Genomic Heterogeneity among French Rhizobium Strains Isolated from Phaseolus vulgaris L.

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Levels of DNA relatedness between strains isolated from root nodules of Phaseolus vulgaris and reference strains of different Rhizobium species were determined by performing DNA-DNA hybridization experiments (S1 nuclease method). The nine strains examined were members of three genic groups previously delineated by a restriction fragment length polymorphism analysis among strains isolated from P. vulgaris at different sites in France. In agreement with the results of the restriction fragment length polymorphism analysis, three genomic species were found. We confirmed that one of these species corresponded to Rhizobium leguminosarum since the strain examined was 100% related to the type strain of this species. The other two species were new genomic species which were less than 21% related to reference strains belonging to other Rhizobium species, including Rhizobium etli and Rhizobium tropici, and were 18% related to each other. As determined by an analysis of partial 16S ribosomal DNA sequences, each of the genomic species was found to belong to a lineage independent from the lineages of previously described Rhizobium species. Nevertheless, they were included in the group formed by the fast-growing Rhizobium species. Both genomic species 1 and genomic species 2 contained a majority of strains which were capable of nodulating both P. vulgaris and Leucaena leucocephala, like R. tropici. However, they also contained strains with a nodulation phenotype restricted to P. vulgaris, like R. leguminosarum bv. phaseoli and R. etli bv. phaseoli. Our data are the first evidence that in Europe species other than R. leguminosarum nodulate P. vulgaris.

The fast-growing bacteria that are able to induce nodules on the roots of leguminous plants are clustered in the genus Rhizobium. Each Rhizobium species is associated with a group of host plants (5, 14, 19, 23, 34, 36). The species Rhizobium leguminosarum is divided into the following three biovars: R. leguminosarum bv. vicieae, which nodulates Vicia, Pisum, Lens, and Lathyrus species; R. leguminosarum bv. trifolii, which nodulates Trifolium species; and R. leguminosarum bv. phaseoli, which nodulates Phaseolus species (14). Plant specificity is encoded by genes located on a large plasmid, the symbiotic plasmid, which therefore, in R. leguminosarum, defines the biovar of its host bacteria. The bacteria that are able to establish a nitrogen-fixing symbiosis with Phaseolus vulgaris are known to be both genotypically diverse and genetically distant (6, 27, 30). In addition to R. leguminosarum, two other species that nodulate this plant have been characterized recently, Rhizobium etli and Rhizobium tropici (23, 36). R. etli includes the former R. leguminosarum bv. phaseoli type I American strains (21), which constitute one biovar, R. etli bv. phaseoli, and genetically related nonsymbiotic soil isolates from Mexico (35). Like R. leguminosarum bv. phaseoli, R. etli bv. phaseoli apparently nodulates only beans and possesses multiple copies of the nitrogenase reductase gene, nifH (22, 28, 36). These plasmid-borne characteristics distinguish these organisms from other Rhizobium species. Partial sequences of 16S rRNA genes have been used to differentiate R. leguminosarum and R. etli (36). The third species, R. tropici, includes American strains (formerly R. leguminosarum bv. phaseoli type II strains) which are able to nodulate effectively both P. vulgaris and Leucaena spp. (23). These organisms have a single copy of nifH (21, 22). Two subgroups with distinctive phenotypic features (types IIA and IIB) have been identified in R. tropici (23).

All strains of R. etli and R. tropici originated in the Americas, most probably in Mexico. In Europe, the genetic structures of several Rhizobium populations isolated from beans have been studied (10, 17, 24, 41). The enzyme electrophoretic types of strains isolated from English fields were typical of R. leguminosarum and weakly polymorphic (41). In contrast, three taxa were delineated among 72 strains isolated from 20 different sites in French fields (10). One of these taxa was assigned to R. leguminosarum bv. phaseoli on the basis of a nodulation phenotype restricted to P. vulgaris associated with multiple copies of nifH, homology to an R. leguminosarum chromosomal DNA probe, and the results of a restriction fragment length polymorphism (RFLP) analysis. Within this taxon, the RFLP analysis revealed two main chromosomal groups, designated genomic groups A and B. Well-studied American strain CFN 42 also exhibited strong homology to the R. leguminosarum chromosomal probe and a restricted nodulation phenotype, but it had a distinct chromosomal type. These results correlated well with the fact that strain CFN 42 has recently been reclassified in the new species R. etli (36). Group A strains had RFLP patterns similar to those found in the predominant types of R. leguminosarum isolates obtained from root nodules of peas, lentils, and clover (17), but group B strains formed a group that was distinct from both typical strains of R. leguminosarum and CFN 42. Thus, the classification of group B strains as members of R. leguminosarum had to be verified. The two other taxa, designated genomic groups D and E, were genomically distinct from the three previously recognized species that nodulate beans. Some of the strains belonging to groups D and E had the same nodulation phenotype as R. tropici since they were able to nodulate both P. vulgaris and Leucaena leucocephala.

