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Rhizobium tropici, a Novel Species Nodulating *Phaseolus vulgaris* L. Beans and *Leucaena* sp. Trees

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A new *Rhizobium* species that nodulates *Phaseolus vulgaris* L. and *Leucaena* spp. is proposed on the basis of the results of multilocus enzyme electrophoresis, DNA-DNA hybridization, an analysis of ribosomal DNA organization, a sequence analysis of 16S rDNA, and an analysis of phenotypic characteristics. This taxon, *Rhizobium tropici* sp. nov., was previously named *Rhizobium leguminosarum* biovar phaseoli (type II strains) and was recognized by its host range (which includes *Leucaena* spp.) and *nif* gene organization. In contrast to *R. leguminosarum* biovar phaseoli, *R. tropici* strains tolerate high temperatures and high levels of acidity in culture and are symbiotically more stable. We identified two subgroups within *R. tropici* and describe them in this paper.

Members of the genus *Rhizobium* nodulate the roots of leguminous plants. The rhizobia that infect peas, clovers, and beans (*Phaseolus vulgaris* L.) are clustered in a single species, *Rhizobium leguminosarum* (29), which has three biovars (*Rhizobium leguminosarum* biovar viciae, *Rhizobium leguminosarum* biovar trifolii, and *Rhizobium leguminosarum* biovar phaseoli); these biovars contain different symbiotic plasmids that encode distinct nodulation specificities. Nevertheless, heterogeneity in *Rhizobium leguminosarum* biovar phaseoli has been identified by using such different criteria as protein pattern (50), antibiotic resistance (2), serological type (49), multilocus enzyme electrophoresis behavior (45), DNA-DNA hybridization data (10, 26, 54), plasmid profile (37), and exopolysaccharide structure (70).

We previously distinguished two different types among isolates obtained from bean nodules and found differences in their symbiotic plasmids (36, 38, 39). Type I strains have multiple copies of nitrogenase *nifH* genes (39, 46), a narrow nodulation host range, and hybridize with the *psi* (polysaccharide inhibition) gene (3). Type II strains have single copies of *nif* genes, nodulate *Leucaena* spp., and do not hybridize with the *psi* gene (36, 39).

Type II strains have received attention because their symbiotic plasmids promote an effective and completely differentiated symbiotic process in *Agrobacterium tumefaciens* recipients (5, 38). They are genetically stable, retaining their symbiotic plasmid after prolonged incubation at 37°C. Some are heat tolerant (31) or acid and aluminum resistant (12, 25, 30, 62). The nodulation genes from one of these strains have been cloned (64). The chemical composition and structure of the extracellular polysaccharides from one type II strain differ from the chemical composition and structure of the extracellular polysaccharides from type I isolates (23).

Type II strains have been less successful in competition for bean nodule occupancy than the type I strains used (41). The former have been reported to occur less frequently in bean nodules (39). Nodule occupancy by type II strains can be enhanced under acid conditions (47, 63).

To define the taxonomic position and the genetic relatedness of type II strains, we analyzed 64 type II strains having different geographical origins and compared them with other species of rhizobia.

For a long time multilocus enzyme electrophoresis has been a standard method used in systematics (44), and this method is perhaps the best approach in large-scale studies to estimate the genetic diversity and structure of related populations (55, 67, 68). The results of multilocus enzyme electrophoresis studies provided the basis for the identification of two previously undescribed species among *Legionella pneumophila* strains (57) and identified two groups of bacteria within *Rhizobium meliloti* (19). Our strategy was to order type II strains by multilocus enzyme electrophoresis and then to characterize these bacteria phenotypically. Representative strains were chosen for total DNA and ribosomal DNA hybridization and for the determination of partial 16S rRNA gene sequences.

On the basis of the criteria analyzed, we propose a new species, *Rhizobium tropici*, which contains two subgroups that correspond to type IIA and type IIB strains.

MATERIALS AND METHODS

Bacterial strains. The strains which we used are listed in Table 1.

Growth conditions. Rhizobia were maintained on yeast extract-mannitol (YM) medium (65), on peptone-yeast extract (PY) medium, (43), or in minimal medium (MM) (17) containing different substrates. Average doubling times were estimated from optical densities recorded at 600 nm every 2 h in PY medium at 30°C. Bacterial swarming was tested by growing strains for 2 days on PY medium supplemented with 0.3% agar.

Nodulation and nitrogen fixation were tested in sterilized Leonard jars (65) containing vermiculite and sand by using *P. vulgaris* cv. Carioca 80 and *L. leucocephala*.

Multilocus enzyme electrophoresis. Cultures derived from single colonies were grown overnight at 30°C in 50 ml of PY

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TABLE 1. Bacterial strains and ETs

