

## Reclassification of American *Rhizobium leguminosarum* Biovar Phaseoli Type I Strains as *Rhizobium etli* sp. nov.

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**A new *Rhizobium* species that nodulates *Phaseolus vulgaris* L. is proposed on the basis of a sequence analysis of 16S ribosomal DNA. This taxon, *Rhizobium etli* sp. nov., was previously named *Rhizobium leguminosarum* biovar phaseoli (type I strains) and is characterized by the capacity to establish an effective symbiosis with bean plants, the reiteration of the nitrogenase structural genes, the organization of the common nodulation genes into two separate transcriptional units bearing *nodA* and *nodBC*, the presence of the polysaccharide inhibition gene, *psi*, and the 16S ribosomal DNA sequence. An analysis of the sequence of a fragment of the 16S rRNA gene shows that this gene is quite different from the gene of *R. leguminosarum*. In addition, all *R. etli* strains have identical sequences. We describe these analyses and discuss additional evidence supporting our proposal.**

The bacteria of the genus *Rhizobium* nodulate the roots of leguminous plants. *Rhizobium* species have been defined primarily on the basis of phenotypic characteristics such as host range, colony morphology, growth on selective media, and certain metabolic attributes. The bacteria that infect peas, clovers, and beans (*Phaseolus vulgaris* L.) have been clustered in a single species, *Rhizobium leguminosarum*, which has three biovars (*R. leguminosarum* biovar viciae, *R. leguminosarum* biovar trifolii, and *R. leguminosarum* biovar phaseoli) (11). This subdivision is based mainly on the presence of different symbiotic plasmids coding for distinct nodulation specificities in a uniform chromosomal background. The uniformity of this background has been supported by different types of data; the symbionts of peas, clovers, and beans cannot reliably be distinguished on the basis of phenotypic characteristics (17). Under laboratory conditions, recombination of chromosomal genes among isolates belonging to the different biovars can be obtained easily (9, 13); symbiotic plasmids conferring different symbiotic capacities can be transferred among strains isolated from peas, clovers, and beans and remain functional (7, 10, 14). DNA-DNA hybridization levels are higher among isolates belonging to the three biovars than to isolates belonging to other related species (3); the nucleotide sequences of a 16S rDNA fragment from different isolates obtained from peas, clovers, and beans have been found to be identical (6, 25).

This classification has been challenged by several lines of evidence. Heterogeneity in *R. leguminosarum* biovar phaseoli has been identified by a variety of methods, including protein patterns in two-dimensional polyacrylamide gel electrophoresis (21), antibiotic resistance profiles, serological types, DNA-DNA hybridization data (8), plasmid profiles, and exopolysaccharide structures (16). The genetic structures of several *R. leguminosarum* populations have been determined. Young's analysis of three enzymes in a collection of strains belonging to the three biovars of *R. leguminosarum* isolated in English fields showed that there is a considerable amount of sharing of enzyme mobility variants

among all strains (mostly between isolates of *R. leguminosarum* biovar viciae and *R. leguminosarum* biovar trifolii). However, the strains assigned to *R. leguminosarum* biovar phaseoli were weakly polymorphic (24). In contrast, Piñero et al. established in a study of the genetic structures of a collection of isolates of *R. leguminosarum* biovar phaseoli obtained mostly from Mexico that there is a very high level of heterogeneity in the chromosomal structural genes (19). The genetic distance and genotypic diversity observed were the highest ever reported for any single species of bacterium.

Two different types of strains can be distinguished among isolates from bean nodules. Some strains exhibit the characteristics expected of *R. leguminosarum* biovar phaseoli strains (type I strains) (15), and others have been characterized as a separate species, *Rhizobium tropici* (formerly type II strains), on the basis of multilocus enzyme electrophoresis data, DNA-DNA hybridization data, ribosomal DNA organization data, data from a sequence analysis of 16S ribosomal DNA, and data from an analysis of phenotypic characteristics (16). As part of an analysis of the structure of a nonsymbiotic population of *R. leguminosarum* isolates by multilocus enzyme electrophoresis, we found that bona fide *R. leguminosarum* biovar phaseoli isolates from our collection formed a cluster separate from *R. leguminosarum* biovar viciae and *R. leguminosarum* biovar trifolii (22).

Recently, Eardly et al. analyzed the phylogenetic position of several symbionts of *Phaseolus vulgaris* L. on the basis of the nucleotide sequence of a fragment of the 16S rRNA gene (6). They found that one *Rhizobium* sp. type I strain, previously classified as *R. leguminosarum* biovar phaseoli type I, showed a conspicuous number of nucleotide sequence mismatches compared with the *R. leguminosarum* type strain. To avoid confusion below, we follow the suggestion of Eardly et al. and refer to all American isolates obtained from *Phaseolus vulgaris* as *Rhizobium* sp. type I. Type I strains were first described by Martínez et al. (15).

