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Classification of Bacteria Nodulating *Lathyrus japonicus* and *Lathyrus pratensis* in Northern Quebec as Strains of *Rhizobium leguminosarum* biovar viciae†

PASCAL DROUIN,[1] DANIELLE PRÉVOST,[2]* AND HANI ANTOUN[1]

Recherche Sciences de la Vie et de la Santé, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4,[1] and Soil and Crops Research Centre, Agriculture and Agri-Food Canada, Sainte-Foy, Québec, Canada G1V 2J3[2]

The diversity of two populations of rhizobia isolated from *Lathyrus japonicus* (30 strains) and *Lathyrus pratensis* (49 strains) growing in northern regions of Quebec, Canada, was determined on the basis of phenotypic characteristics, multilocus enzyme electrophoresis, DNA-DNA homology, and 16S ribosomal DNA sequencing. According to numerical analysis of phenotypic characteristics, strains were divided into four groups. Strains isolated from *L. pratensis* fell in groups I to III; the latter included reference strains of *Rhizobium leguminosarum*. All strains isolated from *L. japonicus* were included in group IV. All strains had nodulation characteristics similar to those of *R. leguminosarum* bv. viciae. Strains isolated from *L. japonicus* originating from an arctic region were usually able to grow at 5°C and were more likely to be tolerant to copper ($CuCl_2 \cdot H_2O$, 100 μg/ml) and lead [$Pb(CH_3COO)_2$, 500 μg/ml] than strains isolated from *L. pratensis* from a boreal zone. However, both populations of *Lathyrus* strains were adapted to the cold in comparison to reference strains from temperate regions. Each population had similar genetic diversity ($H = 0.45$), determined by multilocus enzyme electrophoresis of the loci encoding eight enzymes, but the diversity obtained by analyzing all strains including the reference strains ($H = 0.58$) was higher. Representative strains of both populations showed high levels of DNA homology among themselves and with *R. leguminosarum*. Partial sequences of the 16S ribosomal RNA genes were similar to those reported for *R. leguminosarum* bv. viciae. We conclude that the strains isolated from *L. japonicus* and *L. pratensis* belong to *R. leguminosarum* bv. viciae but are distinguishable by growth at 5°C, which is a characteristic related to their geographic origin.

Many *Lathyrus* species are used for soil cover, green manure, erosion control, and rehabilitation of cutover or burned-over land. Plant species with agronomic potential such as *Lathyrus sylvestris* (flat pea), *Lathyrus tingitanus* (tangier pea), and *Lathyrus sativus* (chickling vetch) used for forage or green manure have been evaluated for their productivity with N input from N_2 fixation (3, 24). Microsymbionts from the legume genera *Lathyrus*, *Pisum*, and *Vicia* were named *Rhizobium leguminosarum* Frank in the first edition of *Bergey's Manual of Determinative Bacteriology* in 1923. The classification of *Lathyrus* rhizobia as *R. leguminosarum* was based on the description of bacteroids and on the host nodulation range. These characteristics were similar for *Lathyrus*, *Pisum*, *Vicia*, and *Lens* species and their rhizobia, and these reciprocal relationships seemed to be restricted within legumes from the tribe Vicieae (1). However, there is no report on the physiological and genetic relationships of *Lathyrus* rhizobia, while *R. leguminosarum* strains from *Vicia* or *Pisum* species have been thoroughly described probably because these symbioses are more agronomically important.

In the last decade, the classification of *R. leguminosarum* was revised with the addition of genetic techniques to the traditional phenotypic characterization. In the Bergey's 1984 edition (9), the three former species *R. leguminosarum*, *Rhizobium phaseoli*, and *Rhizobium trifolii* were grouped in a single species, *R. leguminosarum* comprising the biovars phaseoli, trifolii, and viciae. The division of the species in biovars is based largely on host plant specificity, and accordingly, *R. leguminosarum* bv. trifolii includes strains nodulating clovers, *R. leguminosarum* bv. viciae comprises strains nodulating plant species belonging or related to the tribe Vicieae, such as peas (*Pisum* spp.) and vetches (*Vicia*, *Lens*, and *Lathyrus* spp.), and *R. leguminosarum* bv. phaseoli includes strains that nodulate beans (*Phaseolus* spp.). An example of the complexity and the evolution of this classification has been revealed by the heterogeneity among strains of *R. leguminosarum* bv. phaseoli which resulted in the description of two new species: *Rhizobium tropici* and *Rhizobium etli* (12, 13, 19). The presence of such heterogeneity among strains of biovar viciae has not yet been reported.

The aim of our study was to classify rhizobia isolated from two *Lathyrus* species adapted to the cold conditions in northern Quebec, Canada. One legume species, *Lathyrus japonicus* (beach pea) is perennial and indigenous to the sandy coasts of Quebec, with a growth range extending from temperate to arctic regions (11, 15). The other leguminous species, *Lathyrus pratensis* (yellow vetchling), is an introduced European species that can grow in relatively cold regions of the boreal forest zone of Quebec (11). The rhizobial strains were characterized according to the minimal standards for the description of new genera and species of rhizobia (8).

MATERIALS AND METHODS

Bacterial strains. The two populations of rhizobia consisted of 49 strains from *L. pratensis* growing in a field of the Val d'Or region, Quebec, Canada (48°07′N, 77°47′W) and 30 strains from *L. japonicus* indigenous to Kuujjuarapik (55°20′N, 77°50′W) in the Hudson's Bay arctic zone of Quebec, Canada. Strains were isolated from nodules, and their identities were confirmed by testing their ability to nodulate the trap host species (23). For comparison, 18 reference strains belonging to known species of rhizobia or originating from different plant species were included in the study (Table 1). All strains were maintained on yeast extract-mannitol (YM) agar slants (23) at 4°C or as a dense suspension in 10% (vol/vol) glycerol at −70°C.

* Corresponding author. Mailing address: Soil and Crops Research Centre, Agriculture and Agri-Food Canada, 2560, Hochelaga Blvd., Sainte-Foy (Québec) Canada G1V 2J3. Phone: (418) 657-7980. Fax: (418) 648-2402. Electronic mail address: prevostd@em.agr.ca.

† Contribution 522 of Agriculture and Agri-Food Canada Research Centre at Sainte-Foy.