Strain	Original host plant	ET[a]	Source or reference[b]
Rhizobium leguminosarum biovar phaseoli (type I)			
CFN 42	*Phaseolus vulgaris* L.		46
Viking I	*Phaseolus vulgaris* L.		49
TAL 182	*Phaseolus vulgaris* L.		Ben Bohlool
BR 10027	*Phaseolus vulgaris* L.		CNPBS
BR 10028	*Phaseolus vulgaris* L.	36	CNPBS
BR 10029	*Phaseolus vulgaris* L.		CNPBS
BR 10030	*Phaseolus vulgaris* L.	37	CNPBS
Rhizobium leguminosarum biovar trifolii			
USDA 2046	*Trifolium pratense* L.		USDA
USDA 2152	*Trifolium subterraneum* L.		USDA
Rhizobium leguminosarum biovar viciae			
USDA 2489	*Vicia faba* L.		USDA
Rhizobium meliloti			
RCR 2011	*Medicago sativa*		51
R.me 1	*Medicago sativa*		CFN
Rhizobium fredii			
USDA 191	*Glycine max*		32
HH 103	*Glycine max*		16
Rhizobium galegae 625 (= gal 3)	*Galega officinalis*		66
Rhizobium loti NZP 2037	*Lotus divaricatus*	39	10
Rhizobium spp.			
CFN 234	*Leucaena leucocephala*	40	39
CFN 265	*Leucaena esculenta*		39
NGR 234		38	60
Type IIA			
CFN 299	*Phaseolus vulgaris* L.	1	38
BR 828	*Leucaena leucocephala*	1	CNPBS
BR 829	*Leucaena leucocephala*	1	CNPBS
BR 830	*Leucaena leucocephala*	1	CNPBS
BR 831	*Leucaena leucocephala*	1	CNPBS
BR 832	*Leucaena leucocephala*	1	CNPBS
BR 833	*Leucaena leucocephala*	1	CNPBS
BR 834	*Leucaena leucocephala*	1	CNPBS
BR 835	*Leucaena leucocephala*	1	CNPBS
BR 836	*Leucaena leucocephala*	1	CNPBS
BR 10031	*Phaseolus vulgaris* L.	1	CNPBS
BR 10032	*Phaseolus vulgaris* L.	1	CNPBS
BR 10033	*Phaseolus vulgaris* L.	1	CNPBS
BR 10034	*Phaseolus vulgaris* L.	1	CNPBS
BR 10035	*Phaseolus vulgaris* L.	1	CNPBS
BR 10036	*Phaseolus vulgaris* L.	1	CNPBS
BR 10037	*Phaseolus vulgaris* L.	1	CNPBS
BR 10038	*Phaseolus vulgaris* L.	1	CNPBS
BR 837	*Leucaena leucocephala*	1	CNPBS
BR 838	*Leucaena leucocephala*	1	CNPBS
BR 839	*Leucaena leucocephala*	1	CNPBS
BR 840	*Leucaena leucocephala*	1	CNPBS
BR 10039	*Phaseolus vulgaris* L.	1	CNPBS
BR 841	*Leucaena leucocephala*	1	CNPBS
C-05-I	*Phaseolus vulgaris* L.	1	Tsai
Car 22	*Phaseolus vulgaris* L.	1	Tsai
UMR 1163	*Phaseolus vulgaris* L.	1	Graham
BR 10040	*Phaseolus vulgaris* L	2	CNPBS
BR 10041	*Phaseolus vulgaris* L.	3	CNPBS
BR 10042	*Phaseolus vulgaris* L.	4	CNPBS
BR 842	*Leucaena leucocephala*	5	CNPBS
BR 843	*Leucaena leucocephala*	6	CNPBS
BR 844	*Leucaena leucocephala*	7	CNPBS
BR 845	*Leucaena leucocephala*	8	CNPBS
C-05-35	*Phaseolus vulgaris* L.	9	Tsai
BR 10043	*Phaseolus vulgaris* L.	10	CNPBS
BR 10044	*Phaseolus vulgaris* L.	11	CNPBS
BR 10045	*Phaseolus vulgaris* L.	12	CNPBS
BR 846	*Leucaena leucocephala*	13	CNPBS
UMR 1178 (= IAPAR 47)	*Phaseolus vulgaris* L.	14	Graham

Continued on following page

TABLE 1—*Continued*

Strain	Original host plant	ET[a]	Source or reference[b]
Type IIB			
BR 847	*Leucaena leucocephala*	15	CNPBS
BR 848	*Leucaena leucocephala*	15	CNPBS
BR 849	*Leucaena leucocephala*	15	CNPBS
BR 850	*Leucaena leucocephala*	16	CNPBS
BR 851	*Leucaena leucocephala*	17	CNPBS
BR 852	*Leucaena leucocephala*	18	CNPBS
BR 853	*Leucaena leucocephala*	19	CNPBS
BR 854	*Leucaena leucocephala*	20	CNPBS
UMR 1410 (= CIAT 166)	*Phaseolus vulgaris* L.	21	Graham
CIAT 899[T]	*Phaseolus vulgaris* L.	22	25
AD 822		22	Quinto
AD 4		22	Quinto
BR 855	*Leucaena leucocephala*	23	CNPBS
BR 856	*Leucaena leucocephala*	24	CNPBS
C-05 II	*Phaseolus vulgaris* L.	24	Tsai
BR 857	*Leucaena leucocephala*	25	CNPBS
BR 858	*Leucaena leucocephala*	26	CNPBS
BR 859	*Leucaena leucocephala*	27	CNPBS
BR 860	*Leucaena leucocephala*	28	CNPBS
BR 861	*Leucaena leucocephala*	29	CNPBS
BR 862	*Leucaena leucocephala*	30	CNPBS
CFNE 101	*Phaseolus vulgaris* L.	31	CFN
BR 863	*Leucaena leucocephala*	32	CNPBS
Type II			
UMR 1226 (= IAPAR 70)	*Phaseolus vulgaris* L.	33	Graham
BR 864	*Leucaena leucocephala*	34	CNPBS
UMR 1173 (IAPAR 69)	*Phaseolus vulgaris* L.	35	Graham
Agrobacterium tumefaciens C58			61

[a] ET is the combination of mobility alleles of electromorphs.

[b] Sources: Ben Bohlool, B. Ben Bohlool, NifTAL Project, Paia, Hawaii; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de Mexico, Cuernavaca, Mexico; CNPBS, Centro Nacional de Pesquisa em Biología do Solo, Seropédica 23851, Rio de Janeiro, Brazil; USDA, Beltsville *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, Md.; Graham, P. Graham, Department of Soil Sciences, University of Minnesota, St. Paul; Tsai, M. Tsai, Universidade de Sao Paulo, Sao Paulo, Brazil; Quinto, C. Quinto, Centro de Ingeniería Genética y Biotecnología, Universidad Nacional Autónoma de Mexico, Cuernavaca, Mexico.

medium and then centrifuged, suspended in 1 ml of 10 mM $MgSO_4$, and sonicated twice for 20 s with a 20-s rest by using an MSE sonifier equipped with a microtip at 50% pulse with ice cooling. Lysates were stored at −70°C.

The procedures used for starch gel electrophoresis and activity assays for specific enzymes have been described by Selander et al. (56). The following eight metabolic enzymes were assayed: alcohol dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, xanthine dehydrogenase, indophenol oxidase (superoxide dismutase), hexokinase, and phosphoglucomutase. The buffer system used was Tris-citrate (pH 8). The mobility variants of each enzyme were numbered in order of decreasing anodal mobility. At least five different electrophoretic assays were performed for each of the 65 strains for each enzyme tested. The distinctive combinations of electromorphs (mobility variants of each enzyme) were designated electrophoretic types (ETs) (56). The ET was determined for each strain.

