Numerical Taxonomy of *Rhizobium* Strains from Legumes of the Temperate Zone

NATALIE I. NOVIKOVA,* ELENA A. PAVLOVA, NIKOLAI I. VOROBJEV,
AND ELENA V. LIMESHCHENKO

Research Institute for Agricultural Microbiology, St. Petersburg 189620, Russia

Phenotypic properties (growth characteristics, utilization of carbon and nitrogen sources, and intrinsic antibiotic resistance) of 53 *Rhizobium* strains isolated from root nodules of the temperate-zone legumes *Astragalus* spp. (29 strains), *Oxytropis campanulata* (7 strains), *Hedysarum alpinum* (7 strains), *Ononis arvensis* (3 strains), *Glycyrrhiza* spp. (4 strains), and *Coronilla varia* (3 strains) were compared with those of other *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* strains. The final matrix, containing 83 strains and 56 nonsymbiotic features, was used for computer cluster analysis. The dendrogram showed that the new strains of temperate-zone rhizobia formed a cluster separate from both *Rhizobium* and *Bradyrhizobium* spp. Two large groups of temperate-zone rhizobia were revealed. Group 1 included rhizobial strains originating from different geographical regions with a temperate climate, while group 2 included strains from the same geographical origin, South Siberia. The strains of recognized *Rhizobium* species were clustered, in general, with each other as expected from phylogenetic relatedness.

Root nodule bacteria of the genera *Rhizobium* and *Bradyrhizobium* are a common microflora of temperate soils. These bacteria may interact with the roots of leguminous plants, inducing the formation of nodules in which the fixation of atmospheric nitrogen occurs. These bacteria are commonly used in agriculture for improvement of plant growth. The specificity of the interaction between rhizobia and legumes depends on many factors coded for by the host cultivar and bacterial genomes. The main properties of a bacterial isolate can be described by its taxonomical position in relation to other strains. There are several *Rhizobium* species recognized at this time (10, 13, 24), and knowledge of rhizobial evolution and phylogeny obtained in recent studies allows the proposal of new rhizobial taxa (3, 16, 25). However, the current systematic description of the genus *Rhizobium* does not include many of the root nodule isolates from legumes of tropical (22, 37), arctic (21), and temperate (5, 12, 17) regions.

The host plant specificities and phage sensitivities of *Rhizobium* strains from temperate-zone legume root nodules formed on *Astragalus*, *Hedysarum*, *Glycyrrhiza*, and *Ononis* plants were recently described (17). It was shown that these strains constitute a cross-inoculation group related to *Rhizobium loti*. The aim of the present work was to determine the cultural properties of these strains and to compare these properties with those of other *Rhizobium* species.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this work are listed in Table 1. Bacteria were maintained on yeast extract-mannitol (YEM) (29) agar slants at 4°C.

Growth characteristics. The time necessary for full colony development on YEM agar (estimated here as a colony diameter of 2 mm) was determined. Bacterial growth on meat-peptone agar and reactions with litmus milk were determined as described earlier (11). The ability of strains to grow

in the presence of 0, 1, 1.5, or 2% NaCl was tested by streaking strains on YEM agar containing the salt. Temperature tolerance was determined by plating strains to obtain single colonies on YEM agar and incubating at 33, 35, and 37°C for 7 to 10 days. Unless indicated otherwise, the cultures were incubated at 28°C. Growth of the fast-growing and slowly growing strains was observed after incubation for 3 to 5 and 5 to 7 days, respectively. All tests were performed in duplicate.

Utilization of carbon sources. For analysis of carbon source utilization, the base medium (YEM) was modified so that yeast extract was replaced by 0.01% NH_4NO_3 and mannitol was replaced by one of the following carbohydrates: sucrose, D-maltose, D-lactose, D-glucose, D-arabinose, D-rhamnose, D-mannose, D-xylose, D-raffinose, dulcitol, D-sorbitol, or the dicarboxylic acid Na-succinate. The media were solidified with 0.7% agar, and 0.0025% (wt/vol) bromothymol blue was added as a pH indicator. The pH of the media was adjusted to 7.0. Tests were done in tubes.

