Rhizobium gallicum sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* Nodules

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Thirty-one strains of two new genomic species (genomic species 1 and 2) of rhizobia isolated from root nodules of *Phaseolus vulgaris* and originating from various locations in France were compared with reference strains of rhizobia by performing a numerical analysis of 64 phenotypic features. Each genomic species formed a distinct phenon and was separated from the other rhizobial species. A comparison of the complete 16S rRNA gene sequences of a representative of genomic species 1 (strain R602sp^T) and a representative of genomic species 2 (strain H152^T) with the sequences of other rhizobia and related bacteria revealed that each genomic species formed a lineage independent of the lineages formed by the previously recognized species of rhizobia. Genomic species 1 clustered with the species that include the bean-nodulating rhizobia, *Rhizobium leguminosarum*, *Rhizobium etli*, and *Rhizobium tropici*, and branched with unclassified rhizobial strain OK50, which was isolated from root nodules of *Pterocarpus klemmei* in Japan. Genomic species 2 was distantly related to all other *Rhizobium* species and related taxa, and the most closely related organisms were *Rhizobium galegae* and several *Agrobacterium* species. On the basis of the results of phenotypic and phylogenetic analyses and genotypic data previously published and reviewed in this paper, two new species of the genus *Rhizobium*, *Rhizobium gallicum* and *Rhizobium giardinii*, are proposed for genomic species 1 and 2, respectively. Each species could be divided in two subgroups on the basis of symbiotic characteristics, as shown by phenotypic (host range and nitrogen fixation effectiveness) and genotypic data. For each species, one subgroup had the same symbiotic characteristics as *R. leguminosarum* biovar phaseoli and *R. etli* biovar phaseoli. The other subgroup had a species-specific symbiotic phenotype and genotype. Therefore, we propose that each species should be subdivided into two biovars, as follows: *R. gallicum* biovar gallicum and *R. gallicum* biovar phaseoli; and *R. giardinii* biovar giardinii and *R. giardinii* biovar phaseoli.

Until recently, the rhizobia that nodulate the roots of common bean, *Phaseolus vulgaris*, were classified in a single species, *Rhizobium leguminosarum* (23). This species was subdivided into three biovars differentiated by legume host specificity, and strains recovered from *Phaseolus* spp. nodules were assigned to *R. leguminosarum* bv. phaseoli. Although this biovar has long been recognized as a very heterogeneous group on the basis of a variety of phenotypic (5, 16, 40, 41) and genotypic (7, 15, 21, 38) criteria, it is only with the development of the molecular approach that it gave rise to new taxa. First, based on host range characteristics encoded by genes located on the symbiotic plasmid and on the organization of the symbiotic plasmid (33, 35), two subgroups were distinguished within *R. leguminosarum* bv. phaseoli. Type I strains had multiple copies of the nitrogenase reductase gene, *nifH* (39), and a narrow host range (6) (which recently appeared to be less restricted than first thought [18]), whereas type II strains had a single copy of *nifH* and a broader host range (6, 34). Later, the unusually high level of genetic diversity among Mexican and South American isolates (38) revealed by multilocus enzyme electrophoresis (MLEE) suggested that several species could be delineated among the isolates assigned to *R. leguminosarum* bv. phaseoli and thus brought into question the meaning of this taxonomic unit. The type II strains included in the study formed one of the lineages. A larger sample of type II strains was analyzed further and was classified by MLEE and numerical taxonomy (36). Genotypic characterization of representative strains by

partial sequencing of the 16S rRNA gene, DNA-DNA hybridization, and ribosomal DNA (rDNA) organization led to the creation of a new species of the genus *Rhizobium*, *Rhizobium tropici*, corresponding to the former type II (36). Two subgroups, subgroups IIA and IIB, were described in this species to accommodate the relatively low level of DNA-DNA homology (36%) and differences in phenotypic and genotypic characteristics, differences which were recently emphasized by the observation of a specific megaplasmid in each group (13). Primarily on the basis of differences in a 260-bp nucleotide sequence of the 16S rRNA, type I strains and nonsymbiotic isolates of American origin were later reclassified as *Rhizobium etli* sp. nov. (44). However, the distribution of the *R. etli* 16S rRNA allele is not limited to the Americas, and so far this allele has also been identified in France and Spain (1) and in Indonesia and Africa (49). On the other hand, Eardly et al. (11) have detected the *R. leguminosarum* 16S rRNA allele in isolates from South America. When analyzed by MLEE, the isolates harboring this allele were found to be more closely related to lineages of strains harboring the *R. etli* allele than to each other. It was hypothesized that the *R. leguminosarum* allele had been horizontally transferred and recombined among distantly related chromosomal lineages. This implies that the boundaries between *R. leguminosarum* and *R. etli* are rather loose. The level of DNA-DNA relatedness (45%) and the homology of chromosomal regions, such as the *lac* and lipopolysaccharide gene regions, observed for the two species (26) suggest that other DNA transfers and recombinations between these species might have occurred. Moreover, the symbiotic plasmid genes of the two species are homologous and are organized similarly (8, 52). It has been assumed that *R. leguminosarum* bv. phaseoli might have arisen through horizontal transfer of the symbiotic plasmid from *R. etli* to *R.*

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leguminosarum (44). Whatever their origin, these two species are more closely related to each other than the two subgroups of *R. tropici* are. Phylogenetically, an analysis of the whole sequence of the 16S rRNA genes indicated that *R. etli*, *R. leguminosarum*, and *R. tropici* formed a coherent monophyletic group which also included an independent lineage containing a single isolate from bean nodules (50) and the nonrhizobial species *Agrobacterium rhizogenes*, which is closely related to *R. tropici* (54). However, these three species do not account for all of the genotypic diversity of bean-nodulating rhizobia (14, 38, 50).

Some years ago, in a study of the diversity of field isolates of *R. leguminosarum* at a single site in France, Laguerre et al. (28) detected among bean nodule isolates a group of strains whose genotype differed from that of *R. leguminosarum*. Strains belonging to this group were also detected in another field 300 km away and among a collection of strains isolated from various sites in France (14). In this collection, a second novel group of six strains which could not be assigned to any of the three species or to the first group was also identified. The two groups, which had been differentiated from *R. leguminosarum*, *R. etli*, and *R. tropici* mainly by a restriction fragment length polymorphism analysis of total DNA performed with several DNA probes (14, 28), were later assigned to two new genomic species on the basis of the results of DNA-DNA hybridization and a partial 16S rRNA gene sequence analysis (27). Phylogenetically, the closest relative of genomic species 1 was *R. etli*, and the organisms most closely related to genomic species 2 were *Rhizobium galegae* (30) and *Rhizobium loti* (22). At that time, these new species were not named since additional data on their phenotypic characteristics were required according to the criteria outlined by Graham et al. (17) for the description of new species of the genus *Rhizobium*.