The genetic diversity for an enzyme locus was calculated as follows: $h = (1 - \Sigma x_i^2)n/(n - 1)$, where x_i^2 is the frequency of the ith allele and n is the number of ETs. The mean genetic diversity per locus (H) was the arithmetic average of h values for the eight loci (56). The genetic distance between each pair of ETs was estimated as the proportion of loci at which dissimilar alleles occurred. Clustering from a matrix of pairwise genetic distances was performed by using the average linkage method (58).

DNA-DNA hybridization. DNA was purified from cells that were treated with sodium dodecyl sulfate (1%, wt/vol), Pronase (50 μg/ml), and RNase (10 μg/ml) and then subjected to serial extractions with phenol-chloroform (1:1, vol/vol) and precipitation with NaCl and ethanol. The DNA concentration was estimated spectrophotometrically at 260 nm. Total DNA digested with *Eco*RI was subjected to electrophoresis in 1% agarose gels. The DNA was transferred to nylon filters (59) and hybridized (21) to DNA previously digested with *Eco*RI and labeled with ^{32}P by nick translation (48) (10^8 cpm/μg of DNA). The labeled DNAs were from three reference strains, strains CFN 299 (type IIA), CIAT 899[T] (T = type strain) (type IIB), and *Rhizobium meliloti* RCR 2011. Autoradiography was performed at −70°C for 24 h; filter lanes were cut and counted with a Beckman scintillation counter. The percentage of total homologous hybridization was calculated for each strain tested.

Ribosomal DNA hybridization. The restriction fragment length polymorphisms of the rRNA operons were determined by hybridizing total DNA *Eco*RI, *Xho*I, and *Hin*dIII digests probed with plasmid pKK3535 (7). This plasmid carries a 7.5-kb *Bam*HI fragment containing the *Escherichia coli rrnB* operon cloned in plasmid pBR322.

Numerical taxonomy. A total of 51 strains were characterized, and 118 different characteristics were analyzed. For testing substrate utilization, 5-μl drops of freshly prepared bacterial suspensions (approximately 10^5 bacteria) were ap-

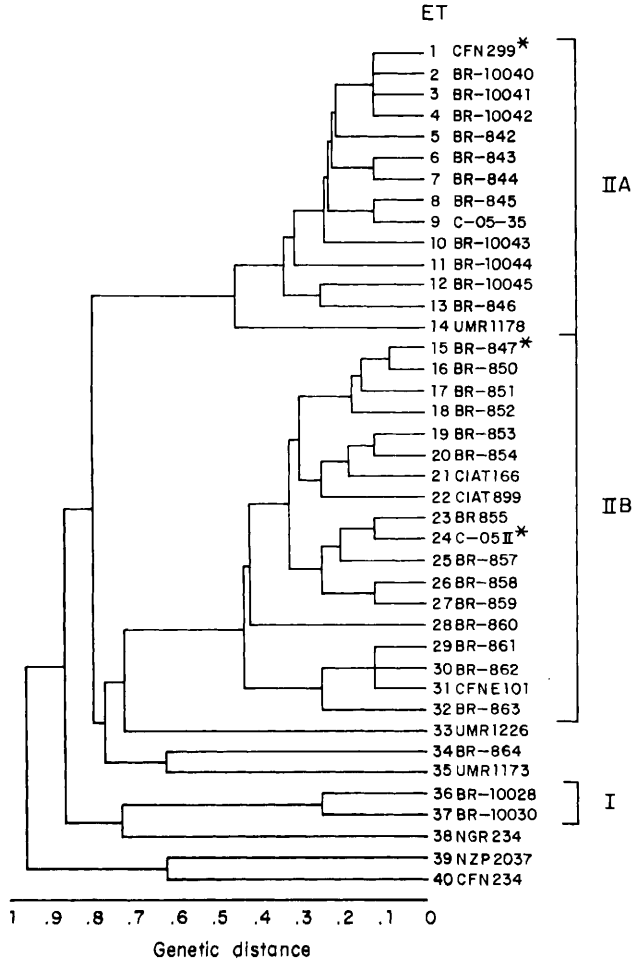

FIG. 1. Dendrogram showing levels of genetic relatedness among 35 ETs of type II strains, 2 ETs of type I strains, and 3 ETs of outgroup reference strains. This dendrogram was based on electrophoretically detectable allelic variation at enzyme loci. The asterisks indicate that other strains having the same ET are included in Table 1.

TABLE 2. Allele profiles at eight enzyme loci of 40 ETs

ET	Type or taxon	No. of isolates	Alleles at the following enzyme loci[a]:							
			HEX	IDH	XDH	MDH	ADH	IPO	G6P	PGM
1	IIA	27	5	5	5	5	5	5	5	5
2	IIA	1	5	5	5	5	5	5	5	5.1
3	IIA	1	5	5	5	5	5	5	5	4.9
4	IIA	1	5	5	5	5	5	5	5	5.5
5	IIA	1	5	5	5	5.5	5	5	5	5
6	IIA	1	5	5	4.8	5	5	5	5	5
7	IIA	1	5	5	5.5	5	5	5	5	5
8	IIA	1	5	4.5	5	5	5	5	5	5
9	IIA	1	5	6	5	5	5	5	5	5
10	IIA	1	4.9	5	5	5	5	5	5	5
11	IIA	1	5.5	5	5	5	5	5	5	6
12	IIA	1	5	4.8	5.1	5	5	5	5	5
13	IIA	1	5	5	5.1	5	6	5	5	5
14	IIA	1	5	5	5.5	5	4.7	5	5.2	5
15	IIB	3	5	4	6	6	4	7	4	5
16	IIB	1	5	4	6	6	5.9	7	4	5
17	IIB	1	5	4	6	6	4	6.5	4	5
18	IIB	1	5	3.5	6	6	4	7	4	5
19	IIB	1	5	4	6.1	6	4	7	4	5
20	IIB	1	5.5	4	6.1	6	4	7	4	5
21	IIB	1	5	4.5	6.1	6	4	7	4	5
22	IIB	1	4.5	3	6.1	6	4	7	4	5
23	IIB	1	5.2	5	6.1	6	4	7	4	5
24	IIB	2	5.2	5	6	6	4	7	4	5
25	IIB	1	5.2	3.9	6.1	6	4	7	4	5
26	IIB	1	5.2	4	6	6	4	7	5	5
27	IIB	1	5.2	4	6	6	4	7	4	5
28	IIB	1	5	5	4.5	6	5.8	7	4	5
29	IIB	1	5	4	7	6	4.5	5	4	5
30	IIB	1	5	4	6.1	6	4.5	5	4	5
31	IIB	1	5	4	6	6	4.5	5	4	5
32	IIB	1	5	4	5	6	4.5	7	4	5
33	II	1	7	6	6.1	4.9	4	5	7	5
34	II	1	5	4.5	6	6	5.9	5	6	4
35	II	1	6	6	4.5	6	5.8	5	6	5.5
36	I	1	5	3	4	4	6	6	7	5.5
37	I	1	3	3	4	4	6	6	7	4
38	*Rhizobium loti*	1	2	3	1	1		2	4.9	5
39	*Rhizobium* sp.	1	6	6	5	4	7	8	6	6
40	*Rhizobium* sp.	1	6	6	7	3	3	4	5.5	6