TABLE 1. Reference strains

Strain	Species	Host	Source[a]
USDA 191	*Rhizobium fredii*	*Glycine max*	USDA
CFN 42	*Rhizobium etli*	*Phaseolus vulgaris* L.	CFN
Viking I	*Rhizobium etli*	*Phaseolus vulgaris* L.	CFN
Hambi 540	*Rhizobium galegae*	*Galega officinalis*	HAMBI
CCBAU 2609	*Rhizobium huakuii*	*Astragalus sinicus*	CCBAU
ATCC 14480	*Rhizobium leguminosarum* bv. trifolii	*Trifolium pratense*	ATCC
ATCC 10004	*Rhizobium leguminosarum* bv. viciae	*Pisum sativum*	ATCC
USDA 2489	*Rhizobium leguminosarum* bv. viciae	*Vicia faba*	USDA
USDA 2445	*Rhizobium leguminosarum* bv. viciae	*Lathyrus latifolius*	USDA
92A3	*Rhizobium leguminosarum* bv. viciae	*Lathyrus hirsutus*	LiphaTech
L3	*Rhizobium loti*	*Lotus corniculatus*	Agriculture Canada
USDA 3471	*Rhizobium loti*	*Lotus corniculatus*	USDA
USDA 1002	*Rhizobium meliloti*	*Medicago sativa* subsp. sativa	USDA
CFN 299	*Rhizobium tropici*	*Phaseolus vulgaris* L.	CFN
CIAT 899	*Rhizobium tropici*	*Phaseolus vulgaris* L.	CFN
N31	*Rhizobium* sp.	*Astragalus alpinus*	Agriculture Canada
32H1	*Bradyrhizobium* sp.	*Crotalaria paulina*	LiphaTech
ATCC 10324	*Bradyrhizobium japonicum*	*Glycine hispida*	ATCC

[a] ATCC, American Type Culture Collection; USDA, U.S. Department of Agriculture, Beltsville Rhizobium Culture Collection, Beltsville, Md.; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Mexico; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland.

Numerical analysis of phenotypic characteristics. Strains were tested for their capacity to nodulate the following legume species: *Glycine max*, *L. japonicus*, *L. pratensis*, *Lens culinaris*, *Lotus corniculatus*, *Macroptilium atropurpureum*, *Medicago sativa*, *Onobrychis viciifolia*, *Phaseolus vulgaris*, *Pisum sativum*, *Vicia sativa*, and *Vicia faba*. Plants were grown in glass tubes (25 by 200 mm) containing a mixture of vermiculite and nitrogen-free nutrient solution (17), except for *Vicia faba*, which was grown in 500-ml Erlenmeyer flasks. Seedlings were inoculated with 10^8 cells of the strain to be tested, and plants were grown under a night-day temperature cycle of 15 and then 20°C with a 16-h light period (300 microeinsteins m^{-2} s^{-1}). After 1 month, plants were examined for nodulation. A strain was considered positive after reisolation from nodules.

Strains were tested for their ability to utilize different substrates for growth. Bacteria were grown in YM broth (23) to the mid-exponential phase, washed twice, and resuspended in NaCl (0.85%). Bacterial suspensions (10^8 cells/ml) were inoculated in triplicate with a replica plater on agar plates of minimal medium (17) containing biotin, thiamine, and pantothenic acid and the appropriate test substrate. The carbohydrates (1 g/liter each) L-(+)-arabinose, dextrin, dulcitol, β-D-fructose, α-D-(+)-fucose, D-(+)-galactose, glucose, glycerol, inositol, α-lactose, maltose, mannitol, D-(+)-mannose, D-(+)-melibiose, raffinose, α-L-rhamnose, D-ribose, D-sorbitol, sucrose, trehalose, and D-(+)-xylose and the organic acids (each at 2, 10, and 20 mM) acetate, α-ketoglutarate, fumarate, glyoxylate, lactate, malate, oxaloacetate, pyruvate, and succinate were each tested for utilization as the sole carbon source. When the amino acids (0.5 g/liter each) L-alanine, L-arginine hydrochloride, L-asparagine, L-glutamate, L-glutamine, glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, and L-valine were each tested as the sole carbon and nitrogen source, ammonium nitrate was not included in the medium. Plates were incubated at 25°C for 3 days, at which time the formation of colonies was recorded.

Resistance to antibiotics was tested by using cell suspensions described above and a replica plater to inoculate the surfaces of YM agar medium plates containing ampicillin (10 μg/ml), chloramphenicol (10 μg/ml), erythromycin (10 μg/ml), gentamicin (25 μg/ml), kanamycin (10 μg/ml), neomycin (10 μg/ml), nystatin (2 μg/ml), penicillin (15 μg/ml), rifampin (120 μg/ml), streptomycin (60 μg/ml), or tetracycline (5 μg/ml). Strains were considered resistant when growth occurred and sensitive when no growth or very poor growth occurred.

Strains were tested for their ability to grow in the presence of heavy metals. Plates containing YM agar medium supplemented with $CuCl_2 \cdot H_2O$ (100 μg/ml), $AlCl_3 \cdot 6H_2O$ (500 μg/ml), $HgCl_2$ (5 μg/ml), $CdCl_2 \cdot 2H_2O$ (20 μg/ml), $ZnCl_2$ (100 μg/ml), or $Pb(CH_3COO)_2$ (500 μg/ml) were inoculated as described above.

Growth at extreme temperatures was determined in liquid YM medium on a rotary shaker (200 rpm) at 5°C for 20 days and at 32°C for 72 h. At the end of incubation, protein content was determined with 1 ml of culture by the modified version (6) of the method of Lowry.

Tolerance to NaCl was evaluated by determining growth on YM agar medium supplemented with 1 to 6% NaCl after 72 h to 7 days of incubation at 25°C.

Tolerance to extreme pH was evaluated by the capacity of strains to grow on YM agar plates adjusted to pH 2.5, 3.5, 5.0, 6.2, 7.5, 8.0, or 9.0.

All results were analyzed using Jaccard's community coefficient and the unweighed pair group method with average (UPGMA) clustering (10).