Utilization of amino acids as sole nitrogen and carbon sources. Tests were performed with the same medium used for the determination of carbon source utilization, but the nitrogen source was replaced by one of the following amino acids (0.1%, wt/vol) as a source of both nitrogen and carbon: L-histidine, DL-valine, L-arginine, L-proline, or DL-serine.

Utilization of mineral nitrogen. The yeast extract in YEM agar was replaced by 0.1% (wt/vol) of one of the following mineral salts: $(\text{NH}_4)_2\text{SO}_4$, $\text{Ca}(\text{NO}_3)_2$, $(\text{NH}_4)_3\text{PO}_4$, or $(\text{NH}_4)_2\text{MoO}_4$. The media contained 0.0025% bromothymol blue as a pH indicator.

Intrinsic antibiotic resistance. Intrinsic antibiotic resistance was tested on YEM agar supplemented with one of the following antibiotics at the indicated concentrations (in micrograms per milliliter): streptomycin, 25, 50, or 100; rifampin, 5, 10, or 20; kanamycin, 10, 25, or 50; penicillin, 5 or 10; or ampicillin, 5 or 10.

Nodulation tests. Legume seeds were obtained from the Vavilov Plant Breeding Institute (St. Petersburg, Russia). Tests were done in tubes with surface-sterilized legume seeds as described earlier (17). Twelve seedlings of each legume were inoculated for each strain tested. Tubes were examined for the presence or absence of nodules after 6 weeks. The ability to fix nitrogen was estimated by measuring acetylene-reducing activ-

* Corresponding author. Present address: Centro de Investigacion sobre Fijacion de Nitrogeno, UNAM, Apdo. Postal 565-A, Cuernavaca, Morelos, Mexico. Fax: (73) 17 55 81. Electronic mail address: novikova@n2.cifini.unam.mx.

