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 (SI nuclease method). The nine strains examined were members of three genomic 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. viciae, 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

Strain	Hybridization patterns ^a		Host plant of original culture	Source ^b
	pBA2-BamHI	pBA2-HindIII		
<i>R. leguminosarum</i> strains				
ATCC 10004 ^T			<i>Pisum sativum</i>	ATCC
8401(pRL1J1)			<i>Phaseolus vulgaris</i>	Young
<i>R. etli</i> bv. phaseoli CFN 42 ^T			<i>Phaseolus vulgaris</i>	Martinez-Romero
<i>R. tropici</i> strains				
UMR 1899 ^T (= CIAT 899 ^T) (type IIB)			<i>Phaseolus vulgaris</i>	Graham
CFN 299 (type IIA)			<i>Phaseolus vulgaris</i>	Martinez-Romero
<i>R. meliloti</i> RCR 2011			<i>Medicago sativa</i>	RCR
<i>R. fredii</i> USDA 205 ^T			<i>Glycine max</i>	USDA
<i>R. loti</i> NZP 2213 ^T			<i>Lotus tenuis</i>	Jarvis
<i>R. galegae</i> HAMBI 540 ^T (= ATCC 43677 ^T)			<i>Galega orientalis</i>	Lindström
<i>Rhizobium</i> spp. strains				
H441 ^c	d	c	<i>Phaseolus vulgaris</i>	MSDJ
R602sp ^c	g	g	<i>Phaseolus vulgaris</i>	MSDJ
PhP222 ^c	g	h	<i>Phaseolus vulgaris</i>	MSDJ
PhD12 ^c	g	i	<i>Phaseolus vulgaris</i>	MSDJ
H152 ^c	h	m	<i>Phaseolus vulgaris</i>	MSDJ
Ro84 ^c	h	o	<i>Phaseolus vulgaris</i>	MSDJ
Ro70 ^c	h	o	<i>Phaseolus vulgaris</i>	MSDJ
Ro68 ^c	h	l	<i>Phaseolus vulgaris</i>	MSDJ
Ro24 ^c	h	p	<i>Phaseolus vulgaris</i>	MSDJ
BR 864			<i>Leucaena leucocephala</i>	Martinez-Romero
ORS 1007			<i>Acacia laeta</i>	Dreyfus
UPMCA 36			<i>Cicer arietinum</i>	Fernandez
522			<i>Cicer arietinum</i>	Fernandez

^a 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.

^b 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 Sols, Dijon, France; Dreyfus, B. Dreyfus, O.R.S.T.O.M., Dakar, Senegal; Fernandez, M. P. Fernandez, Laboratoire d'Ecologie Microbienne, Université Claude Bernard Lyon I, Villeurbanne, France.

^c 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 S1 nuclease-trichloroacetic method (12). DNA-DNA hybridization tests were carried out at 70°C by using labeled DNAs from strains

ATCC 10004^T (T = type strain), CIAT 899^T, R602sp, and H152 as probes.

Nucleotide sequence of 16S rRNA genes. Total DNA was amplified by using primers FGPS6-63 (5'-GGAGAGTTA GATCTTGCTC-3') and FGPS659'-64 (5'-CACCGCTA CACCAGGAATTC-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 *Bgl*II and *Eco*RI, which have sites in the primers (underlined regions), cloned into *Bam*HI-*Eco*RI-cut pBluescript II 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 Quia-gen 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'-CCAGTGTGGCCGGTCGC CCTCTC-3') and a T7 sequencing kit (Pharmacia, St. Quentin-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

TABLE 2. Levels of DNA relatedness between strains of *Rhizobium* species

Strain	% Reassociation at 70°C with labeled DNA from:			
	ATCC 10004 ^T	CIAT 899 ^T	R602sp	H152
<i>R. leguminosarum</i> strains				
ATCC 10004 ^T	100		21	16
8401(pRL1J1)	100			
H441 ^a	100			
<i>R. etli</i> CFN 42 ^T	45		18	14
<i>R. tropici</i> strains				
CIAT 899 ^T (type IIB)	15	100	13	10
CFN 299 (type IIA)	19	36	15	13
<i>R. meliloti</i> RCR 2011			7	15
<i>R. fredii</i> USDA 205 ^T			10	17
<i>R. loti</i> NZP 2213 ^T			5	6
<i>R. galegae</i> HAMBI 540 ^T			11	11
<i>Rhizobium</i> spp. strains				
BR 864	23		11	10
ORS 1007			7	16
UPMCA 36			7	6
522			3	7
Genomic species 1 strains				
R602sp ^a	23	19	100	18
PhP222 ^a	23		91	11
PhD12 ^a			76	14
Genomic species 2 strains				
H152 ^a	10	12	10	100
Ro84 ^a	13		8	88
Ro70 ^a	11		11	93
Ro68 ^a			13	74
Ro24 ^a			11	95

