salta	23°32′S, 64°58′W[b]	350
B6	12	Quebrada del Toro, Salta	24°54′S, 64°40′W[b]	1,590
B7	17	Chorro Blanco, Salta	25°12′S, 65°35′W[b]	1,600
B8	5	Los Laureles, Salta	25°06′S, 65°36′W	1,480
B9	4	Cerrillos, Salta	24°55′S, 65°29′W[b]	1,253
B10	3	Pulares, Salta	25°06′S, 65°36′W[b]	650
C1	1	Burruyacú, Tucumán	26°20′S, 64°57W	720
C2	1	La Higuera, Tucumán	26°20′S, 64°43′W	1,350
C3	1	El Mollar, Tucumán	26°51′S, 65°43′W	890
C4	1	Reartes, Tucumán	26°20′S, 65°35′W	1,520
D1	1	La Banderita, Catamarca	27°21′S, 66°23′W	1,580

[a] Meters above sea level.
[b] Coordinates were determined with a Trimble Scoutmaster global navigation system (global positioning system).

for PCR amplification products of a highly conserved region of the gene with a *nifD* primer pair provided by J. Stoltzfus and F. de Bruijn, Michigan State University, East Lansing (27). Soil isolates were recovered from nodules of plants of common beans or leucaena, which were grown in the laboratory after inoculation with soil suspensions prepared with samples brought from the field sites A1, B5, B6, and B8 (Table 1). Plant tests were conducted with seeds that were stepwise surface sterilized sequentially with 75% ethanol for 1 min and sodium hypochlorite for 4 min and finally washed with water. Seeds were incubated on top of water-agar (1.5%, wt/vol) for about 3 days. Germinated seedlings inoculated with rhizobial suspensions were grown axenically in 500-ml plastic pots filled with sterilized vermiculite and watered twice with N-free mineral nutrient solution (30) and with sterile distilled water as required. Seeds of the wild, primitive bean variety of the Southern Andes, *P. vulgaris* var. aborigineus Burk. (Baudet) (6), collected from plants in various locations in NWA, were provided by Roberto Neumann, Instituto de Tecnología Agropecuaria, Estación Experimental Agropecuaria-Salta (INTA, EEA-Salta), Argentina. Seeds of *P. vulgaris* L. (common beans) cultivar Negro Camilo were obtained from INTA, EEA-Salta, *Leucaena leucocephala* seeds were a gift from Avilio A. Franco, Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), Seropédica, Brazil. The number of indigenous rhizobia in soil samples able to nodulate common beans or leucaena was estimated by the most-probable-number (MPN) method (30). Antibiotic tolerance was determined in TY medium (4) supplemented with 200 μg of streptomycin per ml or 20 μg of nalidixic acid per ml. Melanin production was assayed in TY medium supplemented with 300 mg of tyrosine per ml and 40 mg of $CuSO_4 \cdot 5H_2O$ per ml. Results presented in the following sections refer generally to rhizobial isolates from nodules of wild beans, which have been assigned designations beginning with the letter P. Isolates retrieved in the laboratory from soil samples are coded with the prefix T.

Sixty-nine isolates were obtained from nodules of *P. vulgaris* var. aborigineus collected from 17 different field sites in NWA. The plants were found in virgin lands having no records of previous agricultural management. As shown in Table 1, the sources of the isolates were at different altitudes in the Argentinian provinces of Jujuy, Salta, Tucumán, and Catamarca and extended between coordinates 22°15′ and 27°21′ latitude S and 64°40′ and 66°23′ longitude W.

Except for samples from site A1 (Tilcara), corresponding to the high Andean dry valley of Quebrada de Humahuaca, the samples were mostly collected from a mountain forest ecosystem. At site A1 the mean annual rainfall is 130 mm and the mean annual temperature is 13°C, whereas at the rest of the sites the mean annual rainfall ranges between 900 and 1,200 mm and the mean annual temperatures are between 16 and 22°C. Soils in most sites were near neutral (pH 6.9 to 7.2); the only acid soil sampled was in site A2 (pH 6.2).