[a] HEX, hexokinase; IDH, isocitrate dehydrogenase; XDH, xanthine dehydrogenase; MDH, malate dehydrogenase; ADH, alcohol dehydrogenase; IPO, indophenol oxidase; G6P, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase.

plied to plates containing MM (17) lacking vitamins to which filter-sterilized substrates had been added. When substrates were tested as nitrogen sources, ammonium sulfate was not included and glucose was added at a concentration of 1 g/liter. The plates were incubated at 30°C unless indicated otherwise. The following compounds were tested for utilization as sole carbon sources (at a concentration of 1 g/liter unless indicated otherwise): L-alanine, L-arginine, L-aspartate, L-phenylanine, glycine, L-glutamate, L-glutamine, L-isoleucine, L-leucine, L-lysine, L-histidine, L-methionine, L-proline, L-serine, L-threonine, L-tyrosine, L-tryptophan, L-valine, hypoxanthine, ornithine, nopaline, octopine, α-ketoglutarate, D-fructose, D-galactose, D-glucose, D-glucosamine, D-glucose-6-phosphate, lactose, D-glucuronate, D-mannose, mannitol, D-ribose, sorbose, D-sorbitol, succinate, acetate, anthranilate, casein hydrolysate, citrate, formate, isovalerate, D-malate, nicotinate, oxalate, L-tartrate, starch, sarcosine, urea, glycerol, ethanol, phenol (0.25 g/liter), and methanol. The following compounds were tested for utilization as sole nitrogen sources (at a concentration of 0.5 g/liter): ammonium sulfate, L-aspartate, glycine, L-glutamate, L-glutamine, ornithine, L-tyrosine, and L-tryptophan. We also determined requirements for ascorbic acid (100 μg/ml), biotin (100 μg/ml), folic acid (100 μg/ml), and pantothenate (100 μg/ml).

Tolerance to antibiotics and tolerance to sodium hypochloride were tested by growing organisms on MM containing kasugamycin, lincomycin, oleandomycin, sulfamide, or trimethoprim (each at a concentration of 20 μg/ml) or by growing organisms on PY medium containing carbenicillin (30 or 50 μg/ml), chloramphenicol (30 or 100 μg/ml), erythromycin (100 μg/ml), gentamicin (25 μg/ml), kanamycin (30 μg/ml), neomycin (60 μg/ml), novobiocin (20 μg/ml) polymyxin B (20 μg/ml), rifampin (50 μg/ml), spectinomycin (100 μg/ml), streptomycin (100 μg/ml), tetracycline (1, 5, or 10 μg/ml), or sodium hypochloride (0.12%, wt/vol).

Additional tests included growth on PY medium at 10, 30, 37, and 40°C; growth on PY medium containing 1.0, 1.5, and 2% NaCl; growth on PY medium at pH 4, 5, 6, 8.5, and 10.5; growth on liquid PY medium lacking calcium; growth on

TABLE 3. Genetic diversity at eight enzyme loci among ETs

Enzyme locus[a]	Characteristics of:							
	40 ETs[b]		35 ETs[c]		18 ETs[d]		14 ETs[e]	
	No. of alleles	h[f]	No. of alleles	h	No. of alleles	h	No. of alleles	h
HEX	9	0.615	7	0.537	4	0.576	3	0.275
IDH	8	0.785	8	0.737	6	0.621	4	0.396
XDH	10	0.494	8	0.808	5	0.680	4	0.582
MDH	7	0.646	4	0.543	1	0	2	0.142
ADH	9	0.806	7	0.739	4	0.530	3	0.275
IPO	7	0.641	3	0.532	3	0.386	1	0
G6P	7	0.696	5	0.611	2	0.111	2	0.142
PGM	7	0.391	7	0.309	1	0	5	0.506
Mean	8	0.619	6	0.602	3	0.363	3	0.289

[a] For abbreviations see Table 2, footnote *a*.
[b] The total sample for 40 ETs examined.
[c] The 35 ETs of the *Rhizobium leguminosarum* biovar phaseoli type II strains.
[d] The 18 ETs of the type IIB strains.
[e] The 14 ETs of the type IIA strains.
[f] $h = (1 - \Sigma x_i^2)\, n/(n-1)$, where x_i^2 is the frequency of the ith allele and n is the number of ETs.

Luria broth (LB); colony morphology on PY medium, YM medium, and MM containing various carbon sources; and acid production on YM medium containing bromothymol blue (0.0025%, wt/vol) as an indicator. Plates were incubated at 30°C unless otherwise specified, and growth was recorded at 3 and 5 days after inoculation. The results were analyzed by the mixed parsimony method, using the Wagner criterion (33).