In the present study, phenotypic features of 90 strains isolated from *Phaseolus* spp. nodules and previously assigned to one of the two genomic species or to one of the three previously named species of rhizobia that nodulate beans were compared to the phenotypic features of reference rhizobial strains by numerical taxonomy. The phylogenetic position of each of the genomic species within the α -2 subgroup of the class *Proteobacteria* was assessed by determining the nucleotide sequence of the whole 16S rRNA gene of one representative strain. As a result of the present and previous studies, we propose two new species, *Rhizobium gallicum* and *Rhizobium giardinii*.

MATERIALS AND METHODS

Bacterial strains. A total of 112 strains were used in this investigation (Table 1). Eighty-nine strains were from our collection and had been isolated from root nodules of field-grown *Phaseolus vulgaris* sampled in various regions of France (14), in two fields in Java (Indonesia), and in one field in Burundi (49). The other 23 strains were type or representative strains of *Rhizobium* and *Sinorhizobium* species. All strains were maintained on agar slopes of Bergersen's medium (3) supplemented with 0.2 g of yeast extract per liter. Since isolation they had been stored at -80°C on the same medium containing 20% (vol/vol) glycerol.

Phenotypic characteristics. The following features were analyzed: (i) utilization of L-arabinose, D-fructose, D-galactose, D-glucose, D-glucosamine, D-glucuronate, lactose, maltose, mannitol, D-mannose, raffinose, L-rhamnose, D-ribose, D-sorbitol, sorbose, trehalose, xylose, dulcitol, erythritol, glucitol, inositol, glycerol, mannopine, octopine, soluble starch, citrate, oxalate, tartrate, lactate, and gluconate as sole carbon sources; (ii) utilization of L-arginine, L-alanine, L-asparagine, L-aspartate, L-cysteine, L-cystine, L-glutamine, L-glutamate, glycine, L-histidine, L-proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine as sole nitrogen sources; (iii) tolerance to nalidixic acid (40 $\mu\text{g/ml}$), streptomycin (3 $\mu\text{g/ml}$), spectinomycin (5 $\mu\text{g/ml}$), and kanamycin (10 $\mu\text{g/ml}$) added to TY medium (4); (iv) growth on TY medium containing 0.5, 1, and 2% NaCl; (v) growth on TY medium at pH 4, 5, and 8.5; (vi) growth on TY medium lacking calcium; and (vii) growth on Luria-Bertani medium.

To test for substrate utilization, the different carbon sources were added at a

final concentration of 1 g/liter to a basal medium containing (per liter) 1 g of K_2HPO_4 , 1 g of KH_2PO_4 , 0.01 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of CaCl_2 , 1 g of $(\text{NH}_4)_2\text{SO}_4$, and 15 g of agar. When substrates were tested as nitrogen sources, they were added at a concentration of 0.5 g/liter to a similar medium from which ammonium sulfate had been omitted and to which mannitol had been added at a concentration of 1 g/liter. All of the substrates were filter sterilized before they were added to the basal medium. Growth on the different media was tested in petri dishes by applying approximately 10^5 bacteria from a freshly prepared bacterial suspension with a multipoint inoculator to the surface of the agar. Cultures were incubated at 28°C , and the growth was recorded after 4 or 5 days.

The results were coded as follows: 1 for heavy or light growth and 0 for absence of growth. Similarity values were calculated by using a simple matching coefficient (46). A dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic means (UPGMA) (45). In order to test the goodness of fit in the cluster analysis, cophenetic value matrices were calculated and compared with the original similarity matrix that was UPGMA clustered by using the NTSYS-pc analysis package (version 1.8; Exeter Software, Setauket, N.Y.). Matrix correlation was estimated by using the correlation coefficient r , which is the normalized Mantel test statistic Z .

Plant tests. The ability of isolates to nodulate *Macroptilium atropurpureum* and *Onobrychis viciifolia* was tested in quadruplicate as described by Vincent (53). The effectiveness of symbiosis with *Phaseolus vulgaris* was measured by using plants grown under the greenhouse conditions described by Amarger et al. (2). Four pots were used for each isolate, and the plants were harvested after 7 weeks of growth.

16S rDNA sequencing and data analysis. Bacteria were grown on agar slopes of TY medium at 28°C for 24 h. The nearly full-length 16S rRNA genes were amplified by PCR from lysed cell suspensions with primers fd1 and rd1 as previously described (29). The PCR products were purified with Chroma Spin columns (Clontech, Inc., Palo Alto, Calif.) and were cloned by using a pT7Blue-T vector kit (Novagen, Inc., Madison, Wis.) according to the manufacturers' instructions. Plasmid DNA was purified from *Escherichia coli* DH5 α F' host cells by using a Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany). DNA sequencing was carried out by the dideoxy chain termination method (43) with a T7 sequencing kit (Pharmacia LKB Biotechnology, Orsay, France). Sequencing primers T3 and T7 supplied by Pharmacia and 12 additional primers synthesized by Eurogentec S.A. (Seraing, Belgium) were used to sequence both strands of the 16S rDNA molecules. The forward primers used spanned *E. coli* positions 7 to 26, 480 to 497, 668 to 688, 887 to 904, 1071 to 1088, and 1300 to 1320, and the reverse sequencing primers used spanned positions 297 to 315, 515 to 532, 838 to 857, 1072 to 1092, 1249 to 1267, and 1530 to 1546.

Nucleotide sequences were aligned by using the Clustal program (19) from the Bisance software (10). A matrix of pairwise distances corrected for multiple base substitutions by the method of Kimura (24) was calculated with the program DNADIST of the Phylogenetic Inference Package (PHYLIP) (12), and a phylogenetic tree was constructed with the NEIGHBOR program (42). A bootstrap confidence analysis was performed by using 200 replications and the SEQBOOT and CONSENSE programs of PHYLIP. A parsimony analysis was performed with the computer program PAUP (Phylogenetic Analysis Using Parsimony) (48), and the robustness of the topologies was evaluated through 200 bootstrap replications (heuristic search).

The GenBank accession numbers for the nucleotide sequences of the 16S rRNA genes of the rhizobia and related bacteria used for comparison are as follows: U29386 (*R. leguminosarum* bv. viciae); X67227 (*R. leguminosarum* bv. trifolii); U29388 (*R. leguminosarum* bv. phaseoli); X67233 (*R. tropici* type IIA); X67234 (*R. tropici* type IIB); U28916 and U28939 (*R. etli*); D12793 (*R. galegae*); X67229 (*R. loti*); D12797 (*Rhizobium huakuii*); U07934 (*Rhizobium ciceri*); L38825 (*Rhizobium mediterraneum*); U29387 (*Rhizobium* sp. strain RCR 3618D); X67232 (*Rhizobium* sp. strain LMG 9509); D01271 (*Rhizobium* sp. strain OK50); D01268 (*Rhizobium* sp. strain 113); D12783 (*Sinorhizobium meliloti*); X67231 (*Sinorhizobium fredii*); X68388 (*Sinorhizobium teranga*); X68390 (*Sinorhizobium saheli*); D12781 (*Bradyrhizobium japonicum*); X67224 (*Agrobacterium rhizogenes*); X67223 (*Agrobacterium tumefaciens*); X67228 (*Agrobacterium rubi*); X67225 (*Agrobacterium vitis*); X67221 (*Azorhizobium caulinodans*); M65249 (*Bartonella bacilliformis*); X73041 (*Blastobacter aggregatus*); L26167 (*Brucella neotomae*); D12786 (*Mycoplana dimorpha*); D12794 (*Ochrobactrum anthropi*); D12789 (*Phyllobacterium myrsinacearum*); M73229 (*Rochalimaea henselae*); and M11927 (*Rochalimaea quintana*).