^a French isolates obtained from *Phaseolus* beans.

Jukes-Cantor distances (15). Gap-containing positions were omitted from computations.

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 (*Leucaena* spp.) (GenBank accession number X63824), *R. tropici* type IIB strain CIAT 899^T (GenBank accession number M55233), *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 meliloti* ATCC 9930^T (GenBank accession number M55242), *R. meliloti* 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 10004^T, *R. tropici* CIAT 899^T, 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 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. meliloti*, *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 899^T, a value similar to the value found previously by Martinez-Romero et al. (23). *R. etli* CFN 42^T was found to be 45% related to type strain ATCC 10004^T of *R. leguminosarum*, which also confirmed the relative levels of DNA-DNA hybridization previously obtained between CFN 42^T 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

R. sp. H152	1	11	21	31	41
R. sp. R602sp	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
R. loti	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
R. galegae	AGGCTTAACA	CATGCAAGTC	GAGCG--CCT	CGCAAGAGG-	-AGCGGCAGA
R. Leucaena	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
R. tropici CIAT899	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
R. tropici IIA	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CTCGAGGGG-	-AGCGGCAGA
R. etli Olivia	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
R. sp. medicago 191	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
R. leguminosarum8002	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
R. meliloti ATCC9930	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
R. meliloti CC169	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
R. fredii	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
A. tumefaciens	AGGCTTAACA	CATGCAAGTC	GAACG--CCC	CGCAAGGGG-	-AGTGGCAGA
A. rhizogenes	AGGCTTAACA	CATGCAAGTC	GAGCG--CCC	CGCAAGGGG-	-AGCGGCAGA
A. rubi	AGGCTTAACA	CATGCAAGTC	GAACG--CTC	CGCAAGGAG-	-AGTGGCAGA
A. vitis	AGGCTTAACA	CATGCAAGTC	GAGCG--CCT	CGCAAGAGG-	-AGCGGCAGA
B. japonicum USDA110	AGGCTTAACA	CATGCAAGTC	GAGCGGGCGT	AGCAATACGT	CAGCGGCAGA
Azorhizobium	AGGCTTAACA	CATGCAAGTC	GAACGGGCCC	-TTCG--GG-T	CAGTGGCAGA
Rhodopseudomonas	AGGCTTAACA	CATGCAAGTC	GAACGGGCGT	AGCAATACGT	CAGTGGCAGA
Rhodobacter	AGGCTTAACA	CATGCAAGTC	GAGCGC--CGT	AGCAATACGG	-AGCGGCAGA
Rhodospirillum	AGGCTTAACA	CATGCAAGTC	GAACGCATCC	CTTCG--GGAT	GAGTGGCGCA
R. sp. H152	51	61	71	81	91
R. sp. R602sp	CGGGTGAGTA	ACGCGTGGA	ATCTACCCAT	CTCTACGGAA	TAACCTCAGGG
R. loti	CGGGTGAGTA	ACGCGTGGA	ACGTACCCTT	TACTACGGAA	TAACGCAGGG
R. galegae	CGGGTGAGTA	ACGCGTGGA	ATCTACCCAT	CTCTACGGAA	CAACTCCGGG