The density of *P. vulgaris*-nodulating rhizobia per gram of soil was 2.0×10^3 at site A1, 2.7×10^4 at site B8, and 6.6×10^5 at site B6. In contrast, the soil densities of rhizobia nodulating leucaena were much lower or undetectable: about 4×10^2 rhizobia per gram of soil were detected at sites B5, B6, and B8. The isolates retrieved from leucaena also were able to nodulate beans.

P. vulgaris var. aborigineus inoculated with the reference strains *R. etli* CFN42, *R. leguminosarum* bv. phaseoli RCR3644, and *R. tropici* CFN299 (type A) and CIAT899 (type B) formed effective nodules. The symbiotic effectiveness was assessed by comparing the shoot dry weights with those of noninoculated control plants.

All of the isolates from nodules of wild beans, except P25N1, formed gummy white colonies on YEM-Congo Red medium. P25N1 formed dry colonies that appeared rather opaque as compared to the clear colonies of the other isolates. All isolates were fast growers, produced acid, and were unable to grow on LB medium. All of the bean isolates nodulated *P. vulgaris*, and none, except P25N1, nodulated *L. leucocephala*. Strain P25N1 formed effective nodules with both hosts tested. About 30% of the isolates produced the dark color typical of melanin, and about 10% were nalidixic acid resistant. No association was found between these two characteristics and the origin of the isolates.

Characterization of rhizobial isolates from wild beans. The question of whether the various isolates could be assigned to bean-nodulating rhizobial type I or II was approached by applying two additional tests. Aguilar et al. (1) had shown previously that *R. etli* and *R. leguminosarum* bv. phaseoli type I strains (but not type II strains or other rhizobia) consistently yielded a *nifH* PCR amplification product of 570 bp, indicating that the particular symbiotic gene *nifH* is widely conserved among bean-nodulating strains originating in the Americas. We found that all of the wild isolates—except P25N1—produced a PCR amplification product identical in size to the one observed with the *R. etli* reference strain CFN42, whereas P25N1 produced a 370-bp fragment that was identical in size to that obtained with the *R. tropici* type A strain CFN299.

Second, we examined a representative subsample of 12 type I-like isolates by probing *Bam*HI-restricted total DNA in Southern blots with pCQ15 containing a 270-bp *Sal*I internal *nifH* DNA sequence (24), as they were described by Martínez et al. (18). All of these isolates contained three copies of the *nifH* gene since three restriction fragments ranging in size from 3.5 to 9 kb were found to hybridize to the *nifH* probe. Isolate P25N1 and strain CFN299 tested in the same way each showed only one *nifH* copy on fragments of 4.5 and 2.8 kb, respectively (results not shown).

Further characterization of the isolates from wild beans was attempted by analyzing the respective 16S RNA genes (14, 17, 26, 29, 32). We used the procedure described by Laguerre et al. (14) to identify restriction sites in a PCR-amplified 16S rDNA

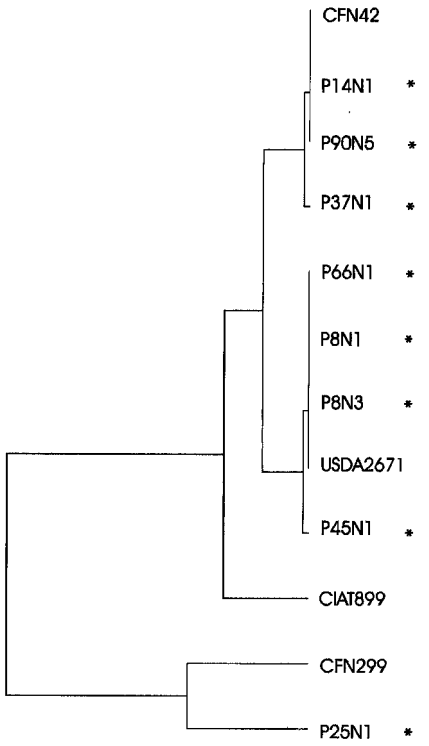

FIG. 1. Dendrogram (UPGMA) of genetic relationships among 16S rDNA genotypes identified by sequence analysis of a 260-bp 16S rRNA gene fragment. Asterisks indicate isolates from wild beans.