Multilocus enzyme electrophoresis. The genetic diversity of strains was investigated by the determination of the electrophoretic types (ETs) of eight enzymes. Strains were grown for 72 h at 25°C in 100 ml of YM medium. After centrifugation at 15,000 × *g* for 10 min, the pellets were suspended in 2 ml of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.5 mM NADP, pH 6.8). The cell suspensions were sonicated twice for 20 s each time with a 20-s interval between the two sonications with a sonic disintegrator (Fisher model 300) and then centrifuged at 30,000 × *g* for 20 min. The supernatants were stored at −70°C in 10% glycerol until electrophoresis.

Polyacrylamide gel electrophoresis was performed by the method of McLellan and Ramshaw (14), and the selective staining of enzymes was done as described by Selander et al. (20). The enzymes assayed were alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, NAD-glutamate dehydrogenase, hexokinase, indophenol oxidase, isocitrate dehydrogenase, NAD-malate dehydrogenase, and xanthine dehydrogenase. The electrophoresis buffer system used was Tris-citrate, pH 8.0.

Distinctive mobility variants (ETs) of each enzyme were numbered in order of decreasing anodal mobility. Each mobility variant was equated with an allele at a structural gene locus, and the ET patterns were considered to be multilocus genotypes. The genetic diversity for an enzyme locus was calculated as follows: $h = 1 - \Sigma x_i^2[n/(n - 1]$, where x_i is the frequency of the *i*th allele and *n* is the number of ETs. The mean genetic diversity per locus (*H*) is the arithmetic average of *h* values for the eight loci (20).

Cluster analysis was performed by using Gower's similarity coefficient (7) calculated by the unweighed pair group method with averages (UPGMA) (10) with the R numerical analysis software package (10).

DNA-DNA hybridization homologies. Eight strains selected in each group formed by numerical analysis were used to determine DNA homology within *Lathyrus* populations of rhizobia and with the reference strains. DNA was extracted and purified from cells of each strain grown in YM broth. Mid-exponential-phase cultures were washed and suspended in STE buffer (50 mM Tris-HCl, 50 mM EDTA, 10% sucrose, pH 8.0). Cells were lysed by incubation on ice with successive additions of lysozyme (2.4 mg/ml), pronase E (450 μg/ml), and *N*-lauryl sarcosinate (0.25% [wt/vol]). The lysates were subjected to serial extractions with successive equal volumes of phenol, chloroform, and ether. The DNA was precipitated first with isopropanol and then with ethanol in the presence of sodium acetate and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The extract of DNA was treated with RNase (10 mg/ml) and denatured in 0.4 M NaOH, and 1.5 μg of the DNA free of RNA was slot blotted onto a nylon membrane (Hybond-N). Hybridization was done with DNA probes previously labelled with ^{32}P by nick translation (10^8 cpm/mg of DNA) (^{T7}QuickPrime Kit; Pharmacia). DNA probes were from eight reference strains (92A3, ATCC 10004, ATCC 10324, CFN 42, L3, N31, USDA 2445, and USDA 2489) and two test strains (Lj 7 and Lp 1006).

Nucleotide sequences of the 16S rRNA genes. The partial nucleotide sequences of the 16S ribosomal RNA (rRNA) genes of strains Lj 3 (from *L. japonicus*) and Lp 1013 (from *L. pratensis*) were determined by directly sequencing double-stranded PCR products with Sequenase (U.S. Biochemical Corp.). A 310-bp region was amplified by using a GenAmp DNA amplification reagent kit (Perkin-Elmer Cetus) with 24-mer primers. The forward primer (5′-TGGCTC AGAACGAACGCTGGCGGC-3′) and the reverse primer (5′-CCCACTGCTG

CCTCCCGTAGGAGT-3′) corresponded to *Escherichia coli* positions 20 to 43 and 361 to 338, respectively. The PCR was carried out as follows: 30 cycles, with 1 cycle consisting of 30 s at 95°C for denaturation, 30 s at 55°C for primer annealing, and 3 min at 72°C for extension. A hot start step preceded the first cycle and consisted of 2 min of denaturation at 95°C, 30 s at 55°C, and 2 min at 72°C after which the polymerase was added. The products were visualized on 1% agarose gel, and the 310-bp band was excised from the gel and incubated in 1 ml of TE buffer overnight at 4°C. This solution was used for an asymmetric PCR with three primer dilutions (1/50, 2/50, and 3/50) to allow the amplification of a single-stranded product. The amplification was done as described above but with 35 cycles. The asymmetric PCR products were purified by using the Magic PCR Preps DNA purification system (Promega) as recommended by the manufacturer. Both strands of three to four independent single-stranded PCR products were sequenced with Sequenase (5).

The sequences were aligned with 18 known sequences of different species by using the program PILEUP (Genetics Computer Group Sequence Analysis). The following sequences were obtained from GenBank (accession number given in parentheses): *Agrobacterium tumefaciens* DMS 30105 (M11223), *Azorhizobium caulinodans* ORS 571 (M55491), *Bradyrhizobium japonicum* USDA 110 (M55485), *Bradyrhizobium* sp. strain BTAi1 (M55492) and NZP 2257 (M55486), *R. etli* OLIVIA (M55235), *Rhizobium fredii* IAM 14142 (D12796), *Rhizobium huakuii* IAM 14158 (D12797), *R. leguminosarum* 162Y13 (M55240), *R. leguminosarum* bv. phaseoli 8002 (M55494) and FL27 (M55234), *Rhizobium loti* NZP 2213 (X63823), *Rhizobium meliloti* ATCC 9930 (M55241) and CC169 (M55242), *Rhizobium* sp. (isolated from *Medicago sativa* and *Phaseolus vulgaris*) OR191 (M55236), and *R. tropici* CIAT 899 (M55233). Nucleotide sequences of *R. leguminosarum* bv. trifolii (USDA 2046) and *R. leguminosarum* bv. viciae (USDA 2370) were obtained from the U.S. Department of Agriculture. Phylogenetic relationships were determined by parsimony analysis performed with an heuristic search algorithm on all sequences followed by a 500-replication bootstrap analysis using the program PAUP (22).

Nucleotide sequence accession numbers. The ribosomal gene sequences of strain Lj 3 isolated from *L. japonicus* and strain Lp 1013 isolated from *L. pratensis* have been deposited in GenBank/EMBL nucleotide sequence database under the accession numbers U08100 and U08101, respectively.