TABLE 1. Rhizobial strains used in this work

Strain ^a	Host plant	Geographical origin and/or reference	Cluster
<i>Bradyrhizobium japonicum</i> strains			
CIAM 2490	<i>Glycine max</i> (L.) Merr.	Georgia (17)	1
CIAM 24100	<i>Glycine max</i> (L.) Merr.	Georgia (17)	1
<i>Bradyrhizobium</i> sp. strains (<i>Desmodium</i>)			
CIAM 3207	<i>Desmodium canadense</i> (L.) DC	Ukraine (this work)	1
CIAM 3208	<i>Desmodium canadense</i> (L.) DC	Ukraine (this work)	1
CIAM 3209	<i>Desmodium canadense</i> (L.) DC	Ukraine (this work)	1
<i>Rhizobium tropici</i> CIAT 899 ^T	<i>Phaseolus vulgaris</i> L.	Colombia (16)	2
<i>Agrobacterium tumefaciens</i> biovar 1 C58		2	2
<i>Rhizobium fredii</i> USDA 191	<i>Glycine max</i> (L.) Merr.	China (34)	3
<i>Rhizobium</i> sp. strain NGR 234	<i>Lablab purpureus</i>	New Guinea (27)	3
<i>Rhizobium huakuii</i> CCBAU 2604	<i>Astragalus sinicus</i> L.	China (3)	3
<i>Rhizobium galegae</i> strains			
HAMBI 540 ^T	<i>Galega orientalis</i> Lam.	Finland (13)	4
CIAM 0703	<i>Galega orientalis</i> Lam.	Estonia (17)	4
<i>Agrobacterium radiobacter</i> biovar 2 K84		2	4
<i>Agrobacterium rhizogenes</i> biovar 2 ATCC 15834		20	4
<i>Rhizobium meliloti</i> strains			
CIAM 1723	<i>Medicago sativa</i> L.	Kazakhstan (17)	5
CIAM 1732	<i>Medicago sativa</i> L.	Latvia (17)	5
SU 47	<i>Medicago sativa</i> L.	Australia (8)	5
<i>Rhizobium fredii</i> 8613	<i>Glycine</i> sp.	India (this work)	5
<i>Rhizobium leguminosarum</i> biovar <i>viciae</i> CB 596	<i>Pisum sativum</i> L.	England (4)	6
<i>Rhizobium loti</i> strains			
NZP 2213 ^T	<i>Lotus tenuis</i> Kit.	New Zealand (9)	6
NZP 2037	<i>Lotus divaricatus</i>	New Zealand (9)	6
<i>Rhizobium huakuii</i> strains			
CCBAU 2605	<i>Astragalus sinicus</i> L.	China (3)	6
CCBAU 2609 ^T	<i>Astragalus sinicus</i> L.	China (3)	6
<i>Rhizobium</i> sp. strain CIAM 1413	<i>Hedysarum alpinum</i> L.	Moscow, Russia (17)	— ^b
<i>Rhizobium leguminosarum</i> biovar <i>trifolii</i> strains			
CIAM 1326	<i>Trifolium pratense</i> L.	St. Petersburg, Russia (17)	7
CIAM 1361	<i>Trifolium hybridum</i> L.	Byelorussia (17)	7
<i>Rhizobium leguminosarum</i> biovar <i>phaseoli</i> strains			
CIAM 2619	<i>Phaseolus vulgaris</i> L.	Armenia (17)	7
CIAM 2620	<i>Phaseolus vulgaris</i> L.	Armenia (17)	7
<i>Rhizobium</i> sp. strains			
CIAM 0205	<i>Astragalus uliginosus</i> L.	Vorkuta, Russia (17)	7
CIAM 0207	<i>Astragalus falcatus</i> Lam.	Stavropol, Russia (17)	7
CIAM 0211	<i>Astragalus uliginosus</i> L.	Altai, Russia (this work)	8
CIAM 1801	<i>Lotus corniculatus</i> L.	Moscow (17)	8
CIAM 3002	<i>Anthyllus arenaria</i> (Rupr.) Juz.	St. Petersburg (17)	8
NZP 5462 (=CC 401)	<i>Coronilla varia</i> L.	United States (4)	8
CIAM 3502	<i>Ononis arvensis</i> L.	Lipetsk, Russia (17)	8
CIAM 3101	<i>Glycyrrhiza glabra</i> L.	Turkmenistan (this work)	8
CIAM 0201	<i>Astragalus politovii</i> Kril.	Novosibirsk, Russia (17)	9
CIAM 0202	<i>Astragalus frigidus</i> Bge.	Byelorussia (17)	9
CIAM 0203	<i>Astragalus cicer</i> L.	Byelorussia (17)	9
CIAM 0204	<i>Astragalus cicer</i> L.	Byelorussia (17)	9
CIAM 0206	<i>Astragalus uliginosus</i> L.	Vorkuta (17)	9
CIAM 0208	<i>Astragalus cicer</i> L.	Ukraine (17)	9
CIAM 0209	<i>Astragalus cicer</i> L.	Ukraine (17)	9
CIAM 0210	<i>Astragalus cicer</i> L.	Ukraine (17)	9
CIAM 1406	<i>Hedysarum alpinum</i> L.	Moscow (17)	9
CIAM 1410	<i>Hedysarum alpinum</i> L.	Moscow (17)	9
CIAM 3503	<i>Ononis arvensis</i> L.	Lipetsk (17)	9
CIAM 3505	<i>Ononis arvensis</i> L.	Lipetsk (17)	9
CIAM 3118	<i>Glycyrrhiza pallidiflora</i> Maxim.	Ukraine (17)	9
CIAM 3119	<i>Glycyrrhiza pallidiflora</i> Maxim.	Ukraine (17)	9
CIAM 3120	<i>Glycyrrhiza pallidiflora</i> Maxim.	Ukraine (17)	9
CIAM 3402	<i>Coronilla varia</i> L.	Daghestan (this work)	9
CIAM 1412	<i>Hedysarum alpinum</i> L.	Moscow (17)	10
CIAM 1414	<i>Hedysarum alpinum</i> L.	Moscow (17)	10
CIAM 1415	<i>Hedysarum alpinum</i> L.	Moscow (17)	10
CIAM 1416	<i>Hedysarum alpinum</i> L.	Moscow (17)	10
CIAM 3401	<i>Coronilla varia</i> L.	Daghestan (this work)	10
CIAM 1802	<i>Lotus corniculatus</i> L.	St. Petersburg (17)	—