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TABLE 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hybridization patterns*</th>
<th>Host plant of original culture</th>
<th>Source*</th>
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<tbody>
<tr>
<td></td>
<td>pBA2-BamHI</td>
<td>pBA2-HindIII</td>
<td></td>
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<tr>
<td><strong>R. leguminosarum strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 10004T</td>
<td></td>
<td>Pseudomonas sativum</td>
<td>ATCC</td>
</tr>
<tr>
<td>USDA 205T</td>
<td></td>
<td>Phaseolus vulgaris</td>
<td>Young</td>
</tr>
<tr>
<td>ATCC 43677</td>
<td></td>
<td>Phaseolus vulgaris</td>
<td>Graham</td>
</tr>
<tr>
<td><strong>R. tropici strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UMR 1899T (= CIAT 899T)</td>
<td></td>
<td>Phaseolus vulgaris</td>
<td>Graham</td>
</tr>
<tr>
<td><strong>R. galegae</strong></td>
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<td></td>
<td></td>
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<tr>
<td>HAMBI 540T (= ATCC 43677T)</td>
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<td>Lotus tenuis</td>
<td>Jarvis</td>
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<td><strong>Rhizobium spp. strains</strong></td>
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<td>Gallisia orientalis</td>
<td>Lindström</td>
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<tr>
<td>H441</td>
<td>d</td>
<td>Phaseolus vulgaris</td>
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<tr>
<td>R602sp</td>
<td>g</td>
<td>Phaseolus vulgaris</td>
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<tr>
<td>522</td>
<td></td>
<td>Cicer arietinum</td>
<td>Fernandez</td>
</tr>
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</table>

* The French isolates obtained from Phaseolus beans were previously characterized by hybridizing endonuclease-restricted DNAs with a 16S rDNA probe, pBA2 (10). Each of the three taxa identified by the RFLP analysis is represented by strains having one of the pBA2-BamHI hybridization patterns (pattern d, g, or h). The pBA2-HindIII patterns characterized variants within groups.

**Sources:** ATCC, American Type Culture Collection, Rockville, Md.; Young, J. P. W. Young, John Innes Institute, Norwich, United Kingdom; Martinez-Romero, E. Martinez-Romero, Centro de Investigacion sobre Fijacion de Nitrogeno, Universidad Nacional Autonoma de Mexico, Cuernavaca, Mexico; Graham, P. Graham, Department of Soil Science, University of Minnesota, St. Paul; RCR, Rothamsted Collection of Rhizobium, Harpenden, Hertfordshire, United Kingdom; USDA, Rhizobium Culture Collection, Beltsville Agricultural Research Center, Beltsville, Md.; Jarvis, B. D. W. Jarvis, Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand; Lindström, K. Lindström, Department of Microbiology, University of Helsinki, Helsinki, Finland; MSDJ, Institut National de la Recherche Agronomique (INRA), Microbiologie des Solis, Dijon, France; Dreyfus, B. Dreyfus, O.R.S.T.O.M., Dakar, Senegal; Fernandez, M. F. Fernandez, Laboratoire d’Ecologie Microbienne, Université Claude Bernard Lyon I, Villeurbanne, France.

**French isolates obtained from Phaseolus beans.**

Broad-host-range strains in group D possessed one copy of nifH, like *R. tropici*, whereas broad-host-range strains in group E did not exhibit homology to nifH. Group E contained about one-third of the bean rhizobia obtained from two natural French field populations (10, 17, 24).

In this study, DNA-DNA hybridization and analysis of partial 16S ribosomal DNA (rDNA) sequences were used to examine the taxonomic status of French *Rhizobium* strains that nodulate *Phaseolus* beans and represent the three groups defined previously (groups B, D, and E) (10, 17).

**MATERIALS AND METHODS**

**Bacterial strains.** The bacterial strains used in this study are listed in Table 1. Cells were grown at 28°C in TY medium (1).