Nucleotide sequence accession numbers. The 16S rDNA sequences of strains R602sp^T and H152^T have been deposited in the GenBank database under accession no. U86343 and U86344, respectively.

RESULTS

Numerical analysis of phenotypic data. The result of the cluster analysis performed on the 112 strains for 64 phenotypic characteristics is shown in Fig. 1. All of the strains examined, including the *Rhizobium* and *Sinorhizobium* reference strains, were linked at a similarity level of 82%. For strains tested in

TABLE 1. Strains used in this study

Strain(s)	Host plant source	Geographical origin	Source ^a	Reference
Genomic species 1 (<i>Rhizobium gallicum</i>) strains				
<i>Rhizobium gallicum</i> bv. <i>gallicum</i> strains				
DC22	<i>Phaseolus coccineus</i>	Côte d'Or, France	MSDJ	14
PhF29	<i>Phaseolus vulgaris</i>	Côte d'Or, France	MSDJ	14
PhP222	<i>Phaseolus vulgaris</i>	Tarn et Garonne, France	MSDJ	14
R602sp ^T	<i>Phaseolus vulgaris</i>	Maine et Loire, France	MSDJ	14
<i>Rhizobium gallicum</i> bv. <i>phaseoli</i> strains				
PhD12	<i>Phaseolus vulgaris</i>	Gers, France	MSDJ	14
PhI21	<i>Phaseolus vulgaris</i>	Gers, France	MSDJ	14
Genomic species 2 (<i>Rhizobium giardinii</i>) strains				
<i>Rhizobium giardinii</i> bv. <i>giardinii</i> strains				
H152 ^T , H153, H231, H236, H241, H325, H342, H412, H423, H462	<i>Phaseolus vulgaris</i>	Côte d'Or, France	MSDJ	14
Ro1, Ro24, Ro53, Ro61, Ro68, Ro7, Ro70, Ro75, Vp2	<i>Phaseolus vulgaris</i>	Yvelines, France	MSDJ	14
<i>Rhizobium giardinii</i> bv. <i>phaseoli</i> strains				
H251	<i>Phaseolus vulgaris</i>	Côte d'Or, France	MSDJ	14
PhF28	<i>Phaseolus vulgaris</i>	Drôme, France	MSDJ	14
Ro52, Ro67, Ro84, Ro88	<i>Phaseolus vulgaris</i>	Yvelines, France	MSDJ	14
<i>Rhizobium</i> sp. (<i>Phaseolus</i>) strain FL27	<i>Phaseolus vulgaris</i>	Puebla, Mexico	CIFN	38
<i>Rhizobium etli</i> strains				
CFN 42 ^T	<i>Phaseolus vulgaris</i>	Guanajuato, Mexico	CIFN	44
CP53, CP91, CP115, CP144, CP145, CP212	<i>Phaseolus vulgaris</i>	Cipanas, Indonesia	MSDJ	49
GD112, GD163, GD261, GD274	<i>Phaseolus vulgaris</i>	Gadag, Indonesia	MSDJ	49
BB13, BC15, BE22, BC45	<i>Phaseolus vulgaris</i>	Burundi	MSDJ	49
Viking I	<i>Phaseolus vulgaris</i>	Belize	CIFN	38
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> ATCC 10004 ^T	<i>Pisum sativum</i>	Illinois	ATCC	
<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> CC2480a	<i>Trifolium subterraneum</i>	Greece	C.S.I.R.O.	
<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i> strains				
8401	<i>Phaseolus vulgaris</i>	England		55
H123, H124, H132, H136, H141, H211, H242, H254, H313, H317, H441	<i>Phaseolus vulgaris</i>	Côte d'Or, France	MSDJ	14
PhC152	<i>Phaseolus vulgaris</i>	Haute Garonne, France	MSDJ	14
PhC202	<i>Phaseolus vulgaris</i>	Gers, France	MSDJ	14
PhF32	<i>Phaseolus vulgaris</i>	Côte d'Or, France	MSDJ	14
PhI10	<i>Phaseolus vulgaris</i>	Tarn et Garonne, France	MSDJ	14
PhN842	<i>Phaseolus vulgaris</i>	Aisne, France	MSDJ	14
PhN852, PhN862	<i>Phaseolus vulgaris</i>	Yonne, France	MSDJ	14
PhP17	<i>Phaseolus vulgaris</i>	Aisne, France	MSDJ	14
<i>Rhizobium tropici</i> strains				
Type IIA strains				
CFN 299	<i>Phaseolus vulgaris</i>	Brazil	CIFN	44
UMR1178	<i>Phaseolus vulgaris</i>	Unknown	CIFN	44
AIREL28, AIREL35	<i>Phaseolus vulgaris</i>	Lot et Garonne, France	MSDJ	2
CARA14, CARA56, CARA72	<i>Phaseolus vulgaris</i>	Landes, France	MSDJ	2
CESTAS114, CESTAS134, CESTAS221, CESTAS224	<i>Phaseolus vulgaris</i>	Gironde, France	MSDJ	2
Type IIB strains				
CIAT 899 ^T	<i>Phaseolus vulgaris</i>	Colombia	CIFN	44
BR859, BR863	<i>Leucaena leucocephala</i>	Brazil	CIFN	44
BA45	<i>Phaseolus vulgaris</i>	Burundi	MSDJ	49
Type II strain BR864	<i>Leucaena leucocephala</i>	Brazil	CIFN	44
<i>Rhizobium galegae</i> ATCC 43677 ^T	<i>Galega orientalis</i>	Finland	ATCC	30
<i>Rhizobium loti</i> strains				
NZP2037	<i>Lotus divaricatus</i>	New Zealand	NZP	7
NZP2234	<i>Lotus corniculatus</i>	New Zealand	NZP	7
<i>Sinorhizobium fredii</i> USDA205 ^T	<i>Glycine max</i>	People's Republic of China	USDA	20
<i>Sinorhizobium meliloti</i> RCR2011	<i>Medicago sativa</i>	Australia		7
<i>Sinorhizobium saheli</i> ORS611	<i>Sesbania grandiflora</i>	Senegal	ORS	9
<i>Sinorhizobium teranga</i> bv. <i>acaciae</i> ORS1007	<i>Acacia laeta</i>	Senegal	ORS	32
<i>Sinorhizobium teranga</i> bv. <i>sesbaniae</i> ORS51	<i>Sesbania rostrata</i>	Senegal	ORS	32
<i>Rhizobium</i> sp. (<i>Acacia</i>) cluster U strains ORS1004 and ORS1010	<i>Acacia senegal</i>	Senegal	ORS	9
<i>Agrobacterium tumefaciens</i> C58				25