Continued on following page

TABLE 1—Continued

Strain ^a	Host plant	Geographical origin and/or reference	Cluster
A11	<i>Astragalus cicer</i> L.	Altai (this work)	11
A12	<i>Astragalus cicer</i> L.	Altai (this work)	11
A14	<i>Astragalus cicer</i> L.	Altai (this work)	11
A23	<i>Astragalus cicer</i> L.	Altai (this work)	11
A74	<i>Astragalus uliginosus</i> L.	Altai (this work)	11
A162	<i>Astragalus danicus</i> Hetz.	Altai (this work)	11
CIAM 0214	<i>Astragalus austrosibiricus</i> B.	Altai (this work)	11
252	<i>Oxytropis campanulata</i> Vass.	Altai (this work)	12
253	<i>Oxytropis campanulata</i> Vass.	Altai (this work)	12
254	<i>Oxytropis campanulata</i> Vass.	Altai (this work)	12
256	<i>Oxytropis campanulata</i> Vass.	Altai (this work)	12
257	<i>Oxytropis campanulata</i> Vass.	Altai (this work)	12
CIAM 2209	<i>Oxytropis campanulata</i> Vass.	Altai (this work)	12
A72	<i>Astragalus uliginosus</i> L.	Altai (this work)	13
A161	<i>Astragalus danicus</i> Hetz.	Altai (this work)	13
CIAM 0219	<i>Astragalus danicus</i> Hetz.	Altai (this work)	13
CIAM 0218	<i>Astragalus uliginosus</i> L.	Altai (this work)	13
CIAM 2210	<i>Oxytropis campanulata</i> Vass.	Altai (this work)	13
CIAM 0212	<i>Astragalus falcatus</i> Lam.	Altai (this work)	14
CIAM 0213	<i>Astragalus danicus</i> Hetz.	Altai (this work)	14
CIAM 0215	<i>Astragalus falcatus</i> Lam.	Altai (this work)	14
A32	<i>Astragalus falcatus</i> Lam.	Altai (this work)	14
A172	<i>Astragalus danicus</i> Hetz.	Altai (this work)	14
A173	<i>Astragalus danicus</i> Hetz.	Altai (this work)	14
A174	<i>Astragalus danicus</i> Hetz.	Altai (this work)	14

^a CIAM, strain obtained from the culture collection of the Research Institute for Agricultural Microbiology, St. Petersburg, Russia. Strains of *R. huakuii* were received from E.-T. Wang (Beijing Agricultural University, Beijing, China). Other *Rhizobium* strains were provided by K. Lindstrom (University of Helsinki, Helsinki, Finland). T, type strains of species.

^b —, Strain not part of a cluster.

ity. In all plant tests, uninoculated plants were included as controls.

Numerical analysis. A computer cluster analysis of 56 phenotypic nonsymbiotic variables for 83 strains was performed by using the weighed complete-linkage method as described earlier (26, 28).

The data were coded for bacterial growth characteristics as follows: 0 for an absence of growth, 1 for light growth, and 2 for heavy growth. Changes in the pH of the culture media were assigned values of -2 for strong acidification, -1 for a weak acidification, 0 for no change, 1 for weak alkalization, and 2 for strong alkalization. Reactions on litmus milk were given values of 0 for the absence of a serum zone and 2 for the presence of a serum zone. Growth rate values were assigned on the basis of the number of days it took for full colony development to occur. Weighting coefficients used were 4 for acidity of culture media, 10 for growth rates, and 2 for other characters.

The characters with a dispersion of 0 were excluded from the calculations. The analysis was performed at the Computer Laboratory, Research Institute for Agricultural Microbiology, St. Petersburg, Russia.

RESULTS

Computer analysis allowed us to group the strains into 14 clusters on the basis of their metabolic properties. The composition of each cluster is shown in Table 1. A dendrogram showing the linkage between clusters is presented in Fig. 1. The strains under investigation formed two large taxonomic groups with a 0.44 level of similarity. These groups were found to be divergent both from other *Rhizobium* species and from *Bradyrhizobium* strains. The level of similarity between these

groups and the *Rhizobium* reference strains was about 0.33, and the level of similarity between *Bradyrhizobium* and *Rhizobium* species was less than 0.3. The metabolic properties of the strains are presented in Table 2.

Cluster 1 included slowly growing *Bradyrhizobium* strains isolated from root nodules of *Desmodium canadense* and *Glycine max*. This cluster has the greatest divergence from the other rhizobia tested.