Nucleotide sequences of 16S rRNA genes. The nucleotide sequences of the 16S rRNA genes of type I strain CFN 42 and type II strains CIAT 899^T, CFN 299, and UMR 1173 were determined by directly sequencing double-stranded polymerase chain reaction products with Sequenase 2 (U.S. Biochemical Corp.). A 491-bp region corresponding to nucleotides 872 through 1,363 of the *A. tumefaciens* 16S rRNA gene was amplified by using a GenAmp DNA amplification reagent kit (Perkin Elmer Cetus) with a 28-mer (CCGCA CAAGCGGTGGAGCATGTGGTTTA) and a 30-mer (CTTG TACACACCGCCCGTCACACCATGGGA) as primers. The reaction was carried out according to the instructions of the manufacturer by using 30 cycles, as follows: 30 s at 95°C for denaturation, 30 s at 55°C for primer annealing, and 3 min for polymerization at 72°C. The polymerase chain reaction products were purified by using QIAGEN tip 20 minicolumns as recommended by the manufacturer.

Both strands of three independent double-stranded polymerase chain reaction products from each strain tested were sequenced with Sequenase by using the method of Casanova et al. (8) and the same primers as those used in the amplification procedure.

We used the program LINEUP to manually align the sequences with the following corresponding sequences obtained from GenBank: *Rickettsia rickettsii* M21293, *Rickettsia typhi* M20499, *Rickettsia prowazekii* M21789, *A. tumefaciens* M11223, *Rochalimea quintana* M11927, and *Brucella abortus* X13695. Phylogenetic distances were determined by using the DISTANCES program of the University of Wisconsin GCG Sequence Analysis Software Package (14). An unweighted pair group method tree was constructed, with the standard errors of branch points determined by using the unweighted pair group method standard error program (42).

TABLE 4. Relative levels of homology at 65°C between DNAs from *Rhizobium* species and reference DNAs from type IIA, type IIB, and *Rhizobium meliloti* strains

Strain	% Of DNA hybridization with the following reference strains:		
	CFN 299 (type IIA)	CIAT 899^T (type IIB)	*R. meliloti* RCR 2011[a]
Rhizobium leguminosarum biovar phaseoli			
Viking I	21	15	
CFN 42	26	19	
Rhizobium leguminosarum biovar trifolii			
USDA 2046	20	30	
USDA 2152	17	23	
Rhizobium leguminosarum biovar viciae USDA 2489	16	27	
Rhizobium meliloti RCR 2011	20	15	100
Rhizobium fredii			
USDA 191	15	24	20
HH 103	20	23	
Rhizobium galegae 625	12	24	25
Rhizobium loti NZP 2037	10	15	19
Rhizobium spp.			
CFN 234	21	38	
CFN 265	16	27	
NGR 234		15	
Type IIA			
CFN 299	100	36	29
C-05-35	96	26	22
UMR 1178	73	14	
BR 10035	98		
Type IIB			
CIAT 899^T	39	100	14
UMR 1410		88	
BR 859		72	
BR 856		85	
BR 863		62	
Type II			
BR 864	18	22	
UMR 1173		38	
UMR 1226		28	
Agrobacterium tumefaciens			
C58	10	17	

[a] *Rhizobium meliloti* RCR 2011 was included only as a reference strain to test the hybridization conditions used in this work.

Nucleotide sequence accession numbers. The ribosomal gene sequences reported below for the different strains have been deposited in GenBank/EMBL nucleotide sequence databases under accession numbers M64317, M64318, M64319, and M64405.

RESULTS

Multilocus enzyme electrophoresis. Figure 1 shows that the type II strains were divided into two groups (types IIA and IIB), both of which differed from type I strains. Type II strains and type I strains were at a genetic distance of 0.86, while type IIA strains and type IIB strains were at a genetic distance of 0.79. Type IIA strains exhibited greater homogeneity than type IIB strains; the mean genetic diversity was 0.289 for the former and 0.363 for the latter. A total of 27 type IIA strains from various geographical origins were identical as determined by the mobilities of the eight metabolic enzymes tested and formed ET 1. The majority of the bean isolates tested could be separated into three groups on

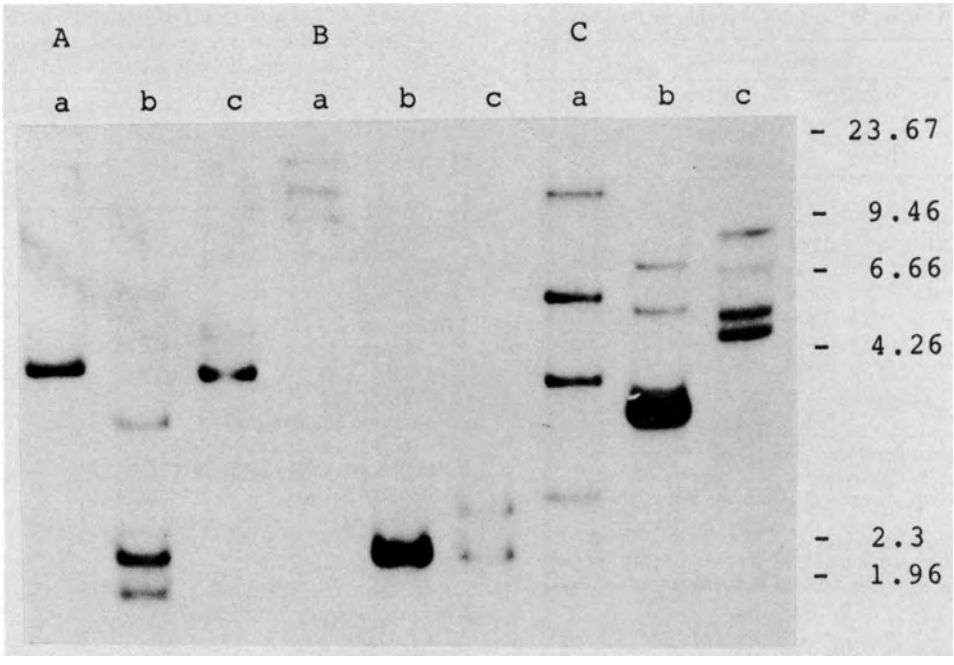

FIG. 2. Autoradiogram of *Eco*RI (A), B) *Hin*dIII (B), and *Xho*I (C) ribosomal restriction fragment length polymorphism patterns of type I strain CFN 42 (lanes a), type IIA strain CFN 299 (lanes b), and type IIB strain CIAT 899^T (lanes c). The positions of the molecular weight markers (in kilobases) are shown on the right.

the basis of the allelic responses at the loci for malate dehydrogenase and, in the majority of the strains, at the loci for indophenol oxidase (Table 2); these groups basically corresponded to type I, type IIA, and IIB type strains. Type IIA and IIB strains shared alleles at the hexokinase and phosphoglucomutase loci, but exhibited very small mobility differences at the glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, and xanthine dehydrogenase loci. Alcohol dehydrogenase activity was difficult to detect in type IIB strains but not in type IIA strains. The genetic diversity at each enzyme locus is shown in Table 3.

