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The Diversity of *Phaseolus*-Nodulating Rhizobial Populations Is Altered by Liming of Acid Soils Planted with *Phaseolus vulgaris* L. in Brazil

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PCR-mediated restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA internally transcribed spacer (ITS) region and the 16S rRNA gene indicated that the rhizobial populations isolated from common bean (*Phaseolus vulgaris* L.) nodules in the unlimed soil from a series of five lime rates applied 6 years previously to plots of an acidic oxisol had less diversity than those from plots with higher rates of liming. Isolates affiliated with *Rhizobium tropici* IIB and *Rhizobium leguminosarum* bv. phaseoli were predominant independent of lime application. An index of richness based on the number of ITS groups increased from 2.2 to 5.7 along the soil liming gradient, and the richness index based on "species" types determined by RFLP analysis of the 16S rRNA gene varied from 0.5 to 1.4. The Shannon index of diversity, based on the number of ITS groups, increased from 1.8 in unlimed soil to 2.8 in limed soil, and, based on RFLP analysis of the 16S rRNA gene, ranged from 0.9 to 1.4. In the limed soil, the subpopulation of *R. tropici* IIB pattern types contained the largest number of ITS groups. In contrast, there were more *R. leguminosarum* bv. phaseoli types in the unlimed soil with the lowest pH than in soils with the highest pH. The number of ITS ("strain") groups within *R. leguminosarum* bv. phaseoli did not change with increased abundance of rhizobia in the soil, while with *R. tropici* IIB, the number of strain groups increased significantly. Some cultural and biochemical characteristics of *Phaseolus*-nodulating isolates were significantly related to changes in soil properties caused by liming, largely due to changes in the predominance of the rhizobial species groups.

The common bean (*Phaseolus vulgaris* L.) is widely cultivated throughout Brazil in soils that often have low pH and problems with aluminum toxicity, in common with many other bean-growing areas of Latin America and Africa. Amendment of acid soils with lime provides nutrients (Ca and Mg) and creates better conditions for growth of bacterial cells in the short term by altering soil pH and increasing the availability of phosphorus and molybdenum. Long-term changes in soil pH due to liming may also have an impact on the diversity of symbiotic rhizobia, by altering the chemical environment of the soil.

P. vulgaris is known to be a relatively permissive host, nodulating effectively with many rhizobial species (24). In Kenya, the dominant types of *Phaseolus*-nodulating rhizobia differ between an acidic soil and a high-pH soil, with *Rhizobium tropici* dominating in the acidic soil (5). Because *R. tropici* is the most acid-tolerant rhizobium species described to date (12), it is tempting to assume that *R. tropici* might generally be better adapted to acidic soils than other species of *Phaseolus*-nodulating rhizobia.

Soil biodiversity and biodiversity in general are usually described according to the two major components of diversity, species richness and species evenness (17), and these indices have been used in several areas, including studies of soil bacteria and rhizobia (17, 27). Although there have been rapid advances in the phylogeny of rhizobia that have changed our view of the relationships between the rhizobial groups and their host legumes (34), we still lack an ecological framework that can aid understanding of the relative environmental tolerances of different rhizobial genera and types. Such a framework may be elusive, given that many important functions for environmental adaptation may be readily transmissible between bacteria in soil. However, at the same time, a framework would allow our rhizobial classification to have more practical use in predicting the ecology of the different bacteria.

A long-term experiment in southern Brazil that had received replicated lime treatments 6 years prior to sampling and that had been planted annually with the common bean (*P. vulgaris)* in the interim period allowed us to study the selective effects of a gradient of increasing soil acidity on populations of *Phaseolus*-nodulating rhizobia under field conditions. In a separate paper, we reported the interactive effects of planting history and soil acidity on the size of rhizobial populations nodulating *P. vulgaris* (4). The study reported here aimed (i) to examine the diversity within rhizobial populations along gradients of increasing acidity stress in a Brazilian soil, which had been limed and had been cultivated with the common bean; and (ii) to assess the distribution of rhizobial genotypes within these populations and to see whether they were correlated with the soil properties from which they originated. Our initial hypothesis was that the abundance and diversity of rhizobial species and of "strain" types within the species would decrease with increasing soil acidity stress.

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TABLE 1. Selected chemical characteristics of soils from which rhizobia were isolated, number of isolates used in phenotypic and genotypic characterization, and MPN of *Phaseolus*-nodulating rhizobia with confidence intervals[a]

Lime treatment (metric tons ha^{-1})	pH (0.01 M $CaCl_2$)[b]	Al saturation (%)[c]	Ca + Mg (cmol kg of $soil^{-1}$)	No. of isolates[d]		Rhizobial MPN (no. of cells g of $soil^{-1}$)	Confidence interval
				ITS	16S rRNA		
0.0	4.12	36	0.81	38	21	1.57×10^3	0.4×10^3–5.9×10^3
2.2	4.25	27	1.83	34	15	4.34×10^3	1.1×10^3–16.5×10^3
4.4	4.45	11	2.50	38	26	8.25×10^3	2.2×10^3–31.4×10^3
6.6	4.60	7	2.93	37	25	1.16×10^4	0.3×10^4–4.4×10^4
8.8	4.68	4	3.39	40	29	7.57×10^4	2.0×10^4–28.8×10^4

[a] The soil had been planted with *P. vulgaris* without inoculation for the 6 years prior to sampling. The values of pH, Al saturation, Ca plus Mg, and MPN of rhizobia are means of four replications in each liming treatment. Soil analysis and MPN results are from reference 4.
[b] pH measured in 0.01 M $CaCl_2$ solution (1:2:5 soil-solution).
[c] Aluminum saturation was calculated as [Al(KCl)/CEC] × 100, where CEC is the sum of the Al(KCl) and exchangeable Ca, Mg, and K.
[d] Number of isolates used for RFLP analysis of 16S-23S rRNA ITS and 16S rRNA-PCR-amplified DNA.