^a ATCC, American Type Collection, Rockville, Md.; CIFN, Centro de Investigacion sobre Fijacion de Nitrogeno, Universidad Nacional Autonoma de Mexico, Cuernavaca, Morelos, Mexico; C.S.I.R.O., Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra City, Australian Capital Territory, Australia; MSDJ, Laboratoire de Microbiologie des Sols, Institut National de la Recherche Agronomique, Dijon, France; NZP, Culture Collection, Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand; ORS, ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement en Coopération, Dakar, Sénégal; USDA, U.S. Department of Agriculture, Beltsville, Md.

replicate experiments, the similarity values were between 98 and 94%. The strains belonging to the same species, or to the same subgroup in the case of *R. tropici*, clustered independent of their geographical origins, at similarity coefficients varying

from 96 to 90%. The only exception was in the species *R. tropici*, in which one type IIA strain, UMR1178, clustered with type IIB. The strains belonging to *Rhizobium* sp. (*Phaseolus*) genomic species 1 clustered at a similarity level of 93%, as did

FIG. 1. UPGMA dendrogram showing phenotypic relationships among rhizobia isolated from *Phaseolus vulgaris* in France and reference strains. The cophenetic correlation (r) value was 0.77.

the 25 strains of genomic species 2. Unclassified strain FL27 was included in the group formed by genomic species 1. *R. leguminosarum*, *R. etli*, and the two subgroups of *R. tropici* each formed a cluster, and each of these clusters was divergent from the three other clusters and from the clusters formed by each genomic species. Most of the levels of similarity between these six clusters and the representative strains of the other *Rhizobium* and *Sinorhizobium* species were less than 90%; the only exception was strain ORS611 of *S. saheli*, which grouped with the genomic species 2 cluster at a similarity coefficient of 92%.

All of the strains tested grew on L-arabinose, D-fructose, D-galactose, D-glucose, D-glucosamine, D-glucuronate, lactose, maltose, mannitol, D-mannose, raffinose, L-rhamnose, D-ribose, D-sorbitol, trehalose, xylose, glucitol, inositol, glycerol, soluble starch, and gluconate as carbon sources. Although they all grew on L-arginine, L-alanine, L-asparagine, L-aspartate, L-cysteine, L-cystine, L-glutamine, L-glutamic acid, L-histidine, L-proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, and L-valine as nitrogen sources, differences in growth were noticed among the isolates belonging to the different bean-nodulating species. Isolates of genomic species 2 grew well on these amino acids, whereas the growth of *R. leguminosarum* and *R. etli* was very slight and the growth of genomic species 1 and *R. tropici* was intermediate. Table 2 shows the most relevant phenotypic features that differentiate the species studied. Utilization of erythritol, utilization of dulcitol, and utilization of lactate are the most useful traits for differentiating the species that nodulate beans. Besides tolerance to nalidixic acid, to which all of the bean-nodulating rhizobia except *R. leguminosarum* were resistant, tolerance to the antibiotics tested, tolerance to salinity, and tolerance to acidity and alkalinity were found to be more strain specific than species specific.

Symbiotic properties. The isolates belonging to genomic species 1 and 2 were tested for their ability to nodulate *Macroptilium atropurpureum* and *Onobrychis viciifolia*. The results are shown in Table 3 along with the results of nodulation tests previously performed with *Phaseolus vulgaris* and *Leucaena leucocephala* (14). All of the isolates nodulated *Macroptilium atropurpureum*, but the time at which the nodules appeared varied with the isolate. For most of the isolates, nodules appeared 10 to 15 days after inoculation, and by 20 days all of the plants were nodulated. For other isolates, nodules appeared only 4 to 6 weeks after inoculation. The isolates in which delayed nodulation of *Macroptilium atropurpureum* was observed were the isolates which had been previously found to be unable to nodulate *L. leucocephala*. Among the isolates which nodulated both *Macroptilium atropurpureum* and *L. leucocephala*, only those belonging to genomic species 1 were able to nodulate *Onobrychis viciifolia*. All of the isolates of genomic species 1, whatever their nodulation phenotype, formed an effective symbiosis with *Phaseolus vulgaris*, whereas for genomic species 2 isolates the effectiveness differed with the nodulation phenotype of the isolate. In this species the isolates which nodulated *Macroptilium atropurpureum* and *L. leucocephala* were totally ineffective with *Phaseolus vulgaris*. The dry matter yield was the same as the dry matter yield of uninoculated control plants, and the nodules formed were numerous, small (1 to 2 mm in diameter), and white or very pale green. The genomic species 2 isolates which did not nodulate *L. leucocephala* and showed delayed nodulation of *Macroptilium atropurpureum* were very weakly effective. The dry matter yield which they gave when they were inoculated onto *Phaseolus vulgaris* was greater ($P = 0.058$) than the dry matter yield of the uninoculated control. The nodules resembled nodules formed

by efficient strains; they were almost as big (2 to 4 mm in diameter), but were dark green instead of red.

Nucleotide sequence analysis of 16S rDNA. The nearly complete 16S rRNA gene sequences of strains R602sp^T and H152^T were determined. The number of nucleotide differences between the two strains was 64, which represented a similarity value of 95.7%. These sequences differed from those of 34 reference strains belonging to the α -2 subclass of the class *Proteobacteria*, including the sequences of 6 rhizobial strains isolated from *Phaseolus vulgaris* (Table 4). The sequence of strain R602sp^T was most similar to that of *Rhizobium* species 3 (*Pterocarpus*) strain OK50 (level of similarity, 98.4%; 23 nucleotide differences). The other most similar 16S rDNAs were those of *R. etli* and *R. leguminosarum* (Table 4). Strain H152^T showed relatively low sequence similarities with the reference strains examined, with similarity values ranging from 96.8 to 88.6%. The highest values were the values obtained with the 16S rDNAs of *S. meliloti* and *S. fredii* (46 and 49 nucleotide differences, respectively).