region of about 1.5 kb that encompasses conserved and variable regions to permit identification of the individual species. Restriction fragment length polymorphism (RFLP) analysis was performed by electrophoresis in 2% agarose gel. It was found that 62 of the 69 isolates from wild beans had RFLP patterns following digestion with enzymes *Alu*I, *Hae*III, *Msp*I, and *Nde*II that were identical to that of reference strain *R. etli* CFN42, and on this basis they were tentatively designated as species *R. etli*. Similarly, six other isolates were assigned to the species *R. leguminosarum* since they showed a 16S rRNA restriction pattern similar to that of *R. leguminosarum* bv. phaseoli reference strain RCR3644. Finally, the RFLP pattern of the broad-host-range isolate P25N1 was identical to that of *R. tropici* type A strain CFN299. Accordingly, P25N1 was designated as species *R. tropici* type A.

These assignments were confirmed for eight isolates by sequencing a 260-bp region of genes encoding 16S RNA and comparing the results with those of reference strains. Young et al. (32) found that this region was highly conserved at the species level but differed among different species. Amplification reactions using Y1 and Y2 primers were performed as described by Young et al. (32). The Y1 and Y2 amplification products were concentrated with isopropanol as described by van Berkum et al. (28), and both strands were sequenced with a model 380 DNA sequencer (Applied Biosystems). The aligned partial 16S rDNA rhizobial sequences were analyzed together with the sequences of reference strains by the Pileup program of the University of Wisconsin Genetics Computer Group package, and the results were used to construct a phylogenetic tree (Fig. 1). The sequences of the 260-bp region from the reference strains *R. etli* CFN42 and *R. leguminosarum* bv. phaseoli 8002 and RCR3644 were found to differ by 9 nucleotides. In the case of *R. etli*-like isolates P14N1 and P90N5, the sequences were identical to the one determined for CFN42 (which in turn was identical to the published sequence). In the case of isolate P37N1, which had also given an RFLP pattern similar to that of *R. etli*, the sequence of the 260-bp region had one base mismatch.

The sequences of the 260-bp DNA fragment from *R. leguminosarum*-like strains P8N1, P8N3, and P66N1 were identical to one another as well as to the published sequence of the reference strains *R. leguminosarum* bv. phaseoli 8002 and RCR3644 (29, 32), thus confirming the occurrence of the 16S rDNA allele of species *R. leguminosarum*. One mismatch was found when P45N1 and reference strains of *R. leguminosarum* were compared. The presence of *R. leguminosarum* is particularly noteworthy since this species had been assigned mostly to European soils (3, 15), whereas *R. etli* and *R. tropici* are believed to represent the American rhizobial strains for beans (25). Although its presence in NWA wild beans in seemingly isolated sites was surprising to us, Eardly et al. (8) also have reported the occurrence of the *R. leguminosarum* 16S rRNA in a bean-nodulating rhizobial population isolated from Colombia. In that study, the *R. leguminosarum* 16S RNA allele was found in groups that were genetically distant with respect to their MLEE profiles (8). Taken together with this earlier observation, our present findings of the *R. leguminosarum* 16S rRNA allele in natural populations in NWA provide further evidence that, in addition to *R. etli* and *R. tropici*, *R. leguminosarum* bv. phaseoli is a natural component in the South American populations of bean-nodulating rhizobia.