MATERIALS AND METHODS

Population sizes and rhizobial isolation. The field experiment was established in 1989 with an acidic oxisol in the state of Paraná, South Brazil, with four replicated treatments of lime at rates of 0, 2.2, 4.4, 6.6, and 8.8 metric tons ha^{-1} added only in the year of establishment (4). The experimental plots had been planted each year with the common bean in the summer and lay fallow in the winter. Selected chemical characteristics of the soils are shown in Table 1. Rhizobia were isolated from nodules of common bean plants inoculated with suspensions of soils sampled from the experiment by standard methods (31). Soil sampling was carried out in October 1995 from a depth of 0 to 20 cm. Eighteen subsamples were taken with disinfected (70% alcohol and fire) spades (trade type Hollander) from the central part of each plot (6 by 5 m), always 1 m from the border. Plant inoculation for rhizobial counts and nodule isolation were done between 1 and 2 weeks after soil sampling. For phenotypic and genotypic studies, the isolates were obtained from nodules on plants grown in an N-free nutrient solution (pH 6.7 to 6.8) and inoculated with soil dilutions of 10^{-1} to 10^{-6}. About 50 to 60 bean plants per liming soil treatment from all dilutions (10^{-1} to 10^{-3} or 10^{-5}) were randomized, and approximately 5 to 8 nodules per seedling were sampled (200 isolates per treatment, giving a total of 1,000 isolates). From these, 1 to 2 isolates from each dilution were randomly selected, giving a total of 10 isolates for each of the four replicate plots, or 40 isolates per soil treatment. Of these two, six, two, and three isolates from unlimed soil and soil treated with 2.2, 4.4, and 6.6 metric tons of lime ha^{-1}, respectively, were lost during the experimental analysis.

The most probable number (MPN) of rhizobia was estimated by a plant infection method with *P. vulgaris* (cv. Negro Argel) as the test host grown in growth pouches (31). The pouches were inoculated with 10-fold soil dilutions with four replicates of each dilution step, and numbers were estimated by using the MPNES program (33).

Colony morphology, nodulation on *Leucaena diversifolia*, growth characteristics, and nitrogen and carbon utilization. Experiments were carried out with the 187 isolates to assess (i) colony morphology, (ii) ability to nodulate *L. diversifolia* L. plants in growth pouches in a controlled-environment room at 20°C night and 25°C day temperatures with a 14-h-light/10-h-dark cycle, (iii) growth in tryptone-yeast (TY) medium minus Ca (TY − CA), (iv) growth in Luria-Bertani (LB) broth, (v) growth on yeast mannitol agar (YMA) containing 2% urea, (vi) melanin production, and (vii) colony fluorescence under UV light on TY agar containing the optical brightener Calcofluor (0.02% [vol/wt]). Utilization of various compounds of nitrogen and carbon was tested as described previously (3). After 3 and 5 days, growth was compared with that of isolates growing on YMA plates.

Acidity and Al tolerance of isolates. Washed cells diluted to a density of about 10^3 viable cells ml^{-1} were inoculated in minimal KM media (13, 18). The KM media at pH 4.0 and 4.5 plus 50 μM Al were buffered with 30 mM Homopiperazine-*N*,*N*′-bis-2-(ethanesulfonic acid) (HomoPIPES) (pK_a, 4.32) (6). Stock solutions of Al ($AlCl_3 \cdot 6H_2O$), phosphate ($K_2HPO_4 \cdot 3H_2O$ and KH_2PO_4), thiamine-HCl, calcium pantothenate, and biotin were adjusted to pH 4.0 and 4.5 and sterilized by membrane filtration (0.22-μm pore diameter). Media lacking either altered pH or Al and strains CIAT899 and CIAT632 were included as controls. After 3 and 5 days, the pH was measured, and the growth was evaluated by counting cells by a drop technique on YMA plates.

Plasmid profile analysis and restriction fragment length polymorphism (RFLP)-PCR analysis of the 16S-23S ITS spacer and 16S rRNA genes. Plasmid profiles were determined by horizontal agarose gel electrophoresis by the in-well lysis method (9), with slight modifications. PCR amplifications were carried out as described elsewhere (19) in a Gene Amp PCR System 9600 (Perkin-Elmer) by using an initial denaturation of the template at 94°C for 3 min; followed by 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C), and extension (45 s at 78°C); followed by a final extension at 72°C for 3 min. Primers fC-16S1493 (5′-GGCTGGATCACCTCCTTTCT-3′), previously described (25), and rD-16S132 (5′-CCGGGTTTCCCCATTCGG-3′) were used to amplify the 16S-23S rRNA internally transcribed spacer (ITS) region. For amplification of part of the 16S rRNA, primers fA-16S(11-32) (5′-GGAGAGTTAGATCTTGGCTCAG-3′) (32) and rC-23S(132′) (5′-AGAAAGGAGGTGATCCAGCC-3′) were used. PCR products were digested with the restriction endonucleases *Hae*III, *Dde*I, *Msp*I, *Mbo*I, and *Hha*I for ITS analysis and *Hin*fI, *Hae*III, *Msp*I, *Mbo*I, and *Rsa*I for 16S rRNA analysis (Promega, Madison, Wis.). The PCR products and the restricted fragments were analyzed by electrophoresis in a 1 to 2.5% (wt/vol) agarose gel that was stained with ethidium bromide (1 mg ml^{-1}). Banding patterns were scored directly from gel photographs (Polaroid type 665-positive/negative films), and the isolates were grouped on the basis of the patterns.

Sequencing of PCR products of 16S rRNA gene fragments. Isolates representative of the RFLP-PCR 16S rRNA groups were selected for direct sequencing of the PCR products. The primers (rG-16S [5′-CCCCACCTTCCTCTCGGCT TATC-3′], fA-16S, and rC-23S) and the PCR cycling profiles and conditions were used as described above. PCR products were purified with Mini Centricon-100 columns (Amicon, Inc., Beverly, Mass.). The sequencing reactions were done with a PRISM Ready Reaction Dye-Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer). First, the samples were held at 96°C for 10 s, cooled to 50°C, and held for 5 s, and then they were warmed to 60°C for 3 min. These steps were repeated for 35 PCR cycles in a Gene Amp PCR Perkin-Elmer system 2400. After ethanol precipitation to remove unincorporated dye-labeled nucleotides, the sequencing reactions were run on an ABI PRISM310 (Perkin-Elmer) Genetic Analyzer. Taxonomic assignments were done by comparing partial sequences of the isolates with sequences in the GenBank/EMBL/DDBJ/PDB non-redundant nucleotide database by using version 2.0 of BLAST (NCBI) Basic (1). The partial sequences (550 bp) were aligned and compared with those of the following organisms (accession numbers of the partial or full sequences published in GenBank/EMBL/DDBJ data library in parentheses): *Rhizobium tropici* IIB SEMIA 4080, PRF81 (AF260274); *Rhizobium leguminosarum* bv. phaseoli ATCC 8002 (M55494); *R. leguminosarum* bv. viciae ATC10004^T (U29386); *R. tropici* IIA LMG9518 (X67233); *Rhizobium etli* CFN42^T (U28916); *Rhizobium* sp. strain OR191 (X91211); *Rhizobium mongolense* USDA 1844^T (U898171); *Rhizobium gallicum* bv. gallicum R602^T (U86343); *R. tropici* CIAT 899^T (U89832); *Azorhizobium caulinodans* ORS571 (M55491); *R. tropici* IIA CFN299 (L21837); *Sinorhizobium fredii* USDA 205^T (M74163); *Agrobacterium* bv. 1 LM G11936 (Aj130721); *Rhizobium galegae* HAMBI 540^T (Y12355); *R. galegae* ATCC 43677 (D11343); *Rhizobium huautlense* S02^T (AF025852); *Rhizobium ciceri* UPM-Ca7 (U07934); *Rhizobium giardinii* bv. giardinii H 152^T (U86344); *Bradyrhizobium japonicum* USDA 110 (Z35330); and *Bradyrhizobium elkanii* USDA 94 (D13429). An unrooted phylogenetic tree was inferred with the unweighted-pair-group method of averages (UPGMA) algorithm by using the Bionumerics program (Applied Mathematics, Kortrijk, Belgium). Confidence limits for phylogenetic trees were estimated from bootstrap analysis (1,000 replications).