We determined the nucleotide sequences of the same region of the 16S rRNA gene from different strains representing the main lineages of *Rhizobium* sp. type I American strains and from a *R. leguminosarum* biovar viciae Mexican isolate. Our results confirm that these strains form a lineage independent from *R. leguminosarum*. We thus propose a

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new species, *Rhizobium etli*, which contains one biovar, *Rhizobium etli* biovar *phaseoli*.

## MATERIALS AND METHODS

**Bacterial strains.** The following *Rhizobium* sp. type I bean isolates were used: CFN 1 (electrophoretic type [ET] 1), CFN 42<sup>T</sup> (T = type strain) (ET 25), Viking 1 (ET 29), CFN 3 (ET 32), and Nitragin 8251 (ET 33). The ETs correspond to those of Piñero et al. (19). In addition, L6, a *Rhizobium* sp. strain isolated from *Pisum sativum* in Huitzilac, Morelos, Mexico, was also analyzed.

**Growth conditions.** Bacteria were maintained in yeast-mannitol (YM) medium and in peptone-yeast extract (PY) liquid medium for DNA isolation.

**DNA isolation.** DNA was purified from cells treated with sodium dodecyl sulfate (1% [wt/vol])–Pronase (50 µg/ml), and then subjected to serial extractions with phenol-chloroform (1:1 [vol/vol]) and precipitation with ethanol and NaCl as described previously (16).

**Nucleotide sequence of 16S rRNA genes.** The nucleotide sequence of the 16S rRNA genes was determined by directly sequencing double-stranded polymerase chain reaction products with Sequenase 2 (U.S. Biochemical Corp.). A 318-bp fragment of the 16S rRNA gene corresponding to positions 20 to 338 of the *Escherichia coli* 16S rRNA sequence was amplified with primers Y1 (5'-TGGCTCA GAACGAACGCTGGCGGC-3') and Y2 (5'-CCCACTGCT GCCTCCCGTAGGAGT-3') as described previously (6, 25). The polymerase chain reaction products were purified by using Magic PCR Preps DNA purification system columns (Promega) and the method suggested by the manufacturer. Direct sequencing with Sequenase was carried out in the presence of 0.5% (vol/vol) Nonidet P-40 (a modification of the method of Casanova et al. [2]).

**Nucleotide sequence analyses.** Nucleotide sequences were aligned by using the PILEUP program of the Genetics Computer Group Sequence Analysis Package (5). J. Felsenstein's PHYLIP 3.4 DNADIST program was used to determine the Jukes-Cantor distances of the aligned sequences (12), and then the unweighted pair group algorithm (UPGMA) was used to determine the phylogenetic relationships (18).

**Nucleotide sequence accession numbers.** We used the following GenBank (release 72) sequences: *R. leguminosarum* 8002, M55494; *R. tropici* CIAT 899, M55233; *Rhizobium* sp. strain FL27, M55234; *Rhizobium* sp. type I strain Olivia 4, M55235; *Rhizobium* sp. strain Or191, M55236; *Agrobacterium tumefaciens*, M11223; *Rochalimea quintana*, M11927; *Brucella abortus*, X13695; *Rhodobacter sphaeroides*, X53855; *Rhodobacter capsulatus*, M34129; *Rhodomicrobium vannielii*, M34127; *Azorhizobium caulinodans*, M55491; *Rhodopseudomonas palustris*, M55496; *Bradyrhizobium* sp. strain NZP 2257, M55486; *Bradyrhizobium* sp. strain BTAi1, M55492; *Bradyrhizobium japonicum* USDA 110, M55485; *Bradyrhizobium japonicum* USDA 59, M55489; *Bradyrhizobium japonicum* USDA 31, M55487; *Rhizobium meliloti* A, M55242; *R. meliloti* B, M55243; and *Rhizobium fredii*, M74163.

## RESULTS

**Nucleotide sequence analysis.** In order to be able to compare previously published *Rhizobium* 16S rRNA gene sequences, we amplified a region of the gene from position 20 to position 338. This region has been extensively used before