To determine the location of the genes coding for these metabolic enzymes in type II strains, derivatives of strains CIAT 899^T, AD 4, and AD 822 lacking either the 200-kb plasmid or the 400-kb plasmid were evaluated. Identical enzyme mobility variants were obtained for all eight enzymes tested, suggesting that, as in *E. coli* (56), these traits are chromosomally determined.

```
          5'--->3'
          1                                                        50
          CtGtAGAGat gCAGGGtcaC TTCGGtggCG aagaACAGGT GCTGCATGGC
Cfn42      T C    GC A     GTT       GAC   GCAC
Cfn299     A C    AG T     TCA       TGG   AAGA
Ciat899    T T    AT G     TCA       TGG   AAGA
Umr1173    T T    AT G     TCA       TGG   AAGA

          51                                                      100
          TGTCGTCAGC TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC
          101                                                     150
          AACCCTCGCC CTTAGTTGCC AGCATTtaGT TGGGCACTCT AAGGGGACTG
Cfn42                                TG
Cfn299                               TA
Ciat899                              CA
Umr1173                              TA

          151                                                     200
          CCGGTGATAA GCCGAGAGGA AGGTGGGGAT GACGTCAAGT CCTCATGGCC
          201                                                     250
          CTTACGGGCT GGGCTACACA CGTGCTACAA TGGTGGTGAC AGTGGGCAGC
          251                                                     300
          GAGCACGCGA GTGTGAGCTA ATCTCCAAAA GCCATCTCAG TTCGGATTGC
          301                                                     350
          ACTCTGCAAC TCGAGTGCAT GAAGTTGGAA TCGCTAGTAA TCGCGGATCA
          351
          GCATGC
```

FIG. 3. Aligned sequences of parts of the 16S rRNA genes from strains CFN 42 (type I), CFN 299 (type IIA), CIAT 899^T (type IIB), and UMR 1173 (type II), corresponding to nucleotides 954 to 1,109 from the *A. tumefaciens* gene. Only the differences from the consensus sequence (at the top) are shown. From nucleotide 151 on the four sequences are identical.

ETs 33, 34, and 35 shared some phenotypic characteristics with type IIB strains but were separated from them by a genetic distance of 0.78, low levels of DNA-DNA hybridization with type IIB reference strain CIAT 899^T (Table 4), and differences in ribosomal gene sequences (see below).

DNA-DNA hybridization. Four type IIA strains and five type IIB strains constituted homogeneous groups with relatively high levels of DNA homology (91.7% for type IIA strains with reference strain CFN 299 and 81.4% for type IIB strains with reference strain CIAT 899^T) (Table 4). DNAs from other *Rhizobium* species, including *Rhizobium leguminosarum* biovar phaseoli, *Rhizobium leguminosarum* biovar trifolii, and *Rhizobium leguminosarum* biovar viciae (27), as well as *Rhizobium galegae* (35, 66), *Rhizobium loti* (28), *Rhizobium meliloti*, *Rhizobium fredii* (9, 53), and unclassified rhizobia, exhibited less than 30% hybridization with total DNA from either strain CFN 299 or strain CIAT 899^T.

Ribosomal gene organization and sequence. Figure 2 shows the restriction fragment length polymorphisms of rRNA operons of strains CFN 42 (type I), CFN 299 (type IIA), and CIAT 899^T (type IIB); the hybridization patterns for these strains were clearly different. Four type IIA strains had patterns identical to the pattern of strain CFN 299 in *Eco*RI digests. Similarly, seven type IIB strains had the same restriction fragment length polymorphisms in *Eco*RI digests as strain CIAT 899^T (data not shown).

Figure 3 shows the DNA sequences of the 16S RNA gene fragments obtained from strains CFN 42 (type I), CFN 299 (type IIA), CIAT 899^T (type IIB), and UMR 1173 (type II, ET 35), and Fig. 4 shows the phylogenetic tree obtained by