**DNA extraction.** DNA was extracted and purified by using the procedure of Brenner et al. (2).

**DNA-DNA hybridization.** Native DNA was labeled by nick translation (29) with tritium-labeled nucleotides (Amersham International, Amersham, England). Levels of DNA relatedness were determined by using the SI nuclease-trichloroacetic method (12). DNA-DNA hybridization tests were carried out at 70°C by using labeled DNAs from strains ATCC 10004T (T = type strain), CIAT 899T, R602sp, and H152 as probes.

**Nucleotide sequence of 16S rRNA genes.** Total DNA was amplified by using primers FGPS6-63 (5'GGAGAGGATGATCCTTGCTC-3') and FGPS69'-64 (5'CACCAGCA CACCGGACTT-3') and a standard protocol (26), which yielded DNA bands of the expected size (650 bp) (data not shown); the amplified fragments were then digested with BglII and EcoRI, which have sites in the primers (underlined). The digested DNA fragments were cloned into BamHI-EcoRI-cut pBluescript SK- vector (Stratagene, La Jolla, Calif.), and transformed into Escherichia coli DH5αF (Bethesda Research Laboratories) as described by Maniatis et al. (20). Ten white clones (i.e., clones with insertions) were pooled and isolated from Qiagen midi columns (Diagen, Hilden, Germany). These double-stranded fragments were sequenced in both directions as described by Sanger et al. (33), using primers FGPS6-63 (see above) and FGPS 305'-78 (5'CCAGTTGGCGGCTCG CCTTC-3') and a T7 sequencing kit (Pharmacia, St. Quent-in-Yvelines, France).

**Alignment of sequences and cluster analysis.** Nucleotide sequences were aligned by using the Clustal program (13) and were visualized by using the MASE software (9) and a Sun workstation, and the neighbor-joining algorithm (32) was used to determine the phylogenetic relationships from
Nucleotide sequence accession numbers. For comparison we used the sequences of *Rhizobium* sp. strain FL27 (*Phaseolus* spp.) (GenBank accession number M55234), *Rhizobium loti* (GenBank accession number X63825), *Rhizobium galegae* (GenBank accession number X63823), *Rhizobium* sp. strain TAL 1145 (*Lewucaena* spp.) (GenBank accession number X63824), *R. tropici* type IIB strain CIAT 899T (GenBank accession number M55235), *R. tropici* type IIA strain CFN 299 (EMBL accession number X67234), *R. etli* Olivia 4 (GenBank accession number M55235), *Rhizobium* sp. strain Or191 (*Medicago* spp.) (GenBank accession number M55236), *R. leguminosarum* bv. phaseoli 8002 (GenBank accession number M55494), *Rhizobium melliloti* ATCC 9930T (GenBank accession number M55242), *R. melliloti* CC169 (GenBank accession number M55243), *Rhizobium fredii* (GenBank accession number M74163), *Agrobacterium tumefaciens* ICPB TT111 (EMBL accession number X67223), *Agrobacterium rhizogenes* ATCC 15834 (EMBL accession number X67224), *Agrobacterium rubi* ATCC 13335 (EMBL accession number X67228), *Agrobacterium vitis* NCPPPB 3554 (EMBL accession number X67225), *Bradyrhizobium japonicum* USDA 110 (GenBank accession number M55485), *Azorhizobium caulinodans* (GenBank accession number M55491), *Rhodopseudomonas palustris* (GenBank accession number M55496), *Rhodospirillum rubrum* (GenBank accession number M55497), and *Rhodobacter sphaeroides* (GenBank accession number M55498). The partial 16S rDNA sequence of *Rhizobium* sp. strain H152 determined in this study was deposited in the GenBank data library under accession number L19662.

**RESULTS**

DNA-DNA hybridizations. The percentages of relative DNA homology at 70°C with reference DNAs from *R. leguminosarum* ATCC 10004T, *R. tropici* CIAT 899T, and strains R602sp and H152 are shown in Table 2 for 22 strains.

Of the nine French strains examined, only one, H441, was 100% related to type strain ATCC 10004 of *R. leguminosarum*. The eight other strains fell into two DNA relatedness groups (genomic species). Genomic species 1 contained three strains which were 76 to 91% related to strain R602sp. The five other French strains, which were 73 to 95% related to strain H152, formed genomic species 2. The genomic species 2 strains exhibited low relative binding ratios (less than 18%) with genomic species 1 strains. Strains belonging to both genomic species were less than 21% related to reference strains belonging to other *Rhizobium* species, including *R. leguminosarum*, *R. etli*, *R. tropici* type IIA and IIB, *R. loti*, *R. galegae*, *R. melliloti*, *R. fredii*, and unclassified field rhizobia isolated from *L. leucocephala*, *Acacia laeta*, and *Cicer arietinum*.