Cluster 2 consisted of only two strains: *Rhizobium tropici* CIAT 899 and *Agrobacterium tumefaciens* C58. Cluster 3 comprised strains having different taxonomical positions: *Rhizobium fredii* USDA 191, *Rhizobium huakuii* CCBAU 2604, and *Rhizobium* sp. strain NGR 234. Cluster 4 included four strains: two strains of *Agrobacterium* and two strains of *Rhizobium galegae*. Cluster 5 is composed of *Rhizobium meliloti* as well as *R. fredii* strains. It should be noted that strains from different geographical origins but belonging to the same taxon were, as a rule, clustered with one another. Cluster 6 comprised the related strains of *R. loti* and *R. huakuii*. Clusters 2 through 6 united into one taxonomic group with a level of similarity of about 0.5. This group included nearly all of the *Rhizobium* and *Agrobacterium* reference strains used in this work.

Two *Rhizobium* strains (CIAM 1413 and CIAM 1802) were not clustered with one another or with other bacteria tested and occupied distinct positions on the dendrogram.

Cluster 7 included two *Rhizobium* strains isolated from root nodules of *Phaseolus vulgaris*, two strains identified as *Rhizobium leguminosarum* bv. trifolii, and two isolates from root nodules formed on an *Astragalus* sp. This cluster was more related to the *Rhizobium* sp. strains under investigation than to the reference strains.

FIG. 1. Dendrogram reflecting phenotypic similarities between *Rhizobium* strains from temperate-zone legumes and other *Rhizobium*, *Agrobacterium*, and *Bradyrhizobium* strains.

TABLE 2. Metabolic properties of clustered strains^a

Characteristic	Cluster (n)													
	1 (5)	2 (2)	3 (3)	4 (4)	5 (4)	6 (5)	7 (6)	8 (6)	9 (16)	10 (5)	11 (7)	12 (6)	13 (5)	14 (7)
Acidification of medium containing the following carbon sources:														
D-Arabinose	-	+	+	+	+	+	+	+	+	+	2	3	1	2
D-Glucose	4	+	+	+	+	+	+	+	14	+	+	+	+	+
D-Lactose	-	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Maltose	3	+	+	3	+	+	+	+	+	+	+	+	+	+
D-Mannose	-	+	+	+	+	+	+	5	+	+	5	+	2	5
D-Rhamnose	-	+	+	+	+	+	+	+	+	+	6	3	1	+
D-Raffinose	-	+	+	+	+	3	-	-	-	-	-	-	-	-
D-Sorbitol	-	+	+	3	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	+	2	3	2	+	2	1	-	2	+	+	4	+
Dulcitol	-	1	-	3	+	4	+	+	+	+	6	+	1	+
Na-succinate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	+	+	2	+	+	+	+	+	+	+	+	+	+
Acidification of medium containing the following nitrogen sources:														
Ca(NO ₃) ₂	-	1	1	+	+	2	+	3	14	2	+	1	+	6
(NH ₄) ₂ SO ₄	-	+	+	+	+	+	+	+	+	+	+	+	+	+
(NH ₄) ₃ PO ₄	-	+	+	+	+	+	+	+	+	+	+	5	+	+
(NH ₄) ₂ MoO ₄	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Alkalinization of medium containing the following amino acids:														
L-Arginine	4	-	-	3	2	-	+	+	+	+	+	+	+	6
L-Histidine	+	1	-	+	3	1	+	+	+	+	+	+	+	+
L-Proline	+	-	-	-	-	-	5	+	+	+	+	+	+	6
DL-Serine	4	1	-	-	-	-	2	+	11	2	6	1	-	-
DL-Valine	-	+	+	-	3	+	-	-	-	-	-	-	-	-
First colony formed after:														
2-3 days	-	+	-	+	+	-	+	-	-	-	-	-	-	-
4-5 days	-	-	+	-	-	+	-	-	-	-	+	-	-	-
5-6 days	-	-	-	-	-	-	-	+	-	-	-	-	-	+
6-7 days	-	-	-	-	-	-	-	-	+	+	-	+	+	-
8 days	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at:														
33°C	+	+	+	+	+	+	+	+	+	-	6	-	-	-
35°C	+	+	+	+	+	+	4	+	6	-	-	-	-	-
37°C	+	+	2	1	+	3	3	5	1	-	-	-	-	-
Growth on MPA ^b	-	+	-	-	+	-	-	-	-	-	-	-	-	-
Intrinsic antibiotic resistance														
Ampicillin														
5 mg/liter	-	+	+	+	+	4	+	4	2	-	-	-	-	3
10 mg/liter	-	1	2	3	+	4	+	3	2	-	-	-	-	-
Kanamycin														
10 mg/liter	+	1	1	+	3	4	2	+	+	+	4	4	2	1
25 mg/liter	+	-	1	+	1	4	-	3	13	+	1	-	-	-
50 mg/liter	-	-	-	-	1	1	-	-	2	-	-	-	-	-
Penicillin														
5 mg/liter	-	1	+	+	+	+	5	2	4	1	-	-	-	3
10 mg/liter	-	1	+	+	+	+	4	1	2	1	-	-	-	-
Rifampin														
5 mg/liter	+	+	-	-	-	+	-	2	1	1	-	-	-	-
10 mg/liter	+	+	-	-	-	+	-	1	-	-	-	-	-	-
20 mg/liter	+	+	-	-	-	2	-	-	-	-	-	-	-	-
Streptomycin														
25 mg/liter	4	+	1	1	1	2	-	1	-	-	-	-	-	-
50 mg/liter	-	+	1	-	-	-	-	-	-	-	-	-	-	-
100 mg/liter	-	1	-	-	-	-	-	-	-	-	-	-	-	-
NaCl tolerance														
1.0%	3	+	+	+	+	4	4	+	15	+	+	+	+	+
1.5%	-	+	2	+	+	3	4	3	6	2	5	1	3	3
2.0%	-	+	-	3	+	2	1	1	-	-	-	-	-	-
Reaction with litmus milk														
Medium alkalinity	+	1	1	2	1	+	5	2	10	2	+	+	+	+
Serum zone	-	+	+	2	+	+	2	+	+	+	+	-	4	+
Utilization of the following amino acids as sole nitrogen and carbon sources:														
L-Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Continued on following page