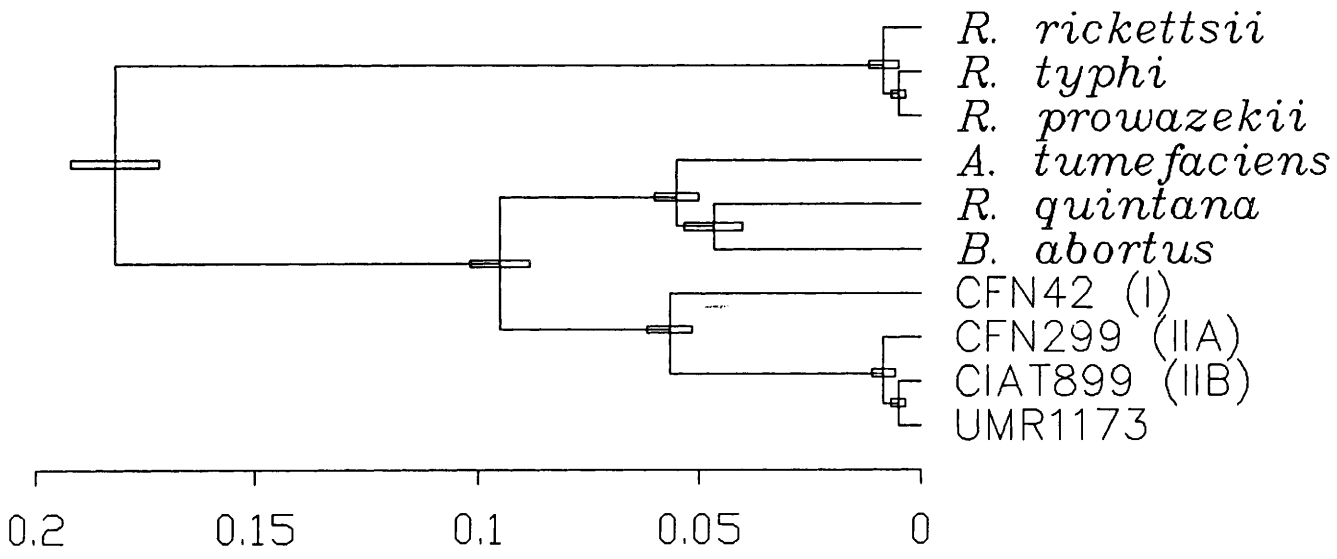

FIG. 4. Unweighted pair group with branching point standard error tree (42) derived from 16S RNA gene fragment sequences of *Rickettsia rickettsii*, *Rickettsia typhi*, *Rickettsia prowazekii*, *A. tumefaciens*, *Rochalimea quintana*, *Brucella abortus*, *Rhizobium leguminosarum* biovar phaseoli, and type II strains.

the unweighted pair group method. The tree is in agreement with the known phylogeny of proteobacteria. The three type II strains formed independent branches that were separated from type I strain CFN 42, and these strains formed a different cluster than the other members of the *Rhizobiaceae*, which in turn were in a different lineage than the rickettsiae. The internal phylogeny of type II strains is not clearly defined, as shown by the overlapping of the standard error bars in Fig. 4.

Numerical taxonomy. We characterized 51 strains, 35 type II strains representing each of the ETs of type II strains and 16 other strains, including *Rhizobium leguminosarum* biovar phaseoli, *Rhizobium leguminosarum* biovar trifolii, *Rhizobium leguminosarum* biovar viciae, *Rhizobium meliloti*, *Rhizobium galegae*, *Rhizobium loti*, and *Rhizobium* sp. strain NGR 234.

The rhizobia did not utilize the following compounds as carbon sources: starch, nicotinate, oxalate, ethanol, methanol, phenol, L-methionine, L-phenylalanine, L-threonine, L-alanine, and L-valine. No strain grew on PY medium at pH 3 or 4 or on PY medium supplemented with 1, 1.5, or 2% NaCl. All of the strains tested grew on α-ketoglutarate, D-fructose, D-galactose, D-glucose, D-glucosamine, glucuronate, D-mannose, mannitol, D-ribose, L-tyrosine, and L-tryptophan as carbon sources and on L-glutamate, L-glutamine, and L-tyrosine as nitrogen sources. Table 5 shows some of the relevant phenotypic characteristics of the strains. The results of the complete-linkage cluster analysis obtained by the mixed parsimony method in which 118 characteristics were considered are shown in Fig. 5; these results are in agreement with the dendrogram derived from multilocus enzyme electrophoresis, but on the basis of the phenotypic characteristics the type IIA and type IIB clusters appear to be more distinct.

TABLE 5. Relevant phenotypic characteristics of *Rhizobium* strains[a]

Characteristic	*Rhizobium leguminosarum* biovar phaseoli	Type IIA strains	Type II strains
Nodulation and nitrogen fixation in *Leucaena* spp.	−	+	+
Colony morphology on PY medium	Gummy	Creamy	Creamy
Growth on LB	−	−	+
Growth on PY medium lacking calcium	−	−[b]	+[c]
Growth on PY medium containing antibiotics[d]	−	−	+[c]
Growth on MM containing arginine as a C source	−	−	+
Growth on MM containing malate	+[c]	−[b]	+[e]
Growth on MM containing hypoxanthine	−[f]	−	+[e]
Growth on MM containing sorbitol	−[f]	−	+[e]
Maximum growth temp (°C)	35	37	40
Colony morphology on YM medium	Wet, translucent	White, opaque	Wet, translucent
Motility on 0.3% agar	+	−	+

[a] The substrate and antibiotic concentrations used are described in Materials and Methods.
[b] More than 90% of the strains were negative.
[c] More than 90% of the strains were positive.
[d] The antibiotic used was carbenicillin, spectinomycin, chloramphenicol, or rifampin.
[e] More than 60% of the strains were positive.
[f] More than 60% of the strains were negative.

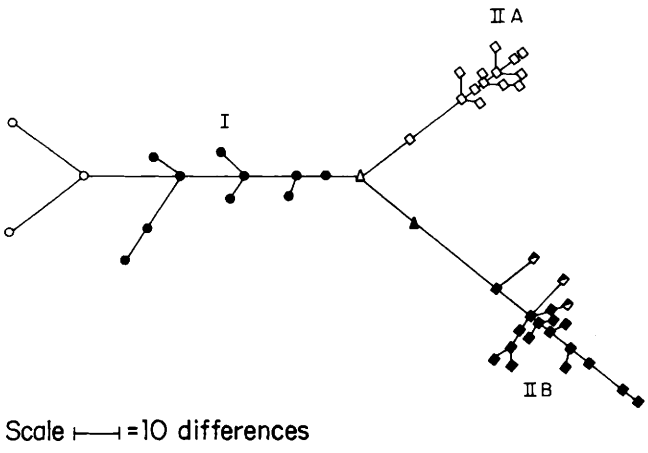

FIG. 5. Cladogram derived from a mixed parsimony analysis of the phenotypic characteristics of *Rhizobium meliloti* and strain NGR 234 (○), *Rhizobium leguminosarum* (●), *Rhizobium loti* (△), and *Rhizobium galegae* (▲), as well as type IIA strains (◇), type IIB strains (◆), and type II unclassified strains (ET 33, 34, and 35) (◈).

General characteristics. Type II strains are gram-negative, rod-shaped, nonsporeforming bacteria that are 1.5 to 2 μm long, are peritrichous, and produce acid in YM medium. The average doubling times are 2 and 1.67 h for type IIA and type IIB strains, respectively, at 30°C in PY medium. These organisms do not produce 3-ketolactose (1) but do grow in MM containing lactose, and they are nalidixic acid resistant, as are most *Rhizobium leguminosarum* biovar phaseoli strains. The type II strains listed in Table 1 nodulate *P. vulgaris* cv. Carioca 80, and some strains are as efficient as the best type I strains.