RESULTS

Biochemical, physiological, and nodulation characteristics. A numerical taxonomic analysis performed on 112 characteristics divided the 79 strains of *L. japonicus* and *L. pratensis* into four distinct groups, and three strains formed three separate clusters at a similarity level of 90% (Fig. 1). The four groups were associated at a similarity level of 83%.

Groups I and II comprised exclusively strains of *L. pratensis* and were related to the three unclustered strains at 85% similarity. Group I strains and the three unclustered strains differed from those of group II by their resistance to chloramphenicol and the utilization of 2 mM acetate. Group III included five strains isolated from *L. pratensis* and three reference strains (*R. fredii* USDA 191 and *R. leguminosarum* bv. viciae USDA 2445 and USDA 2489). Group III strains differed from those of groups I and II by their utilization of 10 mM acetate and their resistance to erythromycin. Groups I and III differed from group II in their tolerance to chloramphenicol. Group IV included only strains of *L. japonicus*, and this group was related to group III at a 87% similarity level and to groups I and II at a 84% similarity level.

Few traits could also distinguish rhizobial strains of *L. pratensis* (groups I, II, and III) from those of *L. japonicus* (group IV) (Table 2). Ribose was catabolized only by strains from *L. japonicus*. In general, strains from *L. japonicus* were tolerant to copper and lead (except for one strain), while strains from *L. pratensis* (except for five strains) were not tolerant. The number of strains showing tolerance to zinc was higher in the *L. japonicus* rhizobial population (18 of 30 strains) than in the *L. pratensis* rhizobial population (5 of 49 strains). Growth at the extreme temperatures of 5 and 32°C clearly indicated an adaptation of the *L. japonicus* rhizobial strains to grow at low temperatures (Table 3). After 20 days of incubation at 5°C, the majority of *L. pratensis* rhizobial strains (groups I, II, and III) grew poorly and produced 0 to 30 μg of protein per ml, while half of the strains of *L. japonicus* produced more than 60 μg of protein per ml (Table 2). At this low temperature, the mean growth yield of the reference strains was significantly lower than that of both populations, suggesting that some strains of *L. pratensis* were also cold adapted (Table 3). At 32°C, the average growth yield of rhizobia from *L. japonicus* was not significantly different from that of rhizobia from *L. pratensis* and from that from reference strains. However, at this temperature many rhizobial strains (13 of 30) from *L. japonicus* had a greater yield (30 to 60 μg of protein per ml) than the majority of rhizobial strains (30 of 49) from *L. pratensis* (<30 μg of protein per ml) (Table 2).

Among the reference strains not included in the groups, the three strains *R. leguminosarum* bv. viciae ATCC 10004 and 92A3 and *R. leguminosarum* bv. trifolii ATCC 14480 were closely related to the four groups at 82, 77, and 75% similarity levels, respectively. The slow-growing reference strains *B. japonicum* ATCC 10324 and *Rhizobium* sp. strain 32H1 (isolated from *Crotalaria paulina*) showed the lowest similarity levels (47 and 36%, respectively) with the *Lathyrus* rhizobia.

Although strains could be subdivided according to their plant origin (*L. pratensis* or *L. japonicus*) by the numerical taxonomic analysis, they shared many similar phenotypic characteristics (Table 2). All strains from the two *Lathyrus* species formed mucoid colonies on YM agar, were motile, and could grow at pH 5.0 to 9.0 (except for three strains). All strains were able to use 14 different sugars as the sole carbon source, and none could use dextrine and fucose. All strains also showed similar utilization of the organic acids fumarate (2, 10, and 20 mM), malate (2 mM), pyruvate (2 and 20 mM), and succinate (10 mM), while acetate (20 mM), α-ketoglutarate, glyoxylate, lactate, malate (10 and 20 mM), and oxaloacetate (10 and 20 mM) were not utilized. They were resistant to ampicillin, nystatin, and penicillin and sensitive to neomycin and rifampin. Sensitivity to the heavy metals aluminium, cobalt, and mercury was observed for all strains. All strains failed to grow on medium containing 1% NaCl, except one which could grow in the presence of 3% NaCl.

Both populations of rhizobia isolated from the two *Lathyrus* species could nodulate *L. pratensis* and *L. japonicus*, as well as *Lens culinaris*, all legume species belonging to the tribe Viciae (Table 2). Among the other legume species from this tribe, *Vicia sativa* was nodulated by all strains from *L. pratensis* and by 90% of strains from *L. japonicus*; *Vicia faba* was nodulated by the majority of strains from *L. pratensis* but by only 14% of strains from *L. japonicus*; *Pisum sativum* was nodulated by only one strain. None of the strains could nodulate *Medicago sativa*, and only 66% of isolates from *L. japonicus* could nodulate *Trifolium pratense* (tribe Trifolieae). None of the strains nodulated members of the tribes Phaseoleae and Hedysareae, and very few strains nodulated *Lotus corniculatus* (tribe Loteae).

Multilocus enzyme electrophoresis. For rhizobial strains isolated from *L. japonicus* and *L. pratensis* and for reference strains, 78 ETs were observed when eight enzymes were used. A mean of nine alleles was observed per enzyme, while only five alleles were detected among *Lathyrus* rhizobial strains (Table 4). A single allele profile was observed for alcohol dehydrogenase in strains from *L. pratensis* and for isocitrate dehydrogenase in strains from *L. japonicus*. Although the number of ETs was lower for strains of *L. japonicus* (21 ETs) than those of *L. pratensis* (40 ETs), the mean genetic diversity was identical between the two populations with a value of 0.451. At a genetic distance of 0.55, eight groups of ETs were identified by cluster analysis (Fig. 2). The majority of strains (40 of 49) from *L. pratensis* were included in group II, which also com-