It should be noted that *R. tropici* type IIA strain CFN 299 was only 36% related to *R. tropici* type IIB strain CIAT 899T, a value similar to the value found previously by Martinez-Romero et al. (23). *R. etli* CFN 42T was found to be 45% related to type strain ATCC 10004T of *R. leguminosarum*, which also confirmed the relative levels of DNA-DNA hybridization previously obtained between CFN 42T and two *R. leguminosarum* strains (35).

**Nucleotide sequence analysis of 16S rDNA.** Partial 16S rDNA sequences containing 246 nucleotides were obtained for genomic species 1 strain R602sp and genomic species 2


strain H152. These sequences were within the region from position 20 to position 338 (E. coli numbering system [4]), which was previously used to determine phylogenetic relationships among rhizobia (8, 36, 42). The sequences were compared with those found for other Rhizobium and Bradyrhizobium species and some other genera belonging to the alpha subdivision (40) of the Proteobacteria (37) (Fig. 1). The sequences of strains R602sp and H152 were included in the group containing fast-growing Rhizobium species (Fig. 2). The sequence of strain R602sp was identical to that of Rhizobium sp. strain FL27, an unclassified strain isolated from Phaseolus beans (27). These two strains formed a lineage independent from the lineages of other Rhizobium strains and more closely related to R. etli than to other Rhizobium species. Genomic species 2 strain H152 also formed an independent lineage; the most closely related organisms to this lineage were R. galegae and Rhodobacter sphaeroides. The underlined sequence TCGA at coordinates 32 through 35 represents the site of the 72-nucleotide insertion unique to R. tropici type IIA strain CFN 299 (TTCTTT CGAAGCT TGAAGG AT). The sequences were within the region from position 20 to position 338 (E. coli numbering system [4]), which was previously used to determine phylogenetic relationships among rhizobia (8, 36, 42). The sequences were compared with those found for other Rhizobium and Bradyrhizobium species and some other genera belonging to the alpha subdivision (40) of the Proteobacteria (37) (Fig. 1). The sequences of strains R602sp and H152 were included in the group containing fast-growing Rhizobium species (Fig. 2). The sequence of strain R602sp was identical to that of Rhizobium sp. strain FL27, an unclassified strain isolated from Phaseolus beans (27). These two strains formed a lineage independent from the lineages of other Rhizobium strains and more closely related to R. etli than to other Rhizobium species. 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with \textit{Rhizobium} I’us relationships below the genus level by using the short leucocephala leguminosarum suggested that reliable conclusions cannot be drawn about itropici IIA, lcies cated that despite its broad host range, genomic species 1 this study were capable of nodulating both \textit{P. vulgaris} and \textit{P. tropici} strain H441; \textit{R. sp. Phaseolus FL27}, \textit{R. sp. Leucaena}, \textit{Rhizobium} sp. strain FL27; \textit{R. sp. R602sp}, \textit{Rhizobium} sp. strain R602sp.

![FIG. 2. Phylogenetic tree showing relationships among Phaseolus bean rhizobia and other strains of \textit{Rhizobium} spp., Agrobacterium spp., and related bacteria based on partial 16S rDNA sequences. The tree was derived from a Jukes-Cantor (15) distance matrix by using the neighbor-joining method (32) and the sequences shown in Fig. 1. The taxa in boxes are the taxa that infect \textit{P. vulgaris}. Abbreviations: R.sp. H152, \textit{Rhizobium} sp. strain H152; R. tropici II A, \textit{R. tropici} type II A strain CFN 299; R. sp. Leucaena, \textit{Rhizobium} sp. strain Tal 1145; R. sp. Medicago 191, \textit{Rhizobium} sp. strain Or191; R. sp. Phaseolus FL27, \textit{Rhizobium} sp. strain FL27; R. sp. R602sp, \textit{Rhizobium} sp. strain R602sp.](image)

Rhizobium leguminosarum (10, 17). Within this taxon, two main groups (groups A and B) of closely related genotypes were found by an RFLP analysis. In this study, we verified that H441 and closely related strains (group B) belonged to the species \textit{R. leguminosarum} since strain H441 was 100% related to ATCC 10004, the type strain of this species. Members of the other group (group A) were not included in this study because their chromosomal types were similar to the predominant types of \textit{R. leguminosarum} isolates obtained from root nodules of peas, lentils, and clover (17) and to the type strain of \textit{R. leguminosarum} (16).