Genetic diversity. The indices of diversity, richness, and evenness were calculated by using the number of isolates belonging to each plasmid profile, ITS, or 16S rRNA RFLP group. Diversity was calculated by using the Shannon index: $H' = \Sigma S\,[(n_1/n)\ln(n_1/n)]$, where n_1 is the number of isolates in each group and n is

the number of isolates in all groups. For richness, the Margalef index was used: $R_1 = S - 1/\ln(n)$, where S in this case is the number of groups and n is the number of isolates in all groups. The Pielou index was used as a measure of evenness: $E_1 = H'/\ln(S)$, where H' is the Shannon index and S is the number of groups.

The number of rhizobia nodulating *P. vulgaris* ($\log_{10}$ MPN) was used to calculate Pearson correlations (r) between the number of ITS groups within each 16S ribosomal DNA (rDNA) type (diversity) and the size of the rhizobial populations in the soil by the CORR procedure of the Statistical Analysis System (SAS Institute, Inc., 1986). A linear regression was fitted to the number of ITS groups and aluminum saturation, and cluster analysis of phenotypic traits was also performed by the SAS procedure (by using the UPGMA and Euclidean distance routines).

RESULTS

Rhizobial numbers, soil properties, and phenotypic characterization of the isolates. All isolates were fast-growing, and 81% were acid producers, independent of the amount of lime originally applied to the soil. Isolates from soil limed with 2.2 metric tons ha^{-1} and unlimed soil (Al saturation from 27 to 36%) had predominantly opaque dry and small (0.5- to 2-mm) colonies. In contrast, most of the isolates from soil limed with 6.6 or 8.8 metric tons ha^{-1} (Al saturation from 4 to 7%) had large colonies similar to that described for *R. tropici* type IIB (23). Colonies of some isolates had a milky appearance, and when there was confluent growth, the white center of the original colony had a marbled appearance (irregular streaks). Only 19 of 124 isolates tested were able to form nodules on *Leucaena diversifolia*, and this ability was strongly correlated ($r = 0.945$, $P < 0.001$) with increasing aluminum saturation and decreasing pH ($r = -0.932$, $P < 0.001$).

All isolates utilized asparagine, L-alanine, L-leucine, L-tryptophan, and L-tyrosine as sole nitrogen sources, and the media were acidified, except with L-tyrosine, for which no change in pH was observed. L-Arginine and lactose were utilized by 91% of the isolates. *R. tropici* type II (A and B) strains are able to grow in minimal medium containing lactose, although they do not produce 3-ketolactose (23). The other compounds tested as C sources (D-serine and sodium tartrate) were utilized by few of the isolates independent of their origin, while phenylalanine and L-isoleucine were utilized by 71% of isolates from soil limed with 8.8 metric tons ha^{-1} and only 30% of the isolates from other plots.

Neither autoclaving; addition of Al, phosphate, or vitamin solutions; nor growth of rhizobia changed the pH of the buffered medium. There was a pH increase of 0.3 U only after 5 days of growth. Approximately 60% of isolates grew at pH 4.0 within 3 days. Of the 78% of isolates from soil limed with 2.2 metric tons ha^{-1} (pH 4.2 and 27% Al) that grew at pH 4.0, only 29% were able to grow at pH 4.5 with 50 μM Al. There was good agreement between growth in LB medium (pH 4.5 with 50 μM Al), growth in TY − Ca medium, formation of fluorescent colonies on TY-Calcofluor, and utilization of glycine as an N source, but none of these phenotypes was clearly related to the soil properties from which they were isolated. Strains of *R. leguminosarum* (RCR3644) and *R. tropici* type IIA (CFN299) failed to stain with Calcofluor and presented dim colonies, whereas colonies of both *R. tropici* IIB (CIAT899) and *R. etli* (CFN42) fluoresced bright blue-green when exposed to UV light. Several of the isolate groups, which had phenotypes that fluoresced on Calcofluor-containing TY medium, corresponded to groups identified by ITS-RFLP pattern analysis, despite the observation that the Calcofluor-dark phenotype of *Sinorhizobium meliloti* strains was found be plasmid borne (22). Similarly, the ability to grow in LB or TY − Ca medium and utilization of glycine as an N source correlated with the ITS group in which isolates were placed. Growth in YMA containing 2% urea and utilization of lactose (which was generally a good carbon substrate), as well as both tartrate and serine (which only a few isolates were able to use), did not show any correlation with the ITS groups.

Colony morphologies (e.g., gum production) showed significant correlations with pH ($r = -0.895$, $P < 0.001$), Al saturation ($r = -0.829$, $P < 0.01$), and Ca concentrations ($r = 0.889$, $P < 0.001$) in the soils from which the colonies were isolated. Utilization of phenylalanine, arginine, and citrate as carbon substrates by isolates was positively correlated with pH and Ca and negatively correlated with Al saturation in the soil from which the isolates originated, while dulcitol utilization was negatively correlated with the same soil properties. In contrast, utilization of glycine as an N source or serine, tartrate, isoleucine, and lactose as C substrates was not significantly correlated with the properties of the soils from which isolates originated. The number of isolates that produced a black pigment was negatively correlated with the pH ($r = -0.754$, $P < 0.001$) and Ca concentration ($r = -0.732$, $P < 0.001$) of the soil from which they were isolated, while production of brown and black pigments combined was not related to soil properties. Production of black pigment was observed in about 60% of isolates from soils with 27 and 36% Al saturation and in only 13% of isolates from soil with 6% Al saturation (data not shown).