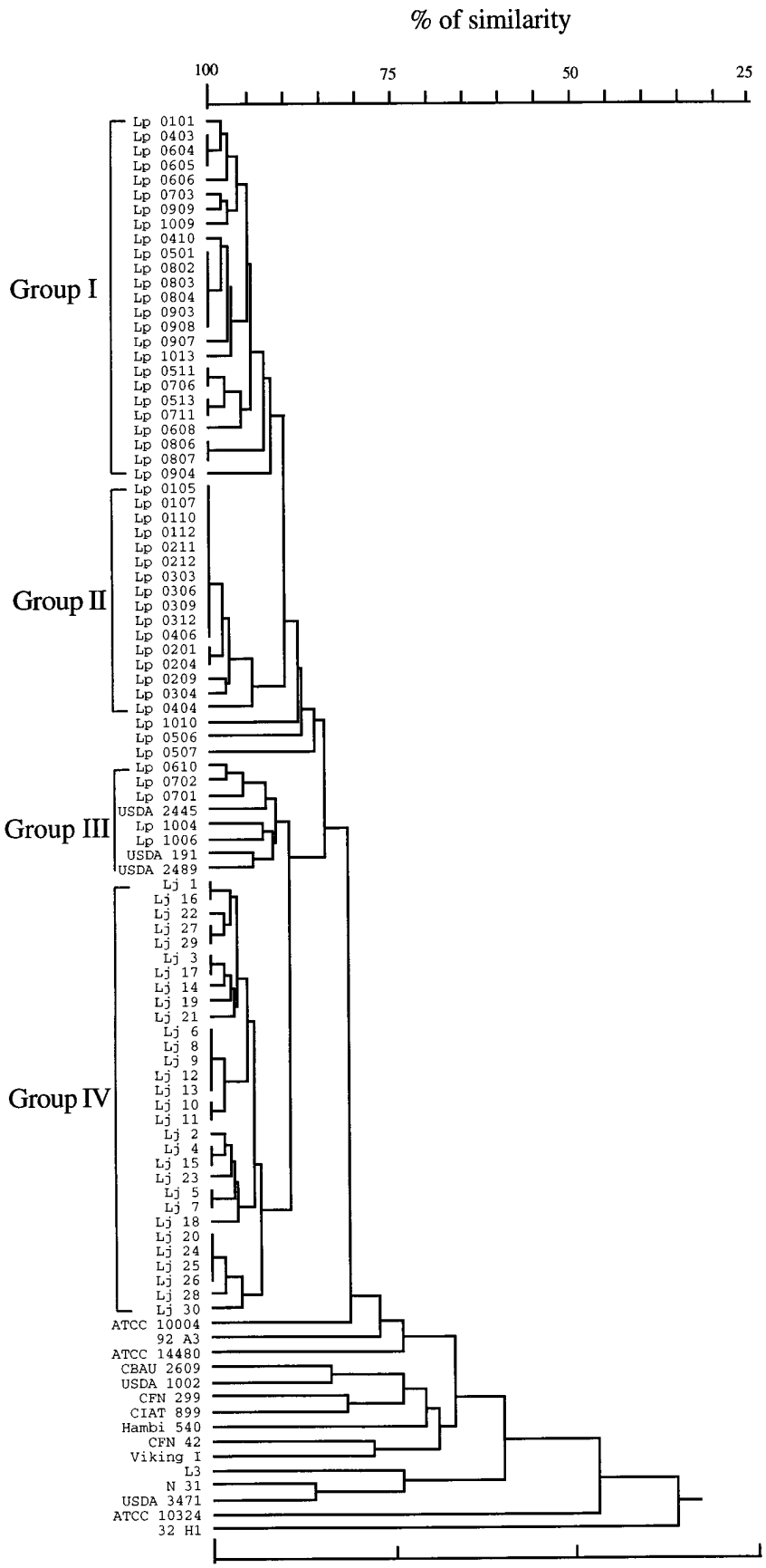

FIG. 1. Dendrogram showing the relationships among *Lathyrus* rhizobial strains, based on physiological, biochemical, and nodulation characteristics. Lp, *L. pratensis*; Lj, *L.* japonicus.

TABLE 2. Phenotypic characteristics of rhizobia isolated from *Lathyrus japonicus* and *Lathyrus pratensis*

Characteristic	Value or result for group[a]			
	L. pratensis			*L. japonicus* group IV
	Group I	Group II	Group III	
No. of strains	25	19	5	30
Growth on:				
Acetate (2 mM)	+	3	+	+
Acetate (10 mM)	5	−	+	+
Acetate (20 mM)	−	−	−	−
α-Ketoglutarate (2, 10, and 20 mM)	−	−	−	−
Fumarate (2, 10, and 20 mM)	+	+	+	+
Glyoxylate (2, 10, and 20 mM)	−	−	−	−
Lactate (2, 10, and 20 mM)	−	−	−	−
Malate (2 mM)	+	+	+	+
Malate (10 and 20 mM)	−	−	−	−
Oxaloacetate (2 mM)	+	+	4	+
Oxaloacetate (10 and 20 mM)	−	−	−	−
Pyruvate (2 mM)	+	+	+	+
Pyruvate (10 mM)	22	18	+	+
Pyruvate (20 mM)	+	+	+	+
Succinate (2 mM)	+	+	4	+
Succinate (10 mM)	+	+	+	+
Succinate (20 mM)	+	16	+	+
Arabinose	24	18	+	+
Dextrin	−	−	−	−
Dulcitol	+	+	+	+
Fructose	+	+	+	+
Fucose	−	−	−	−
Galactose	+	+	+	+
Glucose	+	+	+	+
Glycerol	+	+	+	+
Inositol	+	18	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	+	+	+	+
Mannose	+	+	+	+
Melibiose	+	+	+	28
Raffinose	+	+	+	+
Rhamnose	+	+	+	+
Ribose	−	−	−	+
Sorbitol	+	+	+	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Xylose	+	+	+	+
Alanine	+	+	+	+
Arginine	+	+	+	+
Asparagine	−	−	−	−
Glutamate	+	+	+	+
Glutamine	+	+	+	+
Glycine	−	−	−	−
Histidine	+	+	+	+
Isoleucine	−	−	−	−
Leucine	−	−	−	−
Lysine	−	−	−	−
Methionine	−	−	−	−
Phenylalanine	−	−	−	−
Proline	+	+	+	+
Serine	−	−	1	−
Threonine	−	−	−	−
Valine	−	−	−	−
Resistance to:				
Ampicillin	+	+	+	+
Chloramphenicol	+	3	+	14
Erythromycin	−	−	3	7