The phylogenetic tree constructed by the distance matrix method with the neighbor-joining (NJ) algorithm is shown in Fig. 2. Strains R602sp^T and OK50 were clustered on a monophyletic lineage in 95% of the bootstrap replicates. These strains formed a cluster together with *R. leguminosarum*, *R. etli*, the *R. tropici*-*Agrobacterium rhizogenes* subgroup, and *Rhizobium* sp. (*Phaseolus*) strain RCR3618D (bootstrap value, 99%). Strain H152^T formed an independent lineage and was distantly related to the other bean-nodulating strains. The organisms most closely, but moderately, related to this lineage were *R. galegae* and some *Agrobacterium* species which formed a subgroup together with *Blastobacter aggregatus* in 90% of the bootstrap replicates in the NJ analysis. The branch point of strain H152^T, which was located outside the *R. galegae*-*Agrobacterium* subgroup, was supported by a bootstrap value of 94%. To assess the reliability of the branch points of R602sp^T and H152^T, the data were also analyzed by performing a parsimony analysis (data not shown). While the main groups and the species pair subgroups remained stable, there were some changes in position and branching order, as previously reported by Willems and Collins (54). The bootstrap confidence values obtained with the parsimony method were generally lower than those obtained with the NJ method. However, the results corroborated the phylogenetic positions of strains R602sp^T and H152^T estimated from the NJ analysis.

DISCUSSION

In order to avoid as much as possible inconsistency in bacterial taxonomy, the polyphasic approach is now recommended for the creation of new taxa (51). This approach requires the integration of phenotypic genotypic and phylogenetic information. We used this approach to determine the taxonomic relationships of two new groups of rhizobia nodulating members of the genus *Phaseolus* obtained from several locations in France. The genotypic data were obtained during ecological investigations in which we used DNA-based methods to examine the diversity of rhizobial isolates (14, 28). Plasmid profiling, which is highly discriminatory for many *Rhizobium* species, was used to categorize *Rhizobium* field populations isolated from *Phaseolus* nodules. A sample representative of the plasmid profile diversity was further characterized by whole-genome restriction fragment analysis and by analysis of the patterns of restriction fragments hybridizing to labeled genomic probes. The use of two chromosomal probes specific for *R. leguminosarum* and *R. etli* (26, 28) allowed us to identify a group of isolates which lacked homology to these probes and corre-

TABLE 2. Characteristics that differentiate bean-nodulating rhizobia and other rhizobia

Characteristic	<i>R. gallium</i> (<i>n</i> = 6) ^a	<i>R. gairdneri</i> (<i>n</i> = 25)	<i>R. leguminosarum</i> (<i>n</i> = 37)	<i>R. etli</i> (<i>n</i> = 16)	<i>R. tropici</i> type IIA (<i>n</i> = 10)	<i>R. tropici</i> type IIA strain UMR1178	<i>R. tropici</i> type IIB (<i>n</i> = 4)	<i>R. galegae</i> ATCC 43677 ^b	<i>R. loti</i> (<i>n</i> = 2)	<i>S. meliloti</i> RCR2011	<i>S. fredii</i> USDA205	<i>S. saheli</i> ORS611	<i>S. leranga</i> (<i>n</i> = 2)	Cluster U (<i>n</i> = 2)	<i>Agrobacterium</i> <i>tumefaciens</i> CS8
Utilization of the following compounds as sole carbon sources:															
L-Sorbose	— ^b	—	—	—	—	+	—	—	—	±	—	—	—	—	±
Dl-Tartrate	—	16	59	38	—	—	50	—	—	—	—	—	50	50	±
D-Glucuronic	—	32	—	63	+	+	75	+	—	—	—	—	+	50	+
Erythritol	+	—	+	—	—	+	—	—	50	+	+	±	—	—	—
Dulcitol	±	—	+	44	—	—	—	+	±	+	±	±	—	+	+
Glucosate	83	52	46	±	+	+	50	±	50	+	—	—	50	50	+
Citrate	—	—	—	—	—	±	—	—	—	—	—	—	—	—	—
Dl-Lactate	±	+	—	—	—	±	±	±	+	+	+	+	+	±	+
Utilization of the following compounds as sole nitrogen sources:															
L-Glycine	—	—	—	—	—	+	+	—	—	+	+	±	+	—	+
L-Tryptophan	—	48	—	63	±	+	+	—	+	+	+	+	+	+	+
L-Tyrosine	±	+	+	88	80	±	±	—	50	+	+	+	+	±	+
Tolerance to:															
Nalidixic acid (40 mg/liter)	+	+	—	+	+	+	+	—	—	+	—	—	+	+	+
Streptomycin (3 mg/liter)	50	—	—	75	—	+	+	—	—	—	—	—	—	—	+
Spectinomycin (5 mg/liter)	—	—	—	63	30	+	+	—	+	—	—	—	—	—	+
Kanamycin (10 mg/liter)	+	—	—	44	20	—	—	+	—	+	—	—	—	—	+
NaCl (2%)	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+
NaCl (1%)	—	76	—	—	—	+	±	+	+	+	+	+	+	50	—
NaCl (0.5%)	+	+	32	±	+	+	+	+	+	+	+	+	+	50	+
Growth on:															
TY medium, pH 4	—	33	16	54	±	±	±	—	50	—	—	—	±	—	±
TY medium, pH 8.5	—	±	24	50	50	±	±	—	±	±	±	+	+	—	±
TY medium lacking calcium	67	56	—	50	40	+	+	±	+	+	+	—	+	+	+
Luria-Bertani medium	—	—	—	—	—	±	+	—	—	+	—	—	—	—	+

^a *n* is the number of strains studied.^b —, at least 95% of the strains did not grow; +, at least 95% of strains are positive but show light growth. The values are the percentages of positive strains.

TABLE 3. Nodulation and nitrogen fixation by isolates belonging to *R. gallicum* and *R. giardinii*

Strains	Nodulation of:				Nitrogen fixation with <i>Phaseolus vulgaris</i> ^b
	<i>Phaseolus vulgaris</i> ^a	<i>L. leucocephala</i> ^a	<i>Macroptilium atropurpureum</i>	<i>Onobrychis viciifolia</i>	
<i>R. gallicum</i> strains					
R602sp ^T , DC22, PhP222, PhF29	+	+	+ (<20 days) ^c	+	+
PhI21, PhD12	+	—	+ (30-40 days)	—	+
<i>R. giardinii</i> strains					
H152 ^T , H153, H231, H236, H241, H325, H342, H412, H423, H462, Ro17, Ro24, Ro53, Ro61, Ro68, Ro7, Ro70, Ro75, Vp2	+	+	+ (<20 days)	—	—
H251, PhF28, Ro52, Ro67, Ro84, Ro88	+	—	+ (30-40 days)	—	±

^a Data from reference 14.^b +, the shoot dry matter weight of plants inoculated with each strain was 75 to 105% that of plants inoculated with a reference strain of *R. leguminosarum* bv. *phaseoli*; ±, the shoot dry matter weight was 10 to 25% that of the reference strain and was different from that of the uninoculated control ($P = 0.058$); —, the shoot dry matter weight was less than 10% that of the reference strain and did not differ ($P < 0.01$) from that of the uninoculated control. The shoot dry matter weight of plants inoculated with the reference strain was 4.1 ± 0.3 g.^c The data in parentheses are the times at which nodules appeared.

sponded to isolates in which a megaplasmid (length, more than 1,000 kb) had been observed. As determined by ribotyping, these isolates were found to be distributed in two genomic groups which themselves were polymorphic (14). To further characterize isolates assigned to the two groups, total cellular DNA of a member of each group was used as a probe. Under stringent hybridization conditions, each probe was found to be group specific and did not hybridize with DNAs of *R. leguminosarum*, *R. etli*, or *R. tropici* strains. The levels of DNA relatedness between strains that were representative of the genomic diversity found in each group and the type strains of *Rhizobium* species were less than 21%. The level of DNA relatedness between the two groups was 18%. Each group was thus identified as a new genomic species (27). The minimum levels of DNA relatedness between strains in genomic species 1 and genomic species 2 were 76 and 74%, respectively (27).