TABLE 2—Continued

Characteristic	Cluster (n)													
	1 (5)	2 (2)	3 (3)	4 (4)	5 (4)	6 (5)	7 (6)	8 (6)	9 (16)	10 (5)	11 (7)	12 (6)	13 (5)	14 (7)
L-Histidine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Proline	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DL-Serine	+	+	+	+	+	4	+	+	13	2	+	5	2	5
DL-Valine	+	+	+	3	+	+	2	5	3	1	1	1	—	1
Utilization of the following carbon sources:														
D-Arabinose	+	+	+	+	+	+	+	+	+	+	2	3	1	2
D-Lactose	+	+	+	+	+	+	+	+	+	+	6	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	4	5
D-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	2	+
D-Xylose	+	+	+	+	2	+	3	2	—	2	6	+	4	+

^a +, all strains were positive; —, all strains were negative. Numerical values are the number of positive strains of the total number of strains tested.

^b MPA, meat-peptone agar.

Three clusters (clusters 8, 9, and 10) formed the first taxonomic group of temperate-zone rhizobia, with a 0.57 level of similarity. The central strains of this group were CIAM 0208 and CIAM 0209. Cluster 8 consisted of six strains isolated from root nodules formed on temperate-zone legumes of various genera (*Lotus*, *Anthyllis*, *Glycyrrhiza*, *Astragalus*, *Ononis*, and *Coronilla*). Cluster 9 was the most extensive one and contained 16 strains isolated from diverse legumes from different geographical regions. Cluster 10, bordering cluster 9, included five strains specific for nodulating *Hedysarum alpinum* and *Coronilla varia*.

Four clusters (clusters 11, 12, 13, and 14) made up the second taxonomical group, with a 0.58 level of similarity. This group included only strains isolated from the root nodules of *Astragalus* and *Oxytropis* plants found in Siberia. The central strain of this group is CIAM 0219.

The characteristics that differentiate these newly discovered phenotypic groups from existing *Rhizobium* species are presented in Table 3. The strains under investigation were attributed to the genus *Rhizobium* (they grew rapidly on YEM media, did not produce alkali on sugar media, and utilized a wide range of carbon compounds [10]), while for most of the characteristics studied they had features intermediate between those the fast- and slow-growing root nodule bacteria.