Type IIA strains are nonmotile on soft agar, while type I and IIB strains are motile. Only about 10% of the 64 type II strains analyzed produce melanin, whereas this is a very a common characteristic among *Rhizobium leguminosarum* biovar phaseoli strains (4).

DISCUSSION

Research on rhizobia that nodulate bean plants (*P. vulgaris*) has frequently revealed strains which have behavior that is considered atypical for *Rhizobium leguminosarum* (5, 23, 25, 26, 38, 39, 64, 70). Nevertheless, all of these organisms have been classified as *Rhizobium leguminosarum* biovar phaseoli, which has resulted in a genetically heterogeneous group. Therefore, we propose that a group of these strains should be assigned to a new species, *Rhizobium tropici*. The considerations described below support such an assignment. These bacteria have a wider host range, including *Leucaena* spp., carry single *nif* gene copies, and exhibit low levels of DNA-DNA hybridization with other *Rhizobium* species. Furthermore, the genetic distances as calculated by multilocus enzyme electrophoresis and by 16S rRNA sequence comparisons are well beyond the acceptable threshold that separates bacterial species.

Like other *Rhizobium* species (13, 22), *Rhizobium tropici* sp. nov. strains have two glutamine synthetases (20), and the *nod* and *nif* genes are plasmid borne (5, 38). Our results for the general pattern of utilization of carbon compounds are in accordance with the patterns reported by Dreyfus et al. for *Rhizobium* strains (17). Melanin production was not considered as a phenotypic characteristic in our taxonomic analysis as it is plasmid encoded in *Rhizobium leguminosarum* biovar phaseoli (4) and is widespread among different *Rhizobium* species (11).

Rhizobium leguminosarum biovar phaseoli (type I) strains have been reported to be an assembly of lineages with considerable genetic distances among them (45). *Rhizobium tropici* sp. nov. also encompasses at least two distinct clusters. Strains belonging to one of the groups (type IIA) require calcium for growth on PY medium and do not grow on LB. They form white opaque colonies on YM medium and are nonmotile on 0.3% agar. The maximum temperature for growth is 35 to 37°C. However, type IIB strains do not require calcium on PY medium, do grow on LB, form wet translucent colonies on YM medium, and are motile on 0.3% agar, and their maximum temperature for growth is 40°C. In contrast to type IIA strains, type IIB isolates grow on arginine, malate, hypoxanthine, and sorbitol as carbon sources. They are resistant to chloramphenicol, carbenicillin, spectinomycin, rifampin, and the metals Ni, Pb, Co, Cu, Ag, and Cr (41a). Type IIA strains are susceptible to both the antibiotics and the metals. Taking into consideration these differences, taxonomists in the future may consider it convenient to define the two groups as subspecies.

Isolation of bacteria from *P. vulgaris* nodules does not always provide *Rhizobium leguminosarum* biovar phaseoli (15) or *Rhizobium tropici* sp. nov. strains. Under laboratory conditions, beans nodulate with a wide range of rhizobia (6, 18, 24, 34, 39, 52), in many cases effectively (39). A comprehensive taxonomy of these strains will require further research. ETs 33, 34, and 35 described above did not cluster with type I, type IIA, or type IIB strains, nor did FL strains obtained from nodules of bean plants grown in *Leucaena* fields (39, 45), bean rhizobium isolates from France (source, N. Amarger, INRA, 21034 Dijon Cedex, France), or strain B599 (from E. Schmidt, University of Minnesota, St. Paul) (52a). A high level of diversity among the tree rhizobia has been reported as well (71). Furthermore, *Rhizobium* taxonomy must deal with a large number of diverging lineages that share symbiotic capabilities (40). Additional genera and species of root and stem nodule bacteria will be needed to accommodate this diversity (69).

Description of *Rhizobium tropici* sp. nov. *Rhizobium tropici* (tro' pi. ci. Gr. n. *tropikos*, tropics; N. L. gen. n. *tropici*, from the tropics). These bacteria are aerobic, gram-negative, nonsporeforming flagellated rods that are 0.5 to 0.7 by 1.5 to 2 μm. Colonies are circular, convex, semitranslucent, and usually 2 to 4 mm in diameter within 2 to 4 days on PY agar medium. They grow on YM medium and PY medium, and some strains grow on LB. The optimum pH for growth ranges from 5 to 7, and the temperature at which growth occurs may be as high as 40°C. All strains are nalidixic acid resistant. These strains, which have been isolated from tropical areas, nodulate and fix nitrogen on *P. vulgaris*, *Leucaena esculenta*, and *Leucaena leucocephala*. They are distinguished from other species at the molecular level by the results of whole-DNA hybridization tests, their multilocus enzyme electrophoresis profiles, and their ribosomal gene sequences.

The well-studied type IIB strain CIAT 899 (= ATCC 49672) is designated the type strain. It has the characteristics described above for *Rhizobium tropici* sp. nov. Like other type IIB strains, it grows on LB, and it is resistant to heavy metals and to the antibiotics chloramphenicol, spectinomycin, carbenicillin, and streptomycin.

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ADDENDUM

At the request of our colleagues we classified other strains obtained from *P. vulgaris* nodules as follows: *Rhizobium leguminosarum* biovar phaseoli CIAT 151, CIAT 632, CIAT 652, CIAT 7123, CIAT 7033, CIAT 7100, CIAT 7116, CIAT 7047, CIAT 7052, CIAT 7061, CIAT 7062, CIAT 7064, CIAT 7070, Kim 5 Sm, H2C, Arg 641.2, Arg 634.2, Arg 634.1, Arg 645.1, Arg 637.2, Arg 651.2, Arg 629.2, Arg 640.2, Arg 632.2, Arg 648.1, Arg 651.1, Arg 646.1, and Arg 645.2; and *R. tropici* CIAT 7069, CIAT 2560, Arg 635.2, G348, G522, G763, G842, G867, and G887.

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