R. Leucaena	CGGGTGAGTA	ACGCGTGGA	ATCTACCCAT	CCCTACGGAA	CAACTCCGGG
R. tropici CIAT899	CGGGTGAGTA	ACGCGTGGA	ATCTACCCTT	TTCTACGGAA	TAACGCAGGG
R. tropici IIA	CGGGTGAGTA	ACGCGTGGA	ATCTACCCTT	TTCTACGGAA	TAACGCAGGG
R. etli Olivia	CGGGTGAGTA	ACGCGTGGA	ACGTACCCTT	TACTACGGAA	TAACGCAGGG
R. sp. medicago 191	CGGGTGAGTA	ACGCGTGGA	ACGTACCCTT	TACTACGGAA	TAACGCATGG
R. leguminosarum8002	CGGGTGAGTA	ACGCGTGGA	ATCTACCCTT	GACTACGGAA	TAACGCAGGG
R. meliloti ATCC9930	CGGGTGAGTA	ACGCGTGGA	ATCTACCCTT	TTCTACGGAA	TAACGCAGGG
R. meliloti CC169	CGGGTGAGTA	ACGCGTGGA	ATCTACCCTT	TTCTACGGAA	TAACGCAGGG
R. fredii	CGGGTGAGTA	ACGCGTGGA	ATCTACCCTT	TTCTACGGAA	TAACGCAGGG
A. tumefaciens	CGGGTGAGTA	ACGCGTGGA	ATCTACCGTG	CCCTGCGGAA	TAGCTCCGGG
A. rhizogenes	CGGGTGAGTA	ACGCGTGGA	ATCTACCCTT	TTCTACGGAA	TAACGCAGGG
A. rubi	CGGGTGAGTA	ACGCGTGGA	ATCTACCCAA	CCCTGCGGAA	TAGCTCTGGG
A. vitis	CGGGTGAGTA	ACGCGTGGA	ATCTACCGTA	CCCTACGGAA	TAGCTCCGGG
B. japonicum USDA110	CGGGTGAGTA	ACGCGTGGA	ACGTACCCTT	TGGTTCGGAA	CAACACAGGG
Azorhizobium	CGGGTGAGTA	ACGCGTGGA	ACGTGCCCTT	CAGTTCGGAA	TAACCCAGGG
Rhodopseudomonas	CGGGTGAGTA	ACGCGTGGA	ACGTACCCTT	TGGTTCGGAA	CAACACAGGG
Rhodobacter	CGGGTGAGTA	ACGCGTGGA	ATGTGCCCAA	AGGTACGGAA	CAACCAAGGG
Rhodospirillum	CGGGTGAGTA	ACACGTGGGA	ACGTACCCTT	GAGTGC GGAA	TAATCTTTGG
R. sp. H152	101	111	121	131	141
R. sp. R602sp	AAACTTGTGC	TAATACCGTA	TACGCCCTTC	GGGGGAAAGA	TTTATCGGAG
R. loti	AAACTTGTGC	TAATACCGTA	TGTGCCCTTC	GGGGGAAAGA	TTTATCGGTA
R. galegae	AAACTTGTGC	TAATACCGTA	TACGCCCTTC	GGGAGAAAGA	TTTATCGGAG
R. Leucaena	AAACTTGTGC	TAATACCGTA	TGTGTCCTTC	GGGAGAAAGA	TTTATCGGGG
R. tropici CIAT899	AAACTTGTGC	TAATACCGTA	TGTGTCCTTC	GGGAGAAAGA	TTTATCGGCA
R. tropici IIA	AAACTTGTGC	TAATACCGTA	TGTGTCCTTC	GGGAGAAAGA	TTTATCGGGA
R. etli Olivia	AAACTTGTGC	TAATACCGTA	TGTGCCCTTT	GGGGGAAAGA	TTTATCGGTA
R. sp. medicago 191	AAACGTGTGC	TAATACCGTA	TGTGCCCTTT	GGGGGAAAGA	TTTATCGGTA
R. leguminosarum8002	AAACTTGTGC	TAATACCGTA	TGTGTCCTTC	GGGAGAAAGA	TTTATCGGTC
R. meliloti ATCC9930	AAACTTGTGC	TAATACCGTA	TGAGCCCTTC	GGGGGAAAGA	TTTATCGGGA
R. meliloti CC169	AAACTTGTGC	TAATACCGTA	TAAGCCCTTC	GGGGGAAAGA	TTTATCGGGA
R. fredii	AAACTTGTGC	TAATACCGTA	TGAGCCCTTC	GGGGGAAAGA	TTTATCGGGA
A. tumefaciens	AAACTTGTGC	TAATACCGTA	TACGCCCTAC	GGGGGAAAGA	TTTATCGGGG
A. rhizogenes	AAACTTGTGC	TAATACCGTA	TGTGTCCTTC	GGGAGAAAGA	TTTATCGGGA
A. rubi	AAACTTGTGC	TAATACCGTA	TACGCCCTAC	GGGGGAAAGA	TTTATCGGGG
A. vitis	AAACTTGTGC	TAATACCGTA	TACGCCCTTC	GGGGGAAAGA	TTTATCGGGG
B. japonicum USDA110	AAACTTGTGC	TAATACCGTA	TAAGCCCTTA	CGGGGAAAGA	TTTATCGGCC
Azorhizobium	AAACTTGTGC	TAATACCGTA	TACGTCCGAA	AGGAGAAAGA	TTGATCGCTG
Rhodopseudomonas	AAACTTGTGC	TAATACCGTA	TAAGCCCTTA	CGGGGAAAGA	TTTATCGCCG
Rhodobacter	AAACTTGTGC	TAATACCGTA	TGTGCCCGAG	AGGGGAAAGA	TTTATTGCCT
Rhodospirillum	AAACGAGGAC	TAATACCGCA	TACGCCCTTA	GGGGGAAAGA	TTTATCGCTC