The sequence of the PCR product from the *R. tropici*-like isolate P25N1 differed by four nucleotides from the published sequences of *R. tropici* type A strains CFN299 and USDA2840 (29). As indicated above, isolate P25N1 also differed from the reference strain CFN299 in the size of the single DNA fragment which hybridized with the *nifH* probe. In addition, as for reference strains of *R. tropici*: IIA (3, 28, 29) found by other authors, the PCR product from P25N1 with Y1 and Y2 primers was 72 bp larger than the products observed when DNAs from *R. etli* and *R. leguminosarum* bv. phaseoli were used as template. Definitive species assignments for the isolates in this study must await DNA-DNA homology studies, but these results indicate that, in the NWA region, *P. vulgaris* var. aboriginaeus is nodulated by a diversity of bacteria representing three of the major recognized 16S rDNA alleles identified among the bean-nodulating species *R. etli*, *R. leguminosarum*, and *R. tropici* type A. Of the three, the 16S rDNA allele of *R. etli* was predominant among the wild bean isolates throughout that region.

Heterogeneity of wild bean rhizobial populations at the regional and local levels. Total genomic DNA from each of the wild bean isolates obtained from 17 different sites listed in Table 1 was used as a template for PCR with either repetitive extragenic palindromic (Rep) or enterobacterial repetitive intergeneric consensus (ERIC) primers according to the procedure described by de Bruijn (7). The results (not shown) indicated that in no case did different sites have isolates with identical profiles. These differences were studied in more detail in Fig. 2. The patterns, obtained with ERIC primers and with 20 wild bean isolates from eight sites, together with nine soil isolates from site B8, were ordered by similarity by the unweighted pair group (UPGMA) method of clustering. As before, no two isolates gave the same PCR pattern, and the maximal similarity among sites, 94%, was observed between isolates from sites B6 and B8 (P55N1 and P36N3, respectively). Overall, the majority of isolates could be grouped into two main clusters (P40N1 to PLP1001 and P55N1 to P64N1), each with a limited degree of similarity of 52%.

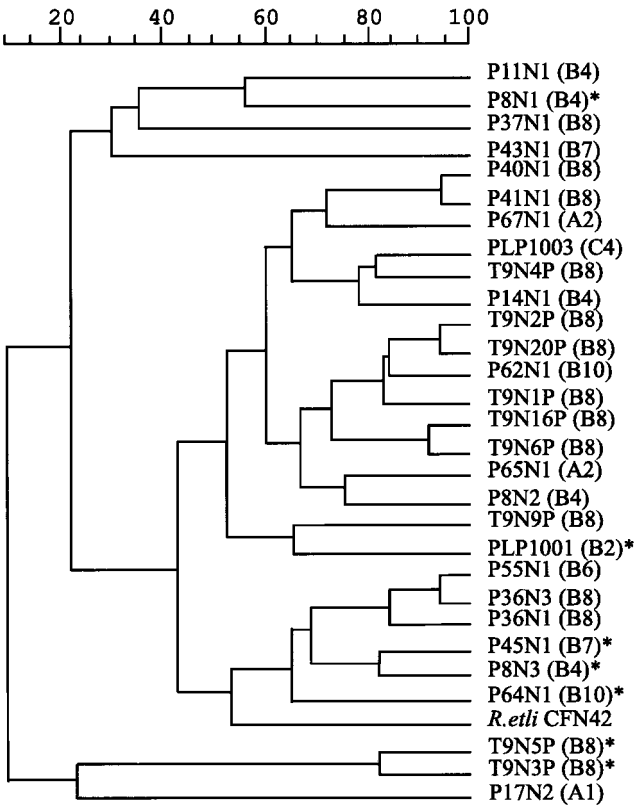

FIG. 2. Genotypic relatedness of isolates from wild beans. The dendrogram is derived from analysis of ERIC-PCR by using the computer-assisted system of analysis GelCompar from Applied Maths, Kortrijk, Belgium. The degree of relative genetic relatedness is indicated above the dendrogram on a scale from 10 to 100%. Each branch is followed by the respective wild bean isolate (preceded by the letter P) or soil isolate (preceded by the letter T) and site of origin according to Table 1 (in parentheses). Asterisks indicate *R. leguminosarum* bv. phaseoli-like isolates; all the other isolates have the *R. etli* 16S rDNA gene.