Plasmid profiles. Some isolates from different liming treatments had the same plasmid profile, but most of the plasmid profiles differed between the treatments. Despite this, the overall number of plasmid profile patterns observed in each treatment was remarkably constant (Fig. 1a).

PCR-RFLP of 16S-23S rRNA (ITS) analysis. PCR amplifications resulted in the detection of a single band, with the exception of a couple of isolates that had an extra band of approximately 800 to 950 bp. Amplification of multiple 16S-23S ITSs reflects heterogeneity among the three *rrn* operons in the *R. leguminosarum* genome (20, 27). Isolates from soil that received the highest rate of lime (8.8 metric tons ha^{-1}) had an average ITS size of about 1,350 bp, while isolates from soil limed with 4.4 metric tons ha^{-1} yielded PCR products of about 1,100 bp (data not shown). However, there was no apparent selection for size of uncut PCR products (1,070 to 1,450 bp). PCR amplification of 16S-23S rDNA ITS *R. leguminosarum* strains representing the biovars viciae, trifolii, and phaseoli produced bands ranging from 1,160 to 1,400 bp (20).

The same ITS type was not found across different soil treatments, but the same ITS patterns were found in a few cases in isolates from different replicates within the same liming treatment for four of the treatments. Isolates with the same plasmid profile did not always have equivalent restriction fragment polymorphisms, as shown by the 16S-23S rRNA analysis. The populations from soils that had received lime were more diverse, as indicated by the number of ITS groups, with the number of groups increasing from around 8 in the control plot to almost 20 in the plot that had received the most lime (Fig. 1a).

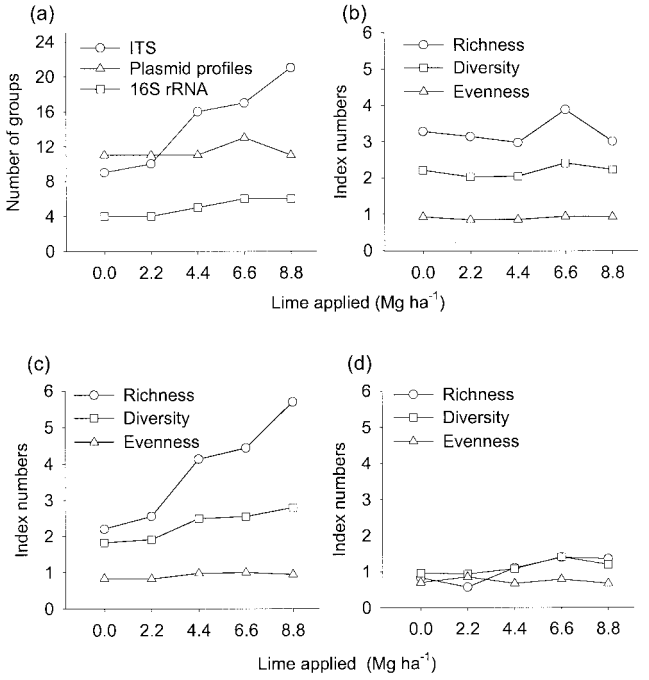

FIG. 1. Diversity of *Phaseolus*-nodulating rhizobial populations from an acidic soil from Brazil as a function of the amount of lime added 6 years earlier. (a) Number of groups assessed by the diversity of ITS profiles, 16S rRNA groups, and plasmid profile groups. (b, c, and d) Indices of richness, diversity, and evenness calculated by using plasmid profiles (b), ITS groups (c), and 16S rRNA patterns (d) revealed by RFLP analysis of 16S-23S rRNA of intergenic spacer (ITS) and 16S rRNA. The soil had been planted with the common bean without inoculation for the 6 years prior to isolation of rhizobia. Data points represent the means from four replicate plots. Mg, metric tons.

16S rDNA PCR-RFLP. The PCR product was a single band of approximately 1,500 bp, as expected from previous reports on bacterial 16S rRNA genes (32), including those from strains of different *Rhizobium* species (20, 21). The number of 16S rDNA PCR-RFLP patterns increased slightly with an increased rate of liming (Table 2 and Fig. 1a). A total of 83% of isolates had profiles similar to those of *R. tropici* type IIB (50%) and *R. leguminosarum* bv. phaseoli (33%) (Table 2).

Partial sequence analysis. The partial 16S rRNA gene sequences of isolates had similarity values ranging from 97 to 100% to type strains of *R. etli*, *R. gallicum*, *R. tropici*, *R. mongolense*, *R. leguminosarum*, *S. meliloti*, *Agrobacterium rhizogenes*, and *Agrobacterium tumefaciens*. A dendrogram built with these partial sequences showed that, in the majority of cases, the partial sequences confirmed the species grouping based on RFLP analysis of the full-length 16S rRNA (Fig. 2). Some of the isolates that were similar to *R. tropici* IIB by RFLP-PCR of the 16S rRNA gene had sequences similar to those of *Agrobacterium*; however, *R. tropici* IIB and *A. rhizogenes* cannot be differentiated by restriction site analysis of the 16S rRNA gene (19, 21). *P. vulgaris* is known to nodulate with *S. meliloti* strains (7, 16).

Diversity indices based on plasmid profiles, ITS, or 16S rRNA groups. The indices of richness, diversity, and evenness calculated by using the number of plasmid profile types did not change with liming (Fig. 1b). In contrast, when based on the number of ITS groups, the Shannon diversity index increased from 1.8 to 2.8 with increasing rates of liming. Similarly, when based on the number of 16S rRNA species types, the Shannon diversity index ranged from 0.9 in unlimed soil to 1.40 with the highest rate of liming (Fig. 1d). The evenness was almost the same in all populations studied. The mean number or richness of different types increased from 2.2 to 5.7 along the soil liming gradient when measured according to the number of ITS groups, while there was an increase from 0.5 to 1.4 when based on the number of 16S rRNA groups (Fig. 1c and d). When the mean was determined across all liming treatments, the bean-nodulating rhizobial populations had a Shannon diversity index of $H' = 3.93$, a richness of $R_1 = 13.95$, and an evenness of $E = 0.91$ based on the number of ITS groups (strains). According to the 16S rRNA analysis, the Shannon index was $H' = 1.29$, and the richness (defined as the mean number of species types within the population) was $R_1 = 1.53$, while the evenness was $E_1 = 0.59$.