The eight other strains examined in this study that belonged to the other RFLP groups defined previously (10) were distributed in two new genomic \textit{Rhizobium} species as revealed by DNA-DNA hybridization and 16S rDNA sequence analysis data. The DNA relatedness data confirmed the previous groupings since strains belonging to the same RFLP group belonged to the same genomic species. This is the first evidence that in Europe species other than \textit{R. leguminosarum} are capable of nodulating \textit{Phaseolus} beans in the field. However, no strains related to \textit{R. etli} and \textit{R. tropici} have been identified in the sample of French isolates obtained from \textit{Phaseolus} beans. So far, members of \textit{R. etli} and \textit{R. tropici} have been found only in the Americas.

Most strains of the two new genomic species identified in this study were capable of nodulating both \textit{P. vulgaris} and \textit{L. leucocephala} (10), a characteristic that they had in common with \textit{R. tropici}. However, the phylogenetic analysis indicated that despite its broad host range, genomic species 1 strain R602sp is more closely related to \textit{R. etli} than to \textit{R. tropici}. Conversely, \textit{R. leguminosarum} is more closely related to \textit{R. tropici} than to \textit{R. etli}. However, it has been suggested that reliable conclusions cannot be drawn about relationships below the genus level by using the short segment of 16S rDNA that was sequenced (42). Nevertheless, some strains belonging to both genomic species 1 and 2 had a nodulation phenotype restricted to \textit{P. vulgaris} associated with the presence of three copies of the \textit{nifH} gene (such as strains PhID12 and Ro84 used in this study), which is characteristic of the pSym of \textit{R. leguminosarum} bv. phaseoli and \textit{R. etli} bv. phaseoli. It was suggested previously that in such strains, the \textit{Phaseolus} restricted phenotype was acquired by horizontal transfer of a pSym from an \textit{R. leguminosarum} bv. phaseoli strain (10). Until now, the recognized \textit{Rhizobium} species that nodulate beans have been clearly associated with specific symbiotic characteristics, in particular the range of hosts that they are able to nodulate. Thus, host range has been considered a helpful diagnostic test to identify such bacteria. The fact that there may be exchanges of symbiotic characteristics between species in the field will hamper the identification of bean isolates.

Since DNA-DNA hybridization is considered the standard arbiter for the designation of species (25, 38), our finding of two new genomic species should lead to the creation of new \textit{Rhizobium} species. The phenotypic characterization of these organisms is currently in progress in our laboratory. \textit{P. vulgaris} is known to be a promiscuous host for \textit{Rhizobium} species, in contrast to other legumes grown in temperate areas. This plant can be nodulated under laboratory conditions by different strains of \textit{Rhizobium} spp. and even by \textit{Bradyrhizobium} spp. isolated from other genera of legumes (3, 6, 7, 11, 18, 22, 31) and in the field by \textit{R. leguminosarum} strains harboring typical \textit{R. leguminosarum} bv. viciea and \textit{R. leguminosarum} bv. trifolii symbiotic plasmids (17). The identification of two additional genomic species of rhizobia able to nodulate \textit{P. vulgaris} further demonstrates the low level of specificity of the \textit{Phaseolus-Rhizobium} symbiosis and helps to explain some of the genetic diversity found in \textit{Rhizobium} species that nodulate beans (6, 27, 30).

Despite the recent creation of two novel species, \textit{R. etli} and \textit{R. tropici}, and the possible creation of two other species, we can expect larger taxonomic diversity among \textit{Rhizobium} strains isolated from \textit{Phaseolus} beans. The two subgroups of the species \textit{R. tropici} were found to be only 36% related (23), and \textit{R. etli} contains genetically distinct strains (27) which were grouped in a single species mainly on the basis of similar sequences in a partial region of the 16S rDNA (36). Moreover, until now, studies of the bean symbionts have been limited to the Americas and a part of occidental Europe. As further isolations are made from other geographic areas, additional new species may be created to reflect, at a taxonomic level, the great diversity of bacteria capable of nodulating beans.

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