	151	161	171	181	191
<i>R. sp.</i> H152	ATGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. sp.</i> R602sp	AGGGATCGGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. loti</i>	ATGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	TGGCCTACCA
<i>R. galegae</i>	ATGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. Leucaena</i>	AAGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. tropici</i> CIAT899	AGAGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. tropici</i> IIA	AAGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. etli</i> Olivia	AAGGATCGGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. sp.</i> medicago 191	AAGGATCGGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. leguminosarum</i> 8002	AAGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. meliloti</i> ATCC9930	AAGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. meliloti</i> CC169	AAGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>R. fredii</i>	AAGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>A. tumefaciens</i>	TATGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>A. rhizogenes</i>	AAGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>A. rubi</i>	ATGGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>A. vitis</i>	TATGATGAGC	CCGCGTTGGA	TTAGCTAGTT	GGTGGGGTAA	AGGCCTACCA
<i>B. japonicum</i> USDA110	AAAGATCGGC	CCGCGTCTGA	TTAGCTAGTT	GGTAGGGTAA	CGGCCTACCA
<i>Azorhizobium</i>	AAGGATCGGC	CCGCGTCTGA	TTAGCTAGTT	GGTGAGGTAA	TGGCTCACCA
<i>Rhodopseudomonas</i>	AAAGATCGGC	CCGCGTCTGA	TTAGCTAGTT	GGTGAGGTAA	TGGCTCACCA
<i>Rhodobacter</i>	TTGGAGCAGC	CCGCGTCTGA	TTAGCTAGTT	GGTGGGGTAA	CGGCCACCA
<i>Rhodospirillum</i>	CAAGATCGGC	CCGCGTCCGA	TTAGCTAGTT	GGCGGGGTAA	TGGCCACCA
	201	211	221	231	241
<i>R. sp.</i> H152	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. sp.</i> R602sp	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. loti</i>	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. galegae</i>	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. Leucaena</i>	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. tropici</i> CIAT899	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. tropici</i> IIA	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. etli</i> Olivia	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. sp.</i> medicago 191	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. leguminosarum</i> 8002	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. meliloti</i> ATCC9930	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. meliloti</i> CC169	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>R. fredii</i>	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>A. tumefaciens</i>	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>A. rhizogenes</i>	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>A. rubi</i>	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>A. vitis</i>	AGGCGACGAT	CCATAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>B. japonicum</i> USDA110	AGGCGACGAT	CAGTAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>Azorhizobium</i>	AGGCGACGAT	CAGTAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>Rhodopseudomonas</i>	AGGCGACGAT	CAGTAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>Rhodobacter</i>	AGGCGACGAT	CAGTAGCTGG	TCTGAGAGGA	TGATCAGCCA	CATTGGGACT
<i>Rhodospirillum</i>	AGGCGACGAT	CGGTAGCTGG	TCTGAGAGGA	TGGCAGCCAC	ACCTGGGACT