DISCUSSION

Rhizobial types, abundance, and soil properties. Soil management practices such as plowing and planting history were the same across the replicated treatments, supporting the con-

TABLE 2. Distribution of 187 *Phaseolus*-nodulating rhizobium isolates among the nine putative species groupings and within each soil population based on 16S rRNA RFLP pattern analysis

16S rRNA group designation	Putative identity[a]	No. of ITS groups with amt of lime applied (metric tons ha^{-1})[b]				
		0.0	2.2	4.4	6.6	8.8
A	*R. tropici* type IIB	1 (7)	4 (15)	9 (23)	7 (14)	12 (24)
B	*R. leguminosarum*	4 (22)	4 (16)	3 (9)	5 (14)	2 (4)
C	*R. etli*	2 (2)		2 (4)	1 (2)	5 (8)
D	*R. tropici* type IIA	2 (7)	2 (3)	1 (1)	1 (1)	
E	Uncertain			1 (1)		1 (2)
F	Uncertain				1 (3)	
G	Uncertain				2 (3)	
H	Uncertain					1 (1)
I	Uncertain					1 (1)

[a] Based on RFLP of 16S rRNA analysis. Uncertain, no definite species affiliation could be made by identification by comparison with type or representative strains of *R. etli* (CFN42^T) and *R. tropici* type IIA (CFN299), which were obtained from P. van Berkum, and *R. tropici* IIB (CIAT899^T) and *R. leguminosarum* bv. phaseoli (RCR3644) which were obtained from Wye College Soil Microbiology collection.

[b] The values in parentheses are the total number of isolates belonging to the putative species (16S rRNA) type within each soil liming treatment.

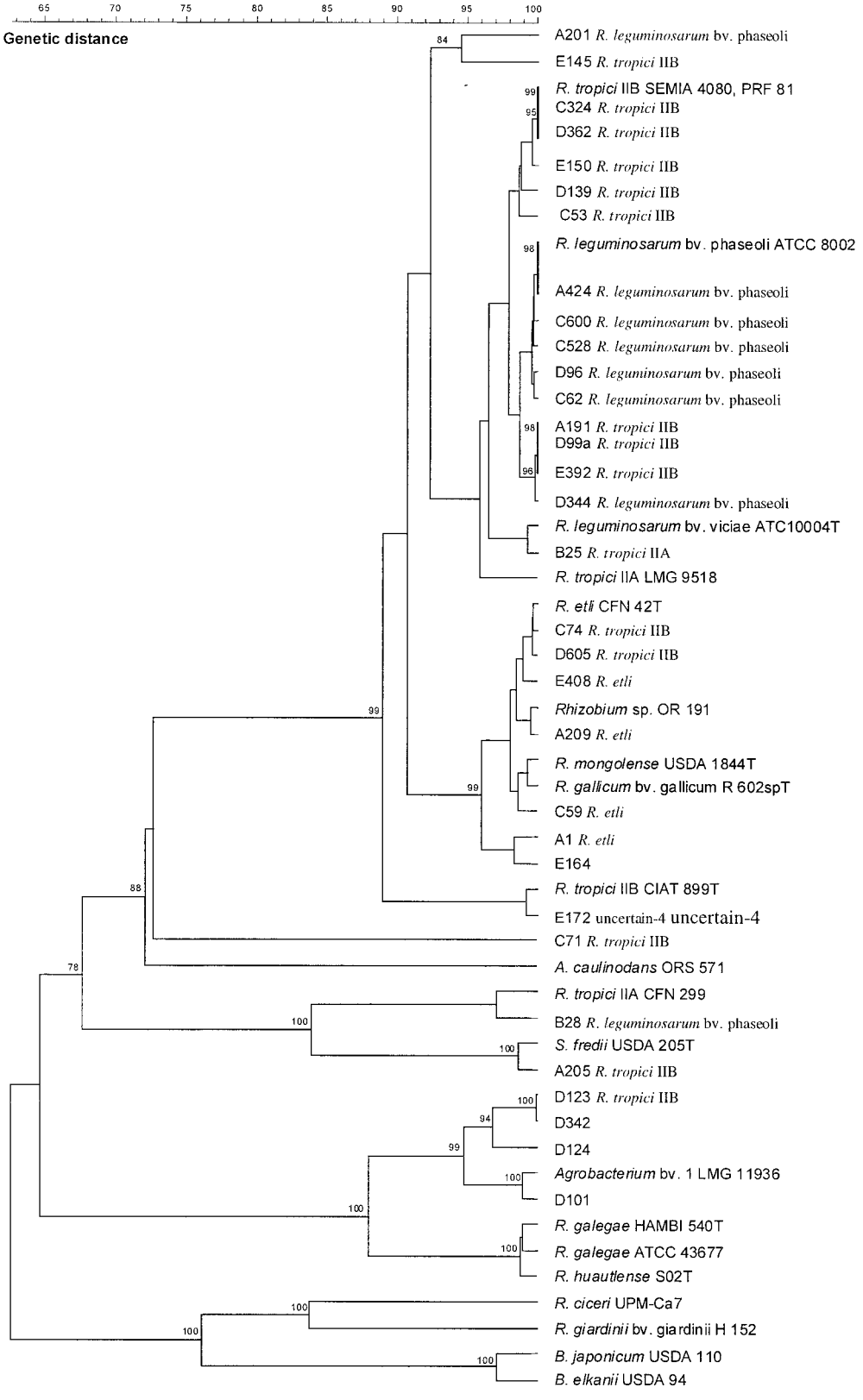

FIG. 2. Dendrogram built with the UPGMA algorithm from multiple alignments of 16S rRNA partial sequences of Brazilian common bean isolates and some related bacteria in the α-subclass of the *Proteobacteria*. For the *Phaseolus*-nodulating isolates, the liming treatment of the soil from which they originate is indicated (A, unlimed soil; B to E, 2.2, 4.4, 6.6, and 8.8 metric tons ha^{-1}, respectively). Numbers at the nodes indicate the percentages of frequencies with which a given branch appeared in 1,000 bootstrapped trees; only values of 75% or greater are shown. From top to bottom, the GenBank/EMBL accession numbers (species name in boldface on the figure) used are AF260274, M55494, U29386, X67233, U28916, X91211, U898171, U86343, U89832, M55491, L21837, M74163, Aj130721, Y12355, D11343, AF025852, U07934, U86344, Z35330, and D13429.