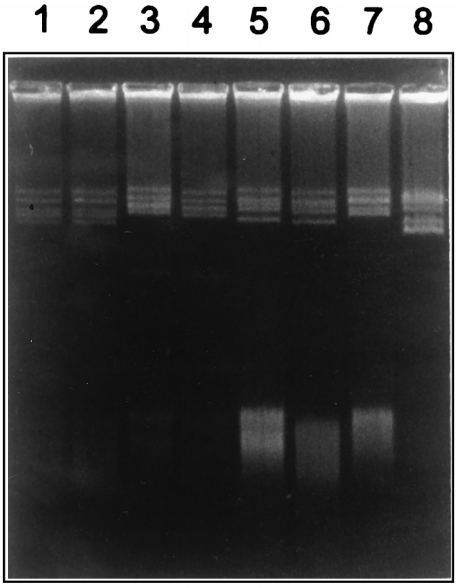

FIG. 3. Representative plasmid profiles of isolates obtained from wild beans and from NWA soils. *R. etli* isolates: lane 1, P40N1 (B8) (geographic origins of isolates according to Table 1 are given in parentheses); lane 2, P41N1 (B8); lane 3, T9N16P (B8); lane 4, P65N1 (A2). *R. leguminosarum* bv. phaseoli isolates: lane 5, P45N1 (B7); lane 6, P8N3 (B4); lane 7, P8N1 (B4). Lane 8, reference strain, *R. etli* CFN42, with plasmids ranging in size from 150 to 600 bp (5).

Heterogeneity also was observed at the level of a single sampling site in all sites where multiple isolates had been obtained. This is exemplified in Fig. 2 by the results of ERIC profiles of isolates obtained from site B8. Of the five *R. etli* wild bean isolates examined (all obtained from adjacent wild plants), only two, each from a different plant, showed a high degree of similarity, 95% (P40N1 and P41N1). Two other isolates (P36N1 and P36N3), obtained from the same plant, were moderately similar (82%) and differed markedly from the first two; finally, the fifth isolate (P37N1) had a very different profile. Despite this general trend, similarities in pattern were found in a few cases of wild bean rhizobia isolated from sites located many kilometers apart. This was particularly the case for isolates *R. etli* P55N1 and P36N3; similarity was also observed, albeit to a lesser degree, in isolates *R. leguminosarum* bv. phaseoli P8N3, P45N1, and P64N1.

Diversity also was found in rhizobia retrieved from soil in the laboratory with common beans as the trapping host. Of 35 soil isolates obtained, 31 had the *R. etli* 16S rDNA allele. Thus, these isolates also reflected the predominance of this allele in the bean-nodulating rhizobia present in these soils. ERIC profiles of these soil isolates showed differences as well as similarities. This is illustrated for site B8 in Fig. 2: of seven *R. etli* soil isolates, two pairs of isolates, each with very similar profiles (93 to 95% similarity), were found, but the similarity between pairs (71%), as well as for the other isolates, was limited. The high similarity within each of these pairs might reflect a common origin of the paired soil strains. The two *R. leguminosarum* bv. phaseoli soil isolates from site B8 had a moderate similarity (82%). It is noteworthy that these populations of isolates retrieved from soil in the laboratory with common beans as the trapping host do not overlap in similarity with any isolates obtained at the same site from field-collected wild bean nodules.