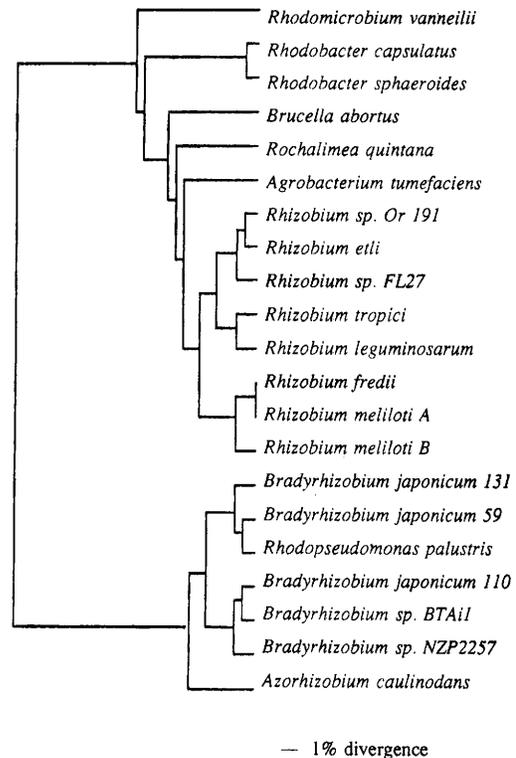


FIG. 1. UPGMA grouping derived from a Jukes-Cantor pairwise distance matrix of the aligned sequences of 16S ribosomal DNA fragments from *Rhizobium* spp. and related bacteria.

to determine taxonomic relationships among rhizobia as it is long and variable enough to permit reliable conclusions (6, 25). The sequences of this 16S rRNA gene fragment in all strains, including the pea isolate, were identical to the sequence previously reported for *Rhizobium* sp. type I strain Olivia 4 (6).

**Phylogenetic analysis.** In order to determine the phylogenetic position of our strains in relation to other *Rhizobium* and *Bradyrhizobium* species, we aligned the sequence obtained with known sequences. We then constructed a pairwise Jukes-Cantor distance matrix (12) based on the multiple alignment obtained. Phylogenetic relationships were determined by the UPGMA grouping method (18). The result of this analysis is shown in Fig. 1.

**Taxonomic relationships.** All *Rhizobium* sp. type I isolates belong to a lineage independent from the *R. leguminosarum* lineage. They are more closely related to strains isolated from beans than to any other *Rhizobium* species, as shown in Fig. 1. All species capable of nodulating beans, even those which have a broader host range, are members of a single group separate from *R. meliloti* and *R. fredii* but included in a larger group of fast-growing *Rhizobium* spp. The slowly growing rhizobia form an independent cluster composed of the different *Bradyrhizobium* species, including the phototrophic strain BTAi1.

## DISCUSSION

It has been shown that the bacteria able to establish an effective symbiosis with beans form a very heterogeneous group on the basis of a variety of criteria, such as protein patterns in two-dimensional polyacrylamide gel electro-

phoresis, antibiotic resistance profiles, serological types, DNA-DNA hybridization data, plasmid profiles, and exopolysaccharide structure (6, 15). We recently proposed a new species, *R. tropici*, comprising the bean isolates previously called *R. leguminosarum* biovar phaseoli type II (16). One of the main characteristics of these bacteria was the capacity to establish an effective symbiosis with both *Phaseolus vulgaris* and *Leucaena* spp. This broad host range distinguished them from the isolates called *R. leguminosarum* biovar phaseoli type I, as the latter have been shown to be restricted to beans (15). *R. leguminosarum* biovar viciae and *R. leguminosarum* biovar trifolii isolates, collected in fields in England, were found to share a considerable number of electrophoretic variants of three metabolic enzymes. Nevertheless, *R. leguminosarum* biovar phaseoli isolates were only weakly polymorphic and shared enzyme genotypes with isolates belonging to the other two biovars (24). The genetic cohesiveness of this group was confirmed by the absence of 16S rRNA sequence variation (6).

In contrast, the genetic diversity of a collection of *Rhizobium* sp. type I isolates, mostly from Mexico, was found to be very high (19). The genetic structure of this collection shows that there are among *Rhizobium* sp. type I strains at least three main lineages separated by a genetic distance greater than 0.6. In another study of genetic variation it was found that *Rhizobium* sp. type I isolates belonged to a different cluster than *R. leguminosarum* biovar viciae and *R. leguminosarum* biovar trifolii reference strains. This was also shown by a lower DNA-DNA hybridization level (22). Eardly et al. sequenced a fragment of the 16S rRNA gene from several bean isolates. They found that the sequence from strain Olivia 4, a *Rhizobium* sp. type I strain, was markedly different from that of *R. leguminosarum*. This indicated that this strain does not belong to *R. leguminosarum* (6).

We determined the sequence of the same fragment of the 16S rRNA gene from *Rhizobium* sp. type I strains CFN 1, CFN 42<sup>T</sup>, Viking 1, CFN 3, and Nitragin 8251. These strains represent the three main lineages found among *Rhizobium* sp. type I strains (19). We also included strain L6, an isolate obtained from *Pisum sativum* in Mexico. In spite of the large genetic distance existing among these strains, we found that all of the sequences are identical to that reported for strain Olivia 4. This indicates that *Rhizobium* sp. type I isolates belong to a single, cohesive genetic group separate from *R. leguminosarum*.