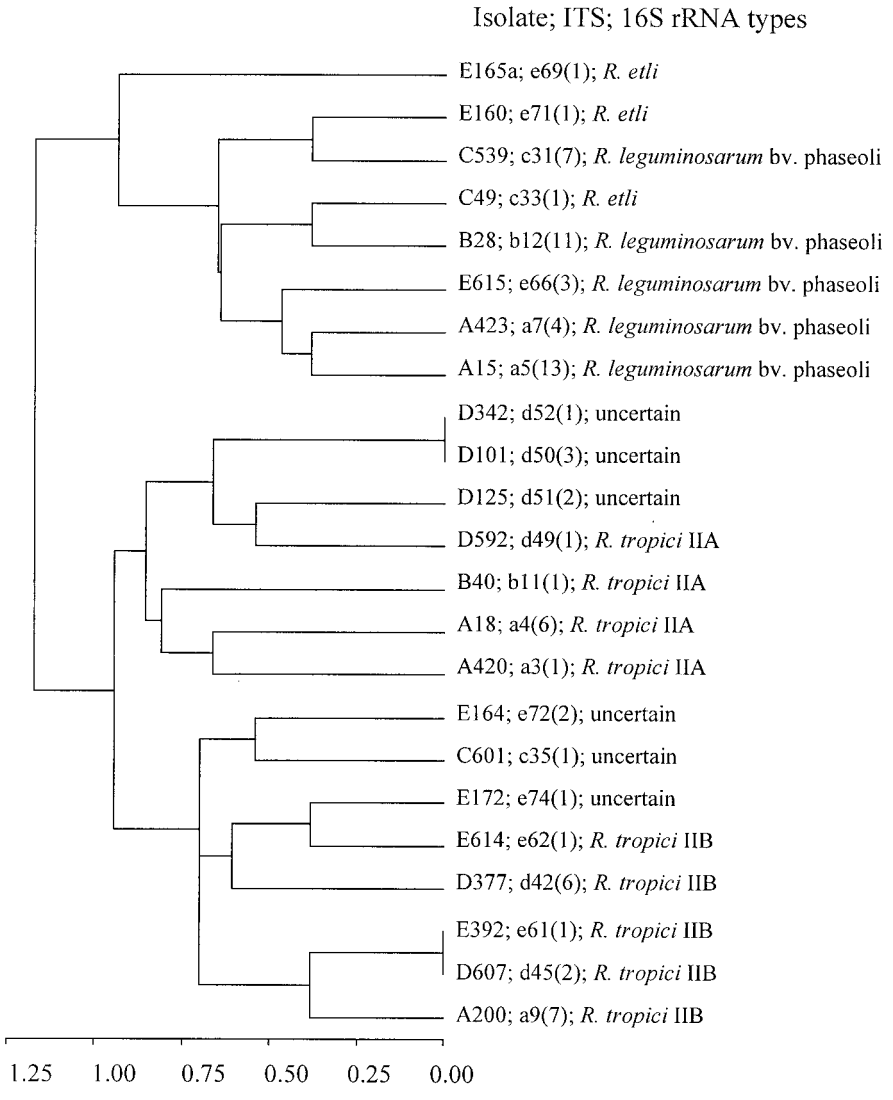

FIG. 3. Dendrogram from cluster analysis (according to matrix and Euclidean distance) of 16 phenotypic traits (growth in LB broth and TY − Ca medium, Calcofluor florescence, urea, carbon and nitrogen utilization, tolerance of Al and pH, and melanin production) of some representative *Phaseolus*-rhizobial isolates of the groupings based on ITS-RFLPs and 16S rRNA RFLPs. The liming treatment of the soil from which the isolates originated is indicated (A, unlimed soil; B to E, 2, 4.4, 6.6, and 8.8 metric tons ha^{-1}, respectively). The values indicated in parentheses are the total number of isolates belonging to the ITS group within each soil liming treatment. Based on RFLP analysis of 16S rRNA DNA, "uncertain" indicates that no definite species affiliation could be made according to identification by comparison with type or representative strains of *R. etli* ($CFN42^T$) and *R. tropici* type IIA (CFN299).

clusion that changes in the abundance of rhizobia and the occurrence of different rhizobial types were due to soil chemical changes after liming. Although the aluminum saturation ranged from 4 to 36% across the soils that had received lime 8 years before, the number of *Phaseolus*-nodulating rhizobia ranged only from 7.6×10^4 cells g of $soil^{-1}$ in the plot with 4% aluminum saturation to 1.57×10^3 cells g of $soil^{-1}$ in the unlimed plot (Table 1). The underlying reason that such large rhizobial populations had been maintained in all cases appeared to be the continuous planting with *P. vulgaris*, because the numbers of rhizobia compatible with *Phaseolus* declined rapidly in the soils with high aluminum saturation when incubated in the laboratory (4). In the same soil, there was no apparent effect of liming on the numbers of rhizobia when counted with *L. diversifolia* as the trap host (4).

The 16S rRNA-RFLP and partial sequence analysis of the 16S rRNA genes indicated that the majority of the rhizobia (83%) were closely related to *R. tropici* IIB or *R. leguminosarum* (Table 2). Changes in the relative abundance of different rhizobial species groupings (Table 2) largely explained the correlations in phenotypic properties across the soil acidity gradient. The predominance of *R. tropici* types in limed soils (Table 2) thus results in the correlations between colony morphology and utilization of C and N substrates. Strains of this species are unable to grow in dulcitol, in contrast with *R. leguminosarum*, *R. etli*, and *R. gallicum* strains (3). Strains be-

longing to *R. tropici* utilized glycine as an N substrate, whereas strains of *R. giardinii*, *R. gallicum*, *R. leguminosarum*, and *R. etli* could not (3).

Only a small proportion of the *R. tropici* isolates from these soils were able to nodulate *Leucaena*, while broad host range was originally described as a phenotype that distinguished *R. tropici* from other *Phaseolus*-nodulating rhizobia (23). It has been reported that some strains of *R. etli* can also nodulate *Leucaena* spp. (15). Ability to nodulate *Leucaena* actually increased with increasing aluminum saturation ($r = 0.945$, $P < 0.001$), although the relative proportion of *R. tropici* in the populations decreased. Cluster analysis based on the 16 phenotypic traits measured showed that these largely supported the genetic groupings based on ITS and 16S rRNA RFLP analyses (Fig. 3).

There was a positive relationship between low soil pH and growth in KM medium at pH 4.0 ($r = 0.857$, $P < 0.001$). In contrast, tolerance of Al in artificial media was apparently not related to the concentration of Al, Ca, or pH in the soil from which the isolates were obtained. It is established that Al tolerance of a rhizobial strain in artificial media does not always reflect its tolerance of Al or acidity in soil (18). It is also important to highlight that there was only a narrow range in pH of 0.6 U among the soils studied, whereas Al saturation varied by 30% between the unlimed soil and the soil that received the highest rate of lime (Table 1).