In the present study, we compared phenotypic traits of isolates belonging to the two genomic species to phenotypic traits of isolates representing the genotypic diversity found in French isolates of *R. leguminosarum* bv. *phaseoli* and *R. tropici* and to phenotypic traits of reference strains. To represent the diversity of *R. etli*, we chose isolates from diverse geographical areas. The phenotypic analysis allowed us to cluster isolates from bean nodules into six different phena. Each phenon contained isolates that had a common genotype. The two phena corresponding to the two genomic species were well-delineated. The genomic species 1 cluster included unclassified strain FL27, a strain which was isolated from a *Phaseolus* nodule in Mexico (38). We previously observed that the 16S rDNA type of this strain was similar if not identical to the 16S rDNA type of genomic species 1 isolates (25, 27). Taken together, these results suggest that strain FL27 may belong to genomic species 1, but more data are needed to definitely assign this strain to the species. The *R. leguminosarum* phenon was well-differentiated from the *R. etli* phenon. This indicates that unlike the MLEE analysis performed by Eardly et al. (11), the phenotypic analysis permitted distinction between the *R. etli* and *R. leguminosarum* 16S rRNA alleles. However, this must be checked with a more diversified sample of strains. Isolates belonging to *R. tropici* formed two phena which corresponded to the two subgroups described by Martinez-Romero et al. (36) and supported the division of the members of the species into two subgroups. The phenotypic analysis showed that the different groups of bean-nodulating rhizobia that we identified as genomic species were also phenotypically

distinct from each other and from the *Rhizobium* species described previously and thus should be assigned to new species.

The results of the 16S rDNA sequence comparison and phylogenetic analysis support the creation of new species. Genomic species 1 clustered with the three previously recognized species of bean-nodulating rhizobia. Mainly on the basis of 16S rRNA gene analysis data, it has been suggested recently that only *R. leguminosarum*, which is the type species of the genus *Rhizobium*, *R. etli*, *R. tropici*, and *Agrobacterium rhizogenes* should represent the genus *Rhizobium* (56). *R. loti* and the other closely related species, *R. huakuii*, *R. ciceri*, and *R. mediterraneum*, should be assigned to a new genus, for which the name "*Mesorhizobium*" has been proposed (31). *R. galegae*, which is more closely related to some *Agrobacterium* species, does not fit into any of the five generic groups of rhizobia (the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium*, and "*Mesorhizobium*"). It has been suggested that *R. galegae* should not be reclassified in a new genus until closely related species are identified on this lineage (56). The levels of 16S rDNA similarity (98 to 96.6%) between strain R602sp^T and the members of the species that form the genus *Rhizobium* sensu stricto as defined above were comparable to the levels of 16S rDNA similarity among these species (99.8 to 97.6%) and were higher than the levels of 16S rDNA similarity between R602sp^T and other rhizobia (<96.2%). In particular, the levels of similarity between R602sp^T and *R. leguminosarum* (97.5%) and between R602sp^T and *R. etli* (98%) indicated that the genomic species represented by R602sp^T should be classified in the same genus as these two species. The analysis of the whole 16S rRNA gene sequence placed R602sp^T on a lineage independent from the lineages formed by *R. leguminosarum*, *R. etli*, and *R. tropici*. On the basis of an analysis of a 246-bp region from the 5' part of the 16S rRNA gene, R602sp^T and *R. etli* were previously found to be tightly clustered (27). This difference between results confirms that the partial 16S rDNA sequences analyzed were not fully representative of the whole gene and that phylogenetic positions inferred from partial 16S rDNA sequence analyses should be considered more indicative than definitive.

Strain R602sp^T branched with an unclassified *Rhizobium* strain, OK50, which was isolated from nodules of *Pterocarpus klemmei* in Japan (37). R602sp^T exhibited the highest level of 16S rDNA sequence similarity with this strain (98.4%). The levels of 16S rDNA homology between R602sp^T and strains which cannot be differentiated by 16S rRNA gene restriction

TABLE 4. Levels of similarity and observed numbers of nucleotide differences in the aligned 16S rDNA sequences of strains of *Rhizobium* and *Agrobacterium* species

Strain	No. of nucleotide differences or % similarity ^a													
	<i>R. etli</i> CFN 42 ^T	<i>R. etli</i> TAL182	<i>R. leguminosarum</i> bv. phaseoli RCR3644	<i>R. tropici</i> type IIA strain CFN 299	<i>R. tropici</i> type IIB strain CIAT 899 ^T	<i>Rhizobium</i> sp. strain RCR3618D	<i>R. galegae</i> ATCC 43677 ^T	<i>Agrobacterium tumefaciens</i> LMG 196	<i>Agrobacterium rubi</i> LMG 156 ^T	<i>Agrobacterium vitis</i> LMG 8750 ^T	<i>R. gallicum</i> R602sp ^T	<i>R. giardinii</i> H152 ^T	<i>R. etli</i> CFN 42 ^T	<i>R. etli</i> TAL182
<i>R. etli</i> CFN 42 ^T	6	17	33	35	31	32	68	80	92	94	29	65		
<i>R. etli</i> TAL182														
<i>R. leguminosarum</i> bv. phaseoli RCR3644	99.6	99.2	12	34	34	26	67	82	96	94	29	64		
<i>R. tropici</i> type IIA strain CFN 299	98.8	97.6	30	30	22	36	65	80	94	92	37	62		
<i>R. tropici</i> type IIB strain CIAT 899 ^T	97.7	97.6	97.9	9	42	54	73	69	82	85	49	54		
<i>Rhizobium</i> sp. strain RCR3618D	97.9	98.2	98.5	99.4	40	52	72	71	85	84	49	55		
<i>R. galegae</i> ATCC 43677 ^T	97.8	98.2	97.5	97.1	38	57	63	72	86	90	47	60		
<i>Agrobacterium tumefaciens</i> LMG 196	95.2	95.3	95.5	96.2	97.4	96.0	95.7	62	86	88	23	65		
<i>Agrobacterium rubi</i> LMG 156 ^T	94.4	94.3	94.4	95.2	95.6	94.8	95.4	98.5	21	51	54	62		
<i>Agrobacterium vitis</i> LMG 8750 ^T	93.6	93.3	93.4	94.3	94.0	94.0	95.4	95.9	95.3	68	82	55		
<i>R. gallicum</i> R602sp ^T	93.4	93.4	93.6	94.0	93.7	93.8	96.4	94.3	93.6	94.0	86	69		
<i>R. giardinii</i> H152 ^T	98.0	98.0	97.5	96.6	96.8	98.4	96.2	96.2	95.7	95.2	95.7	64		
<i>R. etli</i> CFN 42 ^T	95.5	95.5	95.7	96.2	95.9	95.6	95.7	96.2	95.7	95.2	95.7			