Continued

TABLE 2—*Continued*

Characteristic	Value or result for group[a]			
	L. pratensis			*L. japonicus* group IV
	Group I	Group II	Group III	
Gentamicin	−	1	−	−
Kanamycin	13	6	+	+
Neomycin	−	−	−	−
Nystatin	+	+	+	+
Penicillin	+	+	+	+
Rifampin	−	−	−	−
Streptomycin	−	1	−	7
Tetramycin	−	1	1	−
Tolerance to:				
Aluminium	−	−	−	−
Cobalt	1	−	−	−
Copper	2	1	2	+
Lead	2	2	−	+
Mercury	2	−	1	−
Zinc	2	1	1	18
NaCl (1%)	−	1	−	−
NaCl (2%)	−	1	−	−
NaCl (3%)	−	1	−	−
Growth at:				
pH 3.0	−	−	−	−
pH 4.0	−	−	−	−
pH 5.0	23	+	+	+
pH 5.7	23	+	+	+
pH 6.2	+	+	+	+
pH 6.9	+	+	+	+
pH 7.2	+	+	+	+
pH 8.0	+	+	+	+
pH 9.0	24	+	+	+
5°C (<30 mg of protein)	20	+	2	1
5°C (31–60 mg of protein)	3	−	2	14
5°C (>60 mg of protein)	2	−	1	15
32°C (<30 mg of protein)	17	10	3	9
32°C (31–60 mg of protein)	8	9	2	21
32°C (>60 mg of protein)	−	−	−	−
Host nodulation response				
Tribe Vicieae				
Lathyrus japonicus	+	+	+	+
Lathyrus pratensis	+	+	+	+
Lens culinaris	+	+	+	+
Pisum sativum	−	−	−	1
Vicia faba	16	16	3	4
Vicia sativa	+	+	+	27
Tribe Trifolieae				
Trifolium pratense	−	−	−	21
Medicago sativa	−	−	−	−
Tribe Phaseoleae				
Phaseolus vulgaris	−	−	−	−
Macroptillium atropurpureum	−	−	−	−
Glycine max	−	−	−	−
Tribe Hedysareae				
Onobrychis viciifolia	−	−	−	−
Tribe Loteae				
Lotus corniculatus	−	3	−	1

[a] The numbers shown are the numbers of positive strains. Symbols: +, 95% or more of strains are positive; −, 95% or more of strains are negative.

TABLE 3. Average growth yields at extreme temperatures for strains isolated from *Lathyrus japonicus* and *Lathyrus pratensis* and closely related strains

Group of strains	Avg growth yield (μg of protein · ml^{-1}) at the following temp[a]:	
	5°C (20 days of growth)	32°C (24 h of growth)
Groups I, II, and III (*L. pratensis*)	18.7 a	27.4 a
Group IV (*L. japonicus*)	63.5 b	37.5 ab
Reference strains (ATCC 10004, 92A3, ATCC 14480, USDA 191, USDA 2445, and USDA 2489)[b]	1.4 c	47.9 b

[a] Means within a column followed by the same letter are not significantly different at $P < 0.05$ by the Duncan multiple-range test.

[b] The reference strains are *Rhizobium leguminosarum* bv. viciae ATCC 10004, *R. leguminosarum* bv. viciae 92A3 (*Lathyrus hirsutus*), *R. leguminosarum* bv. trifolii ATCC 14480, *Rhizobium fredii* USDA 191, *R. leguminosarum* bv. viciae USDA 2445, and *R. leguminosarum* bv. viciae USDA 2489.

prised half of the rhizobial strains (15 of 30) from *L. japonicus*. All other strains were distributed among all groups, except for groups V, VII, and VIII which comprised only reference strains. Other reference strains were distributed in groups III, IV, and VI. The five reference strains of *R. leguminosarum* were included in groups III and IV and were related to groups I and II (*Lathyrus* rhizobial strains) at a genetic distance of 0.68. Strains of *R. tropici* were included in group VI, which was related to previous groups at a genetic distance of 0.75. Reference strains from group VIII, composed of *R. fredii* USDA 191, *Rhizobium* sp. strain 32H1 (isolated from *Crotalaria paulina*), and *B. japonicum* ATCC 10324 were the most distant, with branching at 0.86.

DNA-DNA hybridization homologies. The three rhizobial strains isolated from *L. japonicus* and the five strains isolated from *L. pratensis* showed relatively high levels of DNA homology (61 to 100%) with each of their two homologous probes (Lj 7 from *L. japonicus* and Lp 1006 from *L. pratensis*), indicating an homogeneity among *Lathyrus* rhizobial strains (Table 5). DNAs from four reference strains of *R. leguminosarum* bv. viciae (92A3, ATCC 10004, USDA 2445, and USDA 2489) exhibited high levels of hybridization with DNAs from all *Lathyrus* rhizobial strains tested. Homology levels were very low between *Lathyrus* rhizobial strains and other reference strains belonging to different species of *Rhizobium*.

Nucleotide sequences of the 16S rRNA gene. The partial 16S rRNA gene sequences of strain Lj 3 (*L. japonicus*) and strain Lp 1013 (*L. pratensis*) were compared with 18 partial sequences of members of the family *Rhizobiaceae*, including five sequences from *R. leguminosarum*. Figure 3 shows the general structure of a phylogenic tree obtained by the heuristic search, which produced four trees with an equal length of 106 base modifications. The calculations were done by defining an outgroup which encompassed *Azorhizobium caulinodans* ORS 571 and all three *Bradyrhizobium* sequences to allow the rooting of the tree. The tree formed two clades: one included *Azorhizobium* and *Bradyrhizobium* strains, and the other contained all *Rhizobium* strains including the two strains isolated from *Lathyrus* species. Sequences of strain Lj 3 from *L. japonicus* were identical to those of one strain of *R. leguminosarum* bv. trifolii, one strain of *R. leguminosarum* bv. phaseoli and one strain of *R. leguminosarum* bv. viciae. Sequences of strain Lp 1013 from *L. pratensis* differed by 2 nucleotides from those grouping Lj 3 with the *R. leguminosarum* strains.