*Phaseolus vulgaris* L. originated in the Americas. Cultivated common beans arose from multiple domestications in Mesoamerica and in Andean South America and are thus an introduced crop in Europe. All of the American *Rhizobium* isolates shown in Fig. 1 share the capacity to establish an effective symbiosis with bean plants although their principal host may be unrelated to beans. Strain Or 191 was originally isolated from field-grown alfalfa (6), strain FL21 was isolated from a bean plant in a field of *Leucaena* trees (19), and *R. tropici* effectively nodulates *Phaseolus vulgaris* and *Leucaena* spp. trees (16). Ostensibly, the only non-American species is *R. leguminosarum*. Although *R. leguminosarum* bv. phaseoli establishes a completely effective symbiosis with bean plants, apparently its only host, this capacity is plasmid borne. It seems that *R. leguminosarum* biovar phaseoli might have arisen from the addition by horizontal transfer of the symbiotic plasmid from a *Rhizobium* sp. type I strain to a *R. leguminosarum* chromosome. Several lines of evidence support this assumption. The genetic diversity of the *Rhizobium* sp. type I strains, which also are specific for

beans, is much higher than that of *R. leguminosarum* biovar phaseoli strains (19, 24). The American origin of the *Rhizobium* sp. type I strains speaks of a much longer history of coexistence with beans. The *R. leguminosarum* biovar phaseoli strains and the *Rhizobium* sp. type I strains share a number of genetic characteristics, and most of them seem to be plasmid borne. Genetic analyses have been focused on two strains, *R. leguminosarum* biovar phaseoli 8002 and *Rhizobium* sp. type I strain CFN 42<sup>T</sup>. The main characteristics distinguishing these strains from strains belonging to other *Rhizobium* species have been the presence of reiterations of the nitrogenase reductase structural genes (20), the existence of the polysaccharide inhibition gene, *psi* (1), and the separation of the *nodA* and *nodBC* genes (4, 23).

The existence of widespread nonsymbiotic *Rhizobium* sp. type I isolates has been shown previously (22). These nonsymbiotic bacteria share a common chromosome with symbiotic *Rhizobium* sp. type I strains, as shown by multilocus enzyme electrophoresis, DNA-DNA hybridization levels, ribosomal gene restriction length polymorphisms, and the sequence of a fragment of the 16S rRNA gene (22). When complemented with a *Rhizobium* sp. type I symbiotic plasmid, they were capable of establishing an effective symbiosis with bean plants. Peas (*Pisum sativum* L.) originated in the Middle East and are thus an introduced crop in America. *Rhizobium* sp. type I pea isolates could also conceivably be the product of the horizontal transfer of a *R. leguminosarum* biovar viciae plasmid to a *Rhizobium* sp. type I chromosomal background.

We therefore propose that *Rhizobium* sp. type I strains be reclassified as a new species, *Rhizobium etli*, which includes the nonsymbiotic isolates and at least one biovar, *Rhizobium etli* biovar phaseoli.

**Description of *Rhizobium etli* sp. nov.** *Rhizobium etli* (et'li. Nahuatl n. *etl*, Bean; N. L. gen. n. *etli*, from bean). Aerobic, gram-negative, nonsporeforming flagellated rods that are 0.5 to 1 by 2 to 3  $\mu$ m. Colonies are circular, convex, opaque, and usually 2 to 4 mm in diameter within 2 to 4 days when they are grown on PY agar medium. They are wet and translucent when they are grown on YM medium. These bacteria are unable to grow on Luria broth medium, on PY medium lacking calcium, or in the presence of carbenicillin, spectinomycin, chloramphenicol, or rifampin. They are able to grow on minimal medium containing malate as a carbon source, but not with arginine, hypoxanthine, or sorbitol as a carbon source. The maximum growth temperature is 35°C. All strains are nalidixic acid resistant. 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.

*R. etli* biovar phaseoli strains nodulate and fix nitrogen on *Phaseolus vulgaris* L. exclusively. They are distinguished from other species at the molecular level by the presence of nitrogenase reductase gene reiterations and the separation of the *nodA* and *nodBC* genes, which form two separate transcriptional units.

Well-studied *R. etli* biovar phaseoli CFN 42 is designated the type strain; it has the characteristics described above for *R. etli* sp. nov. This strain belongs to *R. etli* biovar phaseoli because it is capable of nodulating beans effectively and has the molecular traits described above.

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#### ADDENDUM IN PROOF

Additional DNA-DNA hybridization carried out by G. Laquerre and N. Amarger (Microbiologie des Sols, Institut National de la Recherche Scientifique BV 1540, F-21034 Dijon Cedex, France) gave an average of 45% homology between *R. leguminosarum* bv. *viciae* 10004 and *R. etli* CFN 42.

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