^a Gap-containing and missing information was removed before the numbers of nucleotides were calculated. The values on the lower left are the levels of similarity, and the values on the upper right are the observed numbers of nucleotide differences.

analysis are also probably high. Besides *Rhizobium* sp. (*Phaseolus*) strain FL27, which belonged to the same phenon as R602sp^T, as shown above, three strains isolated from *Onobrychis* spp. and one strain isolated from *Oxytropis riparia* were reported to be indistinguishable from R602sp^T on the basis of 16S rRNA gene restriction analysis data (29). According to Stackebrandt and Goebel (47), organisms exhibiting more than 97% rRNA similarity may belong to the same species. *Rhizobium* sp. strain OK50 and the five strains which had the same 16S rDNA type as R602sp^T might belong to the same species as R602sp^T. However, as a minimum level of DNA-DNA homology between strains of 60 to 70% is needed for the strains to be classified in the same species, we need additional DNA relatedness data to establish whether these unclassified rhizobia belong to the same species as the genomic species 1 isolates. Nevertheless, these results indicate that this species, or at least organisms more closely related to it than to other recognized *Rhizobium* species, may not be limited to France and nodules of *Phaseolus* spp. but may be widely distributed across continents and among various host plant genera.

The phylogenetic trees constructed from the whole 16S rRNA gene analysis indicated that strain H152^T was more closely related to *R. galegae*, to unclassified *Rhizobium* (*Mimosa*) species 1 strain 113 (37), and to several *Agrobacterium* species than to any other rhizobia or related bacteria. In particular, H152^T did not have a close relationship with *R. loti*, which did not corroborate our previous data based on partial 16S rRNA gene sequencing (27). This is another example of the lack of accuracy found when a partial sequence from the 5' part of the 16S rRNA gene is analyzed. Strain H152^T was not closely related enough to *R. galegae* (16S rDNA similarity, 95.7%) to place these organisms in the same genus. However, the species represented by strain H152^T is not phylogenetically closely related to typical members of the genus *Rhizobium*, with 16S rDNA nucleotide sequence similarity values ranging from 96.2 to 95.5%. The levels of similarity between the 16S rDNA nucleotide sequences of H152^T and the *Agrobacterium* species which branched with *R. galegae* ranged from 96.2 to 95.2%. Again, these values are too low to place H152^T in the genus *Agrobacterium*.

Based on the phenotypic and phylogenetic data reported in this paper, we propose that two new *Rhizobium* species should be created for the two genotypically distinct species that we described previously. Genomic species 1 deserves separate species status in the genus *Rhizobium*, and we propose the name *R. gallicum* for it. Genomic species 2 was clearly separated by both phenotypic and molecular characteristics from all of the previously described species and genera of rhizobia. However, before a new genus name is created, it is necessary to identify other species that are closely related to genomic species 2 and also to *R. galegae*, its closest neighbor. As long as the relationships between these rhizobia are not clarified at the genus level, it seems advisable to classify genomic species 2 as a new species of the genus *Rhizobium*, and we propose the name *R. giardinii* for this taxon.

On the basis of the results of a restriction fragment length polymorphism analysis of symbiotic gene regions, we showed previously that genomic species 1 and 2 were each associated with two distinct symbiotic genotypes (14). One of these genotypes was characteristic of the species, and the other was common to the two species. The two symbiotic genotypes specific to the two species were characterized by a lack of homology with a *nodB* gene probe from CFN42, the type strain of *R. etli*, under the high-stringency hybridization conditions which we used for identifying the symbiosis plasmid of *R. leguminosarum* bv. phaseoli and *R. etli* bv. phaseoli (28). However, under less

FIG. 2. Phylogenetic tree obtained by the NJ method showing the relationships among rhizobia and several related taxa belonging to the alpha-2 subgroup of the class *Proteobacteria*. Significant bootstrap probability values are indicated at the branch points (only values greater than 90% are shown). Abbreviations: *R.*, *Rhizobium*; *R. l.*, *Rhizobium leguminosarum*; *Ag.*, *Agrobacterium*; *S.*, *Sinorhizobium*; *B.*, *Bradyrhizobium*; *Az.*, *Azorhizobium*; *Bl.*, *Blastobacter*; *P.*, *Phyllobacterium*; *Ro.*, *Rochalimaea*; *Ba.*, *Bartonella*; *M.*, *Mycoplana*; *O.*, *Ochrobactrum*; *Br.*, *Brucella*. The following unmarked strains were used for the phylogenetic analysis: *Agrobacterium rhizogenes* LMG 152, *Agrobacterium rubi* LMG 156^T, *Agrobacterium tumefaciens* LMG 196, *Agrobacterium vitis* LMG 8750^T, *Azorhizobium caulinodans* LMG 6465^T, *Bradyrhizobium japonicum* IAM 12608^T, *Brucella neotomae* ATCC 23459, *Mycoplana dimorpha* IAM 13154^T, *Ochrobactrum anthropi* IAM 14119^T, *Phyllobacterium myrsinacearum* IAM 13584^T, *R. ciceri* UPM-Ca7^T, *R. galegae* IAM 13631^T (= ATCC 43677^T), *R. huakuii* IAM 1611^T, *R. loti* LMG 6125^T, *R. mediterraneum* UPM-Ca36^T, *Rhizobium* sp. strain LMG 9509 (= BR814), *Rochalimaea quintana* Fuller; *S. fredii* LMG 6217^T (= USDA 205^T), *S. meliloti* IAM 12611^T, *S. saheli* LMG 7837^T, and *S. teranga* LMG 7834^T. The rhizobia that nodulate *Phaseolus vulgaris* are in boldface type. Scale bar = 0.004 substitution per site (s/s).