DISCUSSION

The phenotypic and genotypic characterization of rhizobia isolated from *L. japonicus* and *L. pratensis*, two legume species indigenous to northern Quebec, allowed us to classify them as *R. leguminosarum* bv. viciae. This result confirms earlier studies in which rhizobia from *Lathyrus* species were classified as *R. leguminosarum* because they showed phenotypic and nodulation characteristics similar to those of rhizobia from other legume species belonging to the tribe Vicieae (*Pisum*, *Vicia*, and *Lens*) (1). In our study, physiological characteristics of *Lathyrus* rhizobia such as the inability to grow on 1% NaCl (except for one strain) and to use 20 mM citrate or lactate as the sole carbon source are similar to other strains of *R. leguminosarum* (9). Host plant nodulation range, which is the basis for the identification of biovars in *R. leguminosarum*, is typical of that reported for *R. leguminosarum* biovar viciae (9); the majority of *Lathyrus* rhizobial strains nodulated *Lens culinaris* and *Vicia sativa* of the tribe Vicieae.

Cluster analysis of genotypic characteristics (ET) and levels

TABLE 4. Characteristics of genetic diversity at the loci coding for eight enzymes in the ETs observed in rhizobial strains isolated from *Lathyrus* species

Enzyme locus[a]	All strains tested[b] (78 ETs)		Strains from both *Lathyrus* species (60 ETs)		49 strains from *L. pratensis* (40 ETs)		30 strains from *L. japonicus* (21 ETs)	
	No. of alleles	h[c]	No. of alleles	h	No. of alleles	h	No. of alleles	h
ADH	9	0.349	3	0.144	1	0.000	3	0.343
GDH	10	0.531	5	0.403	3	0.376	5	0.455
G6PH	7	0.779	6	0.746	5	0.678	4	0.609
HEX	16	0.867	9	0.824	8	0.781	3	0.549
IPO	9	0.634	7	0.535	6	0.510	3	0.545
IDH	5	0.247	3	0.099	3	0.156	1	0.000
MDH	6	0.658	4	0.623	4	0.701	4	0.444
XDH	6	0.568	4	0.511	3	0.405	4	0.660
Mean	9	0.579	5	0.485	4	0.451	3	0.451

[a] Abbreviations: ADH, alcohol dehydrogenase; GDH, NAD-glutamate dehydrogenase; G6PH, glucose-6-phosphate dehydrogenase; HEX, hexokinase; IPO, indophenol oxidase; IDH, isocitrate dehydrogenase; MDH, NAD-malate dehydrogenase; XDH, xanthine dehydrogenase.

[b] Reference strains and strains from both *Lathyrus* species.

[c] Genetic diversity; $h = 1 - \Sigma x_i^2[n/(n - 1]$, where x_i is the frequency of the ith allele and n is the number of ETs.

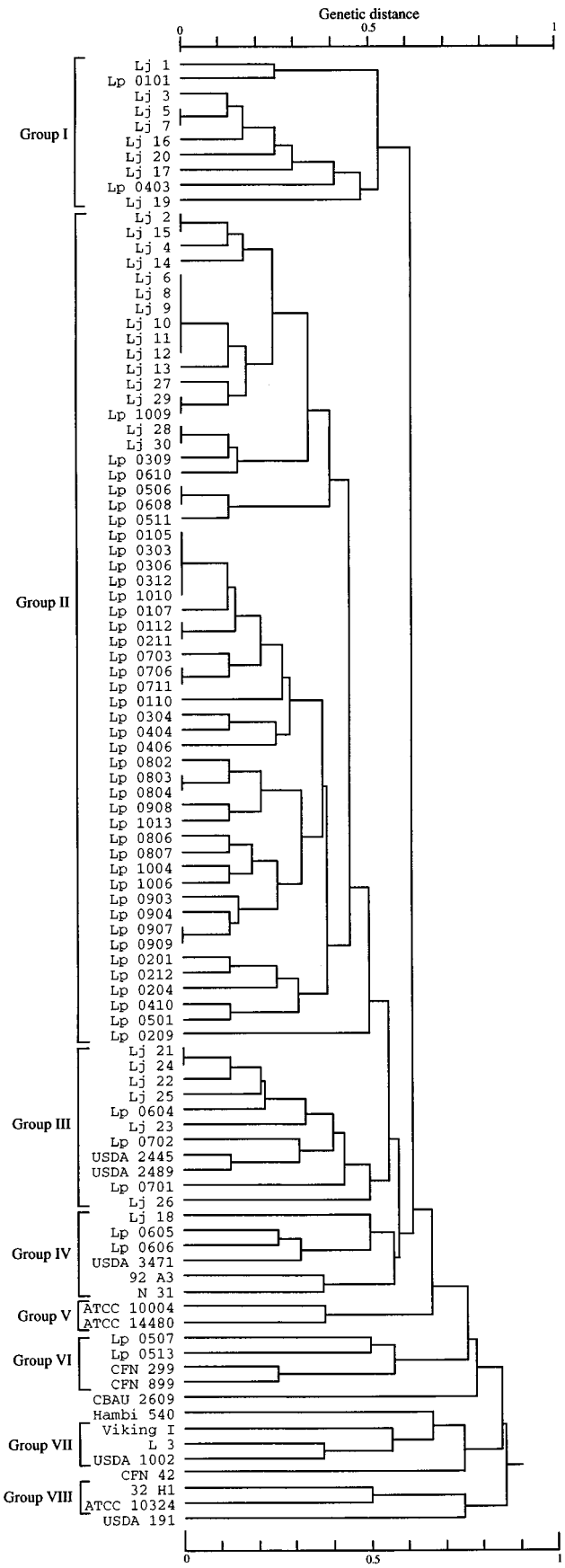

FIG. 2. Dendrogram showing levels of genetic distance among *Lathyrus* rhizobial strains and reference strains based on electrophoretically detectable allelic variation at the loci encoding eight enzymes. Lj, *L. japonicus*; Lp, *L. pratensis.*

of DNA homology also showed a close relationship between reference strains of *R. leguminosarum* bv. viciae and strains of *L. pratensis* or *L. japonicus*. Comparison of partial sequences from the 16S rRNA gene revealed identity between sequences of strain Lj 3 (*L. japonicus*) and those of three strains of *R. leguminosarum* (bv. phaseoli, trifolii, and viciae). Sequences of strain Lp 1013 (*L. pratensis*) differed by 2 nucleotides from those of strain Lj 3, but such slight differences were also found between other strains of *R. leguminosarum* as shown in the phylogram (Fig. 3). Overall, the results demonstrate that rhizobial strains isolated from *Lathyrus* species belong to *R. leguminosarum* bv. viciae.