FIG. 1. 16S rDNA sequences of *Rhizobium* sp. strain H152 (this study), *Rhizobium* sp. strain R602sp (identical to *Rhizobium* sp. strain FL27) (this study), *R. loti*, *R. galegae*, *Rhizobium* sp. strain Tal 1145 (*R. Leucaena*), *R. tropici* CIAT 899^T (type IIB), *R. tropici* CFN 299 (type IIA) (*R. tropici* IIA), *R. etli* Olivia 4, *Rhizobium* sp. strain Or191 (*R. sp. medicago* 191), *R. leguminosarum* bv. phaseoli 8002, *R. meliloti* ATCC 9930^T, *R. meliloti* CC169, *R. fredii* USDA 205^T, *Agrobacterium tumefaciens* ICPB TT111, *Agrobacterium rhizogenes* ATCC 15834, *Agrobacterium rubi* ATCC 13335, *Agrobacterium vitis* NCPPB 3554, *B. japonicum* USDA 110, *Azorhizobium caulinodans*, *Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, 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 (TCCTT CAAGCAAGCT TGAAGGATT TACTCCTTGGGA AAAGAAGATC AAGAGCGCTT CTAAGAAGCT TTCTTGATGGA) (39), which was deleted for clarity.

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 *R. loti* but no other strains isolated from beans (Fig. 2).

DISCUSSION

Of the nine strains examined in this study for their taxonomic status, strain H441 was a member of the taxon which we previously hypothesized as belonging to the spe-

FIG. 2. Phylogenetic tree showing relationships among *Phaseolus* bean rhizobia and other strains of *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 *P. vulgaris*. Abbreviations: R.sp. H152, *Rhizobium* sp. strain H152; R. tropici IIA, *R. tropici* type IIA strain CFN 299; R. sp. Leucaena, *Rhizobium* sp. strain Tal 1145; R. sp. Medicago 191, *Rhizobium* sp. strain Or191; R. sp. Phaseolus FL27, *Rhizobium* sp. strain FL27; R. sp. R602sp, *Rhizobium* sp. strain R602sp.

cies *R. 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 *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 *R. leguminosarum* isolates obtained from root nodules of peas, lentils, and clover (17) and to the type strain of *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 *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 *R. leguminosarum* are capable of nodulating *Phaseolus* beans in the field. However, no strains related to *R. etli* and *R. tropici* have been identified in the sample of French isolates obtained from *Phaseolus* beans. So far, members of *R. etli* and *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 *P. vulgaris* and *L. leucocephala* (10), a characteristic that they had in common with *R. tropici*. However, the phylogenetic analysis indicated that despite its broad host range, genomic species 1 strain R602sp is more closely related to *R. etli* than to *R. tropici*. Conversely, *R. leguminosarum* is more closely related to *R. tropici* than to *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). Neverthe-

less, some strains belonging to both genomic species 1 and 2 had a nodulation phenotype restricted to *P. vulgaris* associated with the presence of three copies of the *nifH* gene (such as strains PhD12 and Ro84 used in this study), which is characteristic of the pSym of *R. leguminosarum* bv. phaseoli and *R. etli* bv. phaseoli. It was suggested previously that in such strains, the *Phaseolus* restricted phenotype was acquired by horizontal transfer of a pSym from an *R. leguminosarum* bv. phaseoli strain (10). Until now, the recognized *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 *Rhizobium* species. The phenotypic characterization of these organisms is currently in progress in our laboratory. *P. vulgaris* is known to be a promiscuous host for *Rhizobium* species, in contrast to other legumes grown in temperate areas. This plant can be nodulated under laboratory conditions by different strains of *Rhizobium* spp. and even by *Bradyrhizobium* spp. isolated from other genera of legumes (3, 6, 7, 11, 18, 22, 31) and in the field by *R. leguminosarum* strains harboring typical *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii symbiotic plasmids (17). The identification of two additional genomic species of rhizobia able to nodulate *P. vulgaris* further demonstrates the low level of specificity of the *Phaseolus*-*Rhizobium* symbiosis and helps to explain some of the genetic diversity found in *Rhizobium* species that nodulate beans (6, 27, 30).

Despite the recent creation of two novel species, *R. etli* and *R. tropici*, and the possible creation of two other species, we can expect larger taxonomic diversity among *Rhizobium* strains isolated from *Phaseolus* beans. The two subgroups of the species *R. tropici* were found to be only 36% related (23), and *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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