stringent conditions, homology to the *nod* probe was obtained and the hybridization signal was found to be located on a plasmid which ranged in size from 250 to 350 kb depending on the isolate, which indicated that the *nod* genes were plasmidic. In genomic species 1, the isolates with low levels of homology to the *nod* gene probe had one copy of the *nifH* gene localized on the same plasmid as the *nodB* gene, which was thus identified as the symbiotic plasmid. These isolates corresponded to those found in the present study to be capable of nodulating *Phaseolus vulgaris*, *L. leucocephala*, and *Onobrychis viciifolia* and to exhibit early nodulation with *Macroptilium atropurpureum*. In genomic species 2, the isolates with low levels of homology to the *nod* gene probe lacked homology to the *nifH*

gene even under low-stringency hybridization conditions. They corresponded to the isolates that nodulated *Phaseolus vulgaris* and *L. leucocephala*, exhibited early nodulation with *Macroptilium atropurpureum*, and did not fix nitrogen with *Phaseolus vulgaris*. Conversely, the isolates with the symbiotic genotype that was common to the two genomic species showed homology to the *nodB* gene probe and contained three copies of the *nifH* gene, which is typical of the symbiotic plasmid of *R. leguminosarum* bv. *phaseoli* and *R. etli* bv. *phaseoli*. This genotype was associated in both genomic species with the nodulation phenotype characterized by nodulation of the genus *Phaseolus* and late nodulation of *Macroptilium atropurpureum*, which is also the nodulation phenotype of *R. leguminosarum*

bv. phaseoli and *R. etli* bv. phaseoli. The genomic species 1 and 2 isolates with the symbiotic genotype and nodulation phenotype similar to the symbiotic genotype and nodulation phenotype of *R. leguminosarum* bv. phaseoli and *R. etli* bv. phaseoli likely received their symbiotic plasmid by horizontal transfer from the *R. leguminosarum* strains which were present in the fields in which the isolates originated. Similar, if not identical, symbiotic plasmids can therefore be harbored by field isolates belonging to four different species, which provides circumstantial evidence that interspecific transfer of a symbiotic plasmid occurred in natural environments. However, when this type of symbiotic plasmid was harbored in the genomic species 2 chromosomal background, it provided only a weak nitrogen fixation capacity to the isolates.

Since strains belonging to the same genomic species can harbor genotypically and phenotypically different symbiotic plasmids, we propose, by analogy to *R. leguminosarum* (23) and *S. teranga* (32), that the new species should be subdivided into biovars. As the genotypic and nodulation characteristics conferred by one of the symbiotic plasmids are common to the two genomic species and to *R. leguminosarum* bv. phaseoli and *R. etli* bv. phaseoli, we propose the epithet phaseoli for this biovar. The host range conferred by each plasmid that is characteristic of its own species is not limited to a single legume genus and has not been fully explored yet; therefore, it would be misleading to give to the biovars names derived from single host plants. Since the two species that we describe here each harbored a specific symbiotic plasmid, we propose that the names of the species should be used for the biovars. *R. gallicum* is thus divided into *R. gallicum* bv. gallicum and *R. gallicum* bv. phaseoli, and *R. giardinii* is divided into *R. giardinii* bv. giardinii and *R. giardinii* bv. phaseoli.

Description of *Rhizobium gallicum* sp. nov. *Rhizobium gallicum* (gal'li.cum. L. adj. *gallicum*, from *Gallia*, the country of origin, France). Gram-negative, aerobic, motile, non-spore-forming rods that are 0.5 to 0.7 μm wide by 1.5 to 2 μm long. Colonies on Bergersen's medium are circular, convex, white, semitranslucent, and usually 2 to 4 mm in diameter within 2 to 3 days at 28°C. These bacteria are unable to grow in Luria broth. They utilize a wide range of carbohydrates as sole carbon sources for growth. They grow on erythritol and are resistant to nalidixic acid. At the molecular level this species can be differentiated from other *Rhizobium* species and related genera by the results of DNA-DNA hybridization experiments, by the sequence of the 16S rRNA gene, and by amplified 16S rDNA restriction analysis data.

R. gallicum bv. gallicum strains nodulate *Phaseolus* spp., *L. leucocephala*, *Macroptilium atropurpureum*, and *Onobrychis viciifolia*. They fix nitrogen with *Phaseolus vulgaris*. At the molecular level this biovar can be differentiated from the other biovar by the presence of a single copy of the nitrogenase reductase gene *nifH*.

R. gallicum bv. phaseoli strains nodulate *Phaseolus* spp. They can also nodulate *Macroptilium atropurpureum* but do so with a delay of at least 1 month after inoculation. They do not nodulate *L. leucocephala* or *Onobrychis viciifolia*. They fix nitrogen with *Phaseolus vulgaris*. At the molecular level this biovar can be differentiated from the other biovar by the presence of three copies of the nitrogenase reductase gene *nifH*.

Strain R602sp, which was isolated from a nodule of *Phaseolus vulgaris*, is the type strain. This strain has the characteristics described above for *R. gallicum* bv. gallicum. Strain R602sp^T and all of the other strains of *R. gallicum* have been deposited in the culture collection of the Laboratoire de Microbiologie des Sols, Institut National de la Recherche Agronomique, Di-

jon, France. Strain R602sp^T has been deposited as strain MSDJ1109^T.

Description of *Rhizobium giardinii* sp. nov. *Rhizobium giardinii* (giar.di'ni.i. N.L. gen. n. *giardinii*, of Giardini, in memory of Roberto Giardini, a Brazilian rhizobiologist who first isolated the organism during a doctoral stay in the Laboratoire de Microbiologie des Sols). Gram-negative, aerobic, motile, non-spore-forming rods that are 0.5 to 0.7 μm wide by 1.6 to 2.2 μm long. Colonies on Bergersen's medium are circular, convex, white, translucent, and usually 2 to 4 mm in diameter within 2 to 3 days at 28°C. These bacteria are unable to grow in Luria broth. They utilize a wide range of carbohydrates as sole carbon sources for growth and a wide range of amino acids as sole nitrogen sources. They grow on lactate but not on erythritol or dulcitol and are resistant to nalidixic acid. At the molecular level this species can be differentiated from other *Rhizobium* species and related genera by the results of DNA-DNA hybridization experiments, by the sequence of the 16S rRNA gene, and by amplified 16S rDNA restriction analysis data.

R. giardinii bv. giardinii strains nodulate *Phaseolus* spp., *L. leucocephala*, and *Macroptilium atropurpureum*. They are not able to fix nitrogen with *Phaseolus vulgaris*. At the molecular level this biovar can be differentiated from the other biovar by the absence of hybridization with a probe containing the nitrogenase reductase gene *nifH*, even under very low-stringency conditions.

R. giardinii bv. phaseoli strains nodulate *Phaseolus* spp. They can also nodulate *Macroptilium atropurpureum* but do so with a delay of at least 1 month after inoculation. They do not nodulate *L. leucocephala*. They are only weakly efficient in nitrogen fixation with *Phaseolus vulgaris*. At the molecular level this biovar can be differentiated from the other biovar by the presence of three copies of the nitrogenase reductase gene *nifH*.

Strain H152, which was isolated from a nodule of *Phaseolus vulgaris* grown in Bretennières, France, is the type strain. This strain has the characteristics described above for *R. giardinii* bv. giardinii. Strain H152^T and all of the other strains of *R. giardinii* have been deposited in the culture collection of the Laboratoire de Microbiologie des Sols, Institut National de la Recherche Agronomique, Dijon, France. Strain H152^T has been deposited as strain MSDJ0144^T.

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