Diversity also was assessed by examining the plasmid profiles by electrophoresis in horizontal agarose gels by the procedure of Wheatcroft et al. (31). Plasmid profiles indicated a diversity in the number of indigenous plasmids per cell, which ranged between three and six, and on this basis most of the isolates could be grouped in a class having five plasmids of variable sizes. Of the plasmid patterns obtained with isolates having the *R. etli* 16S rDNA allele, Fig. 3 shows two, in lanes 1 and 2 and in lanes 3 and 4, corresponding to four isolates from sites B8 and A2, respectively. The six wild bean isolates belonging to *R. leguminosarum* bv. phaseoli yielded two different types of plasmid profiles, one shown in lanes 5 and 6 and the other in lane 7. Profile differences were found among rhizobial isolates from a single site or even from the same wild bean plant. Indeed, for *R. etli*, soil isolate T9N16P from site B8, for instance (Fig. 3), differed from wild bean isolates P40N1 and P41N1 (with similar profiles; see lanes 1 and 2, respectively) obtained from adjacent plants in the same site, B8. *R. leguminosarum* bv. phaseoli isolates P8N3 and P8N1, each obtained from a different nodule on the same plant in site B4, differed in profiles (lanes 6 and 7, respectively). On the other hand, similar patterns were found in isolates from quite distant locations, e.g., *R. etli* T9N16P at site B8 and P65N1 at site A2 (lanes 3 and 4, respectively) and *R. leguminosarum* bv. phaseoli P45N1 at site B7 and P8N3 at site B4 (lanes 5 and 6, respectively). Some of the isolates, which showed similarities in their plasmid profiles (Fig. 3), also are similar in the clustering analysis of Fig. 2, based on a very different criterion. This was the case with isolate pairs *R. etli* P40N1 and P41N1 (Fig. 2, and lanes 1 and

2 in Fig. 3) and *R. leguminosarum* bv. phaseoli P45N1 and P8N3 (Fig. 2, and lanes 5 and 6 in Fig. 3). In the latter case the similar isolates originated from geographically unrelated sites.

The preference for rhizobia with the *R. etli* 16S rDNA allele in the associations with wild beans under field conditions in NWA is shared by common beans (2), perhaps reflecting the presumed predominance of these rhizobia in NWA soils. However, the populations trapped by common beans in the laboratory, which showed diversity in their ERIC-PCR fingerprints and plasmid profiles, differed from the isolates from wild beans collected at the same site. This lack of similarity indicates that the populations which were active in nodulating wild beans in the field differed from those from the same soil which nodulated common beans in the laboratory. Among the possible reasons for this behavior are the following. (i) Environmental conditions for nodulation in the laboratory may have been quite different from those in the field. (ii) The original soil population at the time of nodulation in the field might have changed by the time that the soil samples were brought to the laboratory. (iii) Some of these changes might have been caused by soil sampling, transportation, and laboratory manipulations. (iv) The relative nodulation competitiveness of the strains in the soil might differ for wild beans and common beans. It had been shown that some accessions of *P. vulgaris* are able to restrict nodulation by some rhizobial strains (21). In any case, our results indicate that the rhizobial populations nodulating wild beans in virgin field soils of NWA are not the sole *R. etli* and *R. leguminosarum* bv. phaseoli isolates in those soils that potentially are able to associate with *P. vulgaris*.

As this work was performed on a primitive line of beans found in sites with no history of human disturbance or agricultural management, in a region which is considered to be one center of bean domestication (6, 10), then both the high densities of type I rhizobia detected in the soils and the predominance of *R. etli* occupying root nodules of wild beans induce speculation that *P. vulgaris* in this center of origin might have coevolved in the symbiosis with *Rhizobium* spp.

Nucleotide sequence accession numbers. The nucleotide sequences of 16S rDNA from rhizobial isolates P14N1, P37N1, P90N5, P45N1, P66N1, P8N1, P8N3, and P25N1 determined in this study have been deposited in the GenBank nucleotide sequence database under accession numbers AF071113, AF071114, AF071115, AF071116, AF071117, AF071118, AF071119, and AF071120, respectively.

We are grateful to Aníbal Sánchez-Caro for providing the initial isolates. We also thank Roberto Neumann and Marcelo Salgado for assistance in field collection and for seeds of wild and common beans, Avilio A. Franco for seeds of leucaena, Esperanza Martínez-Romero for the *nifH* probe, Noelle Amarger for assistance in RFLP analysis, and Frans de Bruijn for the use of the GelCompar system.

This research was supported in part by grants from the Volkswagen Foundation and from SECYT/CONICET, Argentina (PID No. 331 BID 802 OC/AR). O.M.A. and G.F. are members of the research career of the National Research Council-CONICET, Argentina. M.V.L. and R.A.G. have been recipients of training studentships from the CIC, Buenos Aires, Argentina.

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