Data on the plant host ranges of representative strains of group 2 as well as bacteria isolated from root nodules of *C. varia* (group 1) are presented in Table 4. Host specificities of other temperate-zone rhizobia included in group 1 have been described previously (17). The results show that the plant host ranges of these rhizobial strains of different temperate-zone geographical origins are similar and include several temperate-zone legumes (Table 4), but no nodules were formed on roots of *Medicago sativa*, *Trifolium pratense*, or *Galega orientalis* plants inoculated with these strains.

DISCUSSION

The family Leguminosae, one of the largest plant families, contains about 20,000 different species distributed from arctic to tropical latitudes. Only a small portion of legume species (less than 15%) have been screened for ability to be nodulated (1, 6). Therefore, many bacterial legume microsymbionts remain uninvestigated.

In recent years, progress towards the development of a more complete *Rhizobium* taxonomy has been made, and new species have been described: *R. tropici* (16), *R. huakuii* (3), and

Rhizobium etli (25). Genomic relatedness between members of the family *Rhizobiaceae* has been revealed by modern approaches and has allowed the construction of phylogenetic trees and the proposal of possible modes of rhizobial evolution (7, 14, 31, 33, 35). The data gathered to date show that the family *Rhizobiaceae* is composed of phylogenetically heterogeneous genera. Thus, *Bradyrhizobium* spp. are more related to nonsymbiotic bacteria such as *Rhodopseudomonas*, *Azospira*, and *Nitrobacter* spp. (19, 31–33); *Rhizobium* spp. are related to *Agrobacterium*, *Brucella*, and *Bartonella* spp. (33). These facts indicate the need for a revision of existing taxa of the family (31, 33) as well as the likelihood of discovery of new forms of root nodule bacteria.

The present work centered on the study of root nodule bacteria isolated from the temperate-zone legumes of the genera *Astragalus*, *Oxytropis*, *Glycyrrhiza*, *Ononis*, *Hedysarum*, and *Coronilla*. Previous analyses of the phage sensitivities and plant host ranges of these isolates indicated that they are related to strains isolated from root nodules formed on *Lotus corniculatus*, and so they were classified as *R. loti* (17). In this work, a comparison of the metabolic properties of the bacteria was performed, and cluster analysis was used to construct a dendrogram reflecting the phenotypic relationships among these strains. A significant distance was found between the *Rhizobium* strains under investigation and the reference strains included in the study. In addition, two large groups were identified among the temperate-zone rhizobia studied. Group 1 included rhizobial strains originating from different geographical regions of the temperate zone, while group 2 included strains from the same geographical origin, South Siberia.

None of the rhizobial isolates obtained from temperate-zone legumes (including bacteria from *L. corniculatus* nodules) were clustered with the *R. loti* type strain NZP2213 or with other *R. loti* or *R. huakuii* strains. These strains, however, have spectra of phage sensitivity similar to those of the temperate-zone rhizobia (data not presented).

It should be mentioned that taxonomic group I included strain NZP 5462, which was isolated from the root nodules of *C. varia*. This strain was used earlier in DNA-DNA hybridization studies and was demonstrated to have not more than 10% DNA homology with the *R. loti* type strain and about 20% homology with strains of the *R. leguminosarum* group (4). The present work shows that the *Rhizobium* strains most closely related to the temperate-zone rhizobia described here were *R. leguminosarum* strains of the same geographic origin.