Indices of diversity and soil characteristics. The three molecular typing methods used to examine diversity among the rhizobial isolates give various degrees of resolution: plasmid profiles are based on the number and size of the plasmids carried by the bacteria, ITS-RFLP patterns are essentially strain groupings based on chromosomal DNA, and the 16S rRNA gene RFLPs are a coarse species grouping. The plasmid profiles essentially showed no change in diversity across the gradient of soil acidity (Fig. 1a and b). The number of plasmid profiles in rhizobial populations was found elsewhere to be negatively correlated with pH (14), although in the earlier study, rhizobia from different locations were compared.

The number of ITS strain groups increased markedly as aluminum saturation decreased from around 8 groups in the control treatment to over 20 groups in the treatment that had received the highest rate of liming (Fig. 4a), with relatively strong increases in both the richness and diversity indices (Fig. 1a and c). Despite the strong increase in the number of ITS groups, the evenness within the populations was relatively constant and close to unity. Thus, none of the groups showed strong dominance in any of the treatments (Fig. 1c). Twelve of 17 phenotypic characteristics evaluated showed significant correlations with the chromosomally based strain groups as defined by the ITS patterns (data not shown). There was also a good agreement between growth at pH 4.0 and ITS groups. The results thus suggest that a decrease in Al was an important factor leading to increases in the diversity of rhizobia (Fig. 1a). Soil acidity accounted for 90% of the genetic diversity of the isolates based on the number of ITS groups, with Al saturation used as a measure of soil changes due to liming (Fig. 4a). The number of species groupings based on 16S rRNA RFLPs increased only slightly with small increases in the diversity indices (Fig. 1a and d). This reflects the presence of RFLP patterns in the soils that had been subject to liming that did not definitely match any of the type strains.

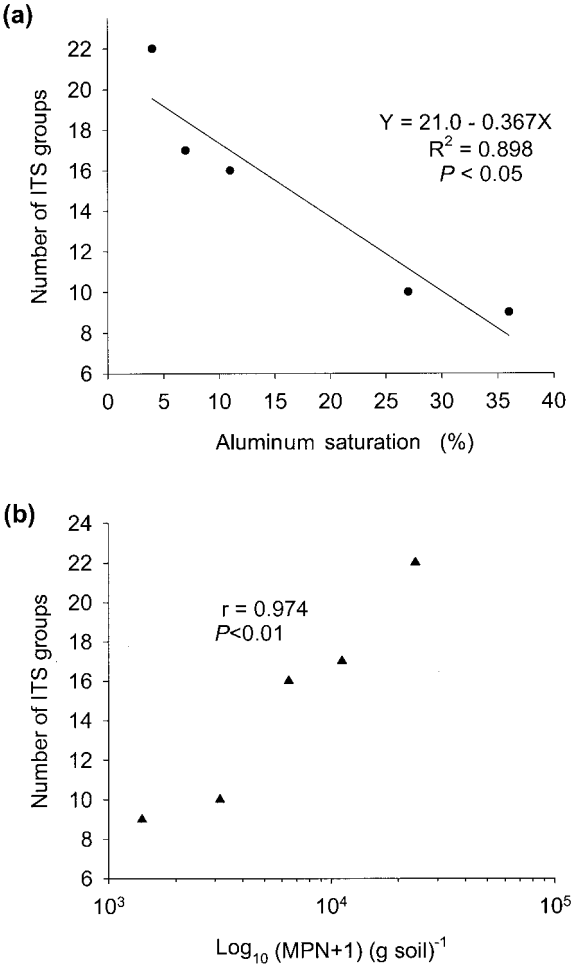

FIG. 4. Relationships between diversity based on the number of ITS groups measured by PCR-RFLP of 16S-23S rRNA and percent aluminum saturation in the soil from which the rhizobia were isolated (a) and diversity based on the number of ITS groups and the size of the rhizobial population (b). The values for the number of rhizobia (MPN) and Al saturation are means of four replications. The numbers of ITS groups were based on isolates from all four replications in each liming treatment.

Genetic diversity and rhizobial abundance. Overall, there was a positive relationship ($r = 0.974$; $P < 0.01$) between the diversity assessed as the number of ITS groups and the abundance of rhizobia (Fig. 4b). This could be attributed to a combination of factors, such as (i) the improved soil conditions leading to better survival of rhizobia, (ii) changes in the soil microbial community overall, and (iii) biotic and abiotic factors that affect plasmid transfer from nonsymbiotic to symbiotic bacteria in the soil. Fernandez-Astorga et al. (10) found a direct relationship between the initial parent cell density and the number of transconjugants of *Escherichia coli* and that the maximum frequency of recombination increased as soil pH increased from 4.7 towards neutrality (28). Harrison et al. (14) found less diversity of *R. leguminosarum* bv. trifolii in acidic soils (pH 4.2 to 4.4), where rhizobial populations were present