The distinction between the two populations composed of 49 strains of rhizobia isolated from *L. pratensis* and 30 strains from *L. japonicus* was not reflected by the genetic diversity which was identical for each population (0.45), even though the number of ETs observed among strains from *L. japonicus* was lower than that among strains from *L. pratensis*. Two populations of *R. leguminosarum* bv. viciae found in two Oregon soils under different *Vicia* species also showed similar genetic diversities (0.49 and 0.51) (21). The two rhizobial populations from *Lathyrus* species were also similar in many phenotypic characteristics such as growth morphology, the utilization of carbohydrates and organic acids, and the nodulation patterns. The patterns of intrinsic antibiotic resistance were not specific to either of the two populations isolated from different soil conditions. This contrasts with a study of the diversity of *R. leguminosarum* bv. viciae from two different microclimates where the intrinsic antibiotic resistance of isolates was reported to be related to topographic position (4). However, in our study, few phenotypic traits could differentiate the two populations, although they could be separated by numerical analysis. The three distinct groups formed by *L. pratensis* rhizobial strains suggest a greater variability among *L. pratensis* rhizobial strains than among *L. japonicus* rhizobial strains which formed one group with less than 10% phenotypic differences. Strains from *L. japonicus* were more likely to grow in the presence of copper and lead and grew better at 5 and 32°C than those from *L. pratensis*. Differences in growth characteristics might be attributed to the adaptation of *L. japonicus* to the harsh environment of Hudson's Bay, located in the arctic zone, where the site of collection, a sandy beach, is subject to sharp temperature gradients during the growing season and over the year. Strains from *L. pratensis*, an introduced legume in the boreal region of Abitibi, were less adapted to low temperatures, but they still grew better than reference strains from temperate regions. Previous studies also indicated that rhizobia from a Canadian arctic zone were cold adapted for growth and nitrogen fixation (16). These arctic rhizobia (from *Astragalus* and *Oxytropis* spp.) and the strains of *Lathyrus* of the present study do not constitute a specific species because of their cold adaptation. In fact, most rhizobia from *Astragalus* and *Oxytropis* spp. of different geographic origins were classified in 16S rRNA gene types similar to or closely related to *R. loti* and *Rhizobium ciceri* (18), and these species, as well as *R. leguminosarum* bv. viciae, are widely found in temperate areas.

The ability of *L. japonicus* rhizobial strains to tolerate various metals requires further investigation. Metal concentrations in sampled soils were not determined. We can suppose that

TABLE 5. DNA-DNA relatedness between *Lathyrus* rhizobial strains and reference strains

Strain used as probe	DNA-DNA relatedness (%) of the strain tested							
	Lj 3	Lj 7	Lj 29	Lp 0110	Lp 0702	Lp 0904	Lp 1006	Lp 1013
Lp 1006	100	67	61	100	100	75	100	100
Lj 7	100	100	91	100	98	90	82	100
R. etli CFN 42	44	31	26	49	39	27	33	30
R. fredii USDA 191	9	28	19	30	33	17	25	30
R. galegae HAMBI 540		23					37	
R. leguminosarum bv. viciae 92A3	95	89	74	87	71	84	76	81
R. leguminosarum bv. viciae ATCC 10004	92	87	53	85	77	79	49	91
R. leguminosarum bv. viciae USDA 2489	88	57	82	88	80	69	61	91
R. leguminosarum bv. viciae USDA 2445	95	64	93	100	100	90	75	93
R. loti L3	31	27	43	41	48	37	44	51
R. meliloti USDA 1002	44	34	33	39	42	36	45	49
R. tropici CIAT 899		28					27	
Rhizobium sp. strain N31	15	25	13	13	22	19	18	31
B. japonicum ATCC 10324	13	11	19	9	15	21	12	16

these strains possess operative mechanisms for detoxification, as was suggested for an arctic *Rhizobium* strain (2).

Although rhizobial strains isolated from *L. japonicus* and *L. pratensis* share genotypic and phenotypic characteristics typical of reference strains of *R. leguminosarum* bv. viciae, they differ in their capacity to grow at low temperatures. This cold adaptation is worthy of further investigation with respect to the improvement of nitrogen fixation under cool climates and for studies on the mechanisms of cold adaptation. A study system composed of two strains of *R. leguminosarum* biovar viciae differing only in their adaptation to temperature would have the potential to allow the identification of genes involved in cold adaptation.

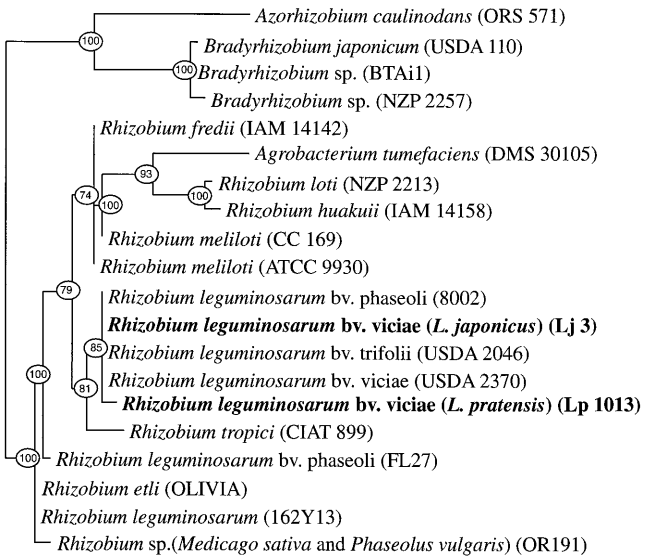

FIG. 3. Phylogenetic tree derived from partial 16S rRNA gene sequences for strains Lj 3 (*L. japonicus*) and strain Lp 1013 (*L. pratensis*) and related bacteria. Sequences were compared by parsimony analysis to form the trees. Tree number 1 is presented and was verified by consensus. The circled numbers represent the number of times (percentage) the group consisting of the strains which are to the right of the fork occurred among 500 replications in a bootstrap analysis. The sequence accession numbers of the related rhizobia can be found in Materials and Methods. Tree length (total base modifications), 106 bp; consistency index, 0.821.

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