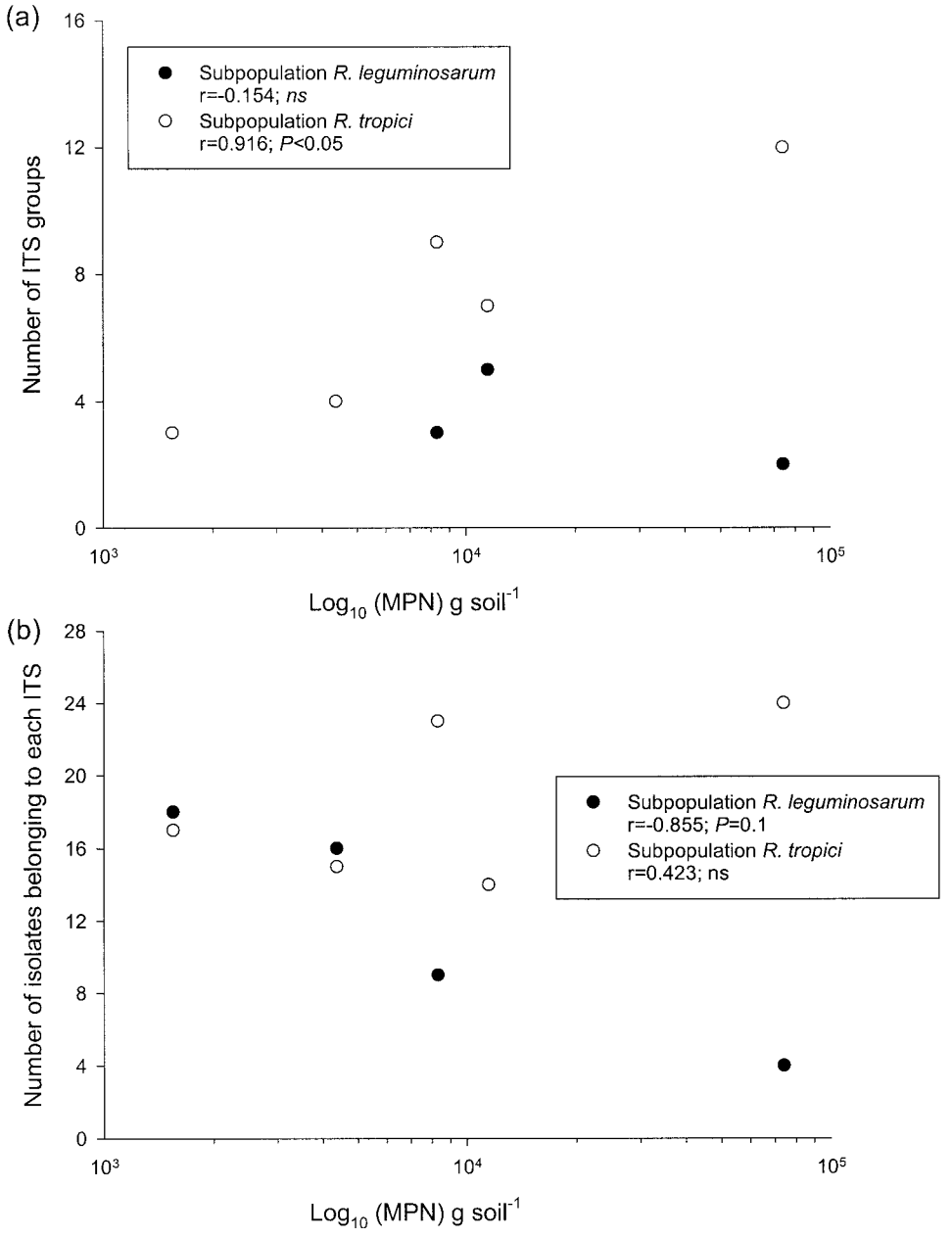

FIG. 5. Relationships between species diversity and the size of the native rhizobial population in the soil from which they were isolated. (a) Number of ITS groups within each subpopulation (*R. leguminosarum* bv. phaseoli profiles and *R. tropici* IIB profiles) in relation to the number of rhizobia estimated by the MPN method. (b) Number of isolates belonging to each ITS group within *R. leguminosarum* bv. phaseoli and *R. tropici* IIB subpopulations in relation to the MPN of rhizobia. *ns*, not significant.

at less than 10^2 per g of soil, than in soils of higher pH, where densities were greater. In contrast to our findings, Strain et al. (29) found that genetic diversity in low-density ($<10^2$ rhizobia) populations of *R. leguminosarum* bv. viciae and trifolii was similar to that in high-density ($>10^5$ rhizobia) populations on the same sites.

Surprisingly, the relationship between diversity as the number of ITS groups and the size of rhizobial populations was species specific (Fig. 5). The number of ITS groups for *R. leguminosarum* profiles changed little with rhizobial population density (MPN) (Fig. 5a). The number of isolates within each ITS group decreased with the overall rhizobial MPN as the proportion of *R. leguminosarum* isolates decreased ($r = -0.855$, $P < 0.1$) (cf. Table 2 and Fig. 5b). In contrast, significant positive relationships ($r = 0.916$, $P < 0.05$) were found between the number of rhizobia and the number of ITS groups for *R. tropici* IIB genotypes (Fig. 5a). The distribution of isolates with profiles similar to those of *R. etli* and *R. tropici* type IIA did not show any clear association with the soil properties from which they were isolated, presumably due to the low number of these types, representing only 17% of the isolates (Table 2). The small proportion of isolates belonging to *R. etli*

in this acid soil may be related to the lesser tolerance of this species to acidic conditions compared with that of *R. tropici* (5, 12). When these soils were incubated in the absence of the legume for 12 months, the numbers of rhizobia declined strongly in the soils with high aluminum saturation (4). The possibility remains that large numbers of *R. leguminosarum* may have survived in acidic soils due to a protective effect of the rhizosphere of *P. vulgaris.*

Is a predictive ecology of rhizobia a realistic goal? The predominance of *R. tropici* was no surprise, because this species has been found in many regions, including temperate and tropical soils (3, 5). Although *R. tropici* type IIB isolates were frequent, few of them could nodulate *Leucaena*, as would have been expected from earlier studies that described this species as a broad-host-range rhizobium (23). The larger number of *R. leguminosarum* types in soil with the lowest pH than in soil with the highest pH was particularly surprising, since several reports have suggested that *R. tropici* is the species best adapted to acid conditions (5, 12). Albeit, Vargas and Graham (30) did not find significant differences between the numbers of *R. tropici* strain CIAT899 (acid tolerant) and *R. leguminosarum* bv. phaseoli strain CIAT632 (acid sensitive) in the rhizosphere of beans at pH 4.5. Likewise, Amarger et al. (2) found that *R. leguminosarum* bv. phaseoli was dominant in soils with low numbers of rhizobia. Nodules with mixed infections, detected first in the isolation step by colony morphology and later confirmed by other phenotypic and genotypic traits, were observed only in populations from soils in which both rhizobial abundance and diversity were highest.

Thus, the decrease in diversity of rhizobial strain types with increasing soil acidity stress based on ITS-RFLP analyses (Fig. 1c) supported our initial hypothesis. However, the detailed analysis showed that the situation was more complex than anticipated, because effects on diversity of rhizobial species types were less clear, with shifts in the dominance of the main species groups and effects on diversity differing between subpopulations.

In other studies, changes in genetic diversity found among populations of *S. meliloti*, *R. leguminosarum* bv. viciae, and *Phaseolus* rhizobia have been attributed to chemical and physical differences between soils (26), history of N fertilization (8), and land management (27). It is difficult to assess and interpret the effects of liming on diversity of rhizobia under field conditions due to the complexity of the soil-plant-microorganism interactions and the number of factors that operate concurrently. Further studies are needed to clarify why these shifts in rhizobial strain diversity and rhizobial species dominance occurred. Maintaining rhizobial diversity could be key to ensuring resilience to further environmental stress or disturbance, as appears to be the case for other organisms (11). Although the common bean appears to be a relatively permissive host, this character does not guarantee effectiveness in N_2 fixation and may actually lead to formation of less effective symbioses with strains not highly evolved to fix N_2 with this legume host. Because little has been done to measure potential benefits of rhizobial diversity, nitrogen fixation, and common bean productivity, we believe that research in this area may be useful in the future.

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