TABLE 3. Metabolic features differentiating rhizobial species^a

Characteristic	Group 1	Group 2	<i>R. leguminosarum</i>	<i>R. meliloti</i>	<i>R. loti</i> + <i>R. huakuii</i>	<i>R. galegae</i>	<i>R. fredii</i>	<i>R. tropici</i>	<i>Bradyrhizobium</i> spp.
Acidification of medium containing the following carbon sources:									
D-Arabinose, D-ramnose, or D-mannose	+	d	+	+	+	+	+	+	-
D-Xylose	d	+	d	d	+	d	d	+	-
Acidification of medium containing mineral nitrogen sources	+	+	+	+	+	+	+	+	-
Alkalinization of medium containing the following amino acids:									
L-Histidine	+	+	+	d	d	+	d	+	+
L-Proline	+	+	d	-	-	-	-	-	+
DL-Serine	d	d	d	-	-	-	-	-	d
First colony formed after:									
2-3 days	-	-	+	+	-	+	+	+	-
4-7 days	+	+	-	-	+	-	+	-	-
8 days	-	-	-	-	-	-	-	-	+
Growth on MPA ^b	-	-	-	+	-	-	d	+	-
Intrinsic antibiotic resistance									
Ampicillin, 10 mg/liter	d	-	+	+	d	+	d	d	-
Kanamycin, 25 mg/liter	d	-	-	-	d	+	-	-	+
Penicillin, 10 mg/liter	d	-	d	+	+	+	+	-	-
Rifampin, 5 mg/liter	d	-	-	-	+	-	-	+	+
Streptomycin, 25 mg/liter	-	-	-	d	d	d	d	+	+
NaCl (1.5%) tolerance	d	d	+	+	d	+	d	+	-
Reaction with litmus milk									
Acidification of medium	-	-	-	d	-	-	d	+	-
Serum zone	+	d	-	+	+	+	+	+	-
Growth at 37°C	d	-	d	+	d	d	+	+	+
Utilization of the following amino acids as sole nitrogen and carbon sources:									
DL-Valine	d	d	d	+	+	+	+	+	+
DL-Serine	d	d	+	+	d	+	+	+	+
Utilization of the following carbon sources:									
D-Arabinose	+	d	+	+	+	+	+	+	+
D-Xylose	d	+	d	d	+	+	+	+	+

^a +, at least 95% of the strains are positive; -, at least 95% of the strains are negative; d, less than 95% but more than 5% of the strains are positive.

^b MPA, meat-peptone agar.

The present work also shows that, in general, the numerical classification of the *Rhizobium* species used in this study placed them into the same groups as methods based on genomic relatedness. Thus, on the basis of its phenotypic characters, the broad-host-range strain NGR 234 was closely related to the fast-growing soybean microsymbiont *R. fredii* USDA 191 (cluster 3), consistent with the results obtained by others from a comparison of 16S rRNA sequences (7). The other *R. fredii* strain, which originates from India, was grouped with the *R. meliloti* strains (cluster 5), and this is also in agreement with the published phylogenetic data for these two species (7, 30, 35). Strains of *R. loti* showed a close phenotypic relatedness to strains of *R. huakuii* (cluster 6, excepting strain CCB AU 2604), which also in accordance with results obtained previously by using 16S rRNA sequence comparisons (20, 31). The type strains of *R. loti* and *R. huakuii* however, had no more than 33% similarity to the *Rhizobium* strains isolated from *L. corniculatus* nodules used in this analysis.

In contrast to the similarities between the present work and the other work cited above, our dendrogram showed that *R. tropici* CIAT 899 was linked with *A. tumefaciens* C58 (biovar 1) (cluster 2). 16S rRNA sequence analysis indicates that this

strain is more related to *Agrobacterium* sp. biovar 2 or *R. leguminosarum* than it is to *Agrobacterium* sp. biovar 1 (14, 16, 23, 31). Nevertheless, the symbiotic plasmid of CIAT 899 can be expressed in an *A. tumefaciens* C58 genetic background (15), and this fact suggests a degree of phenotypic relatedness between these two strains.

The metabolic properties of the *R. galegae* strains were closely related to those of *Agrobacterium* strains (cluster 4). The precise taxonomic position of the *R. galegae* strains is not yet clear. There is some evidence of their phylogenetic linkage to *R. loti* strains (36), but other authors showed them to be divergent from *R. loti* and demonstrated that they were more closely related to *Agrobacterium* species (23, 31). The results of mobilization of symbiotic genes from *R. galegae* into *Agrobacterium* strains would seem to support the second finding (18). In any case, formal taxonomic conclusions must be supported with data obtained by different systematic approaches. To conclude whether the temperate-zone legume *Rhizobium* strains should be assigned to a new species of the genus or to *R. loti* will require further phylogenetic study.

TABLE 4. Plant host ranges of *Rhizobium* strains isolated from root nodules of *Astragalus*, *Oxytropis*, and *Coronilla* plants

Host genus and <i>Rhizobium</i> sp. strain	Nodulation ^a of the following plant host:										
	<i>Lotus corniculatus</i>	<i>Lotus pedunculatus</i>	<i>Onobrychis viciifolia</i>	<i>Macropitium atropurpureum</i>	<i>Desmodium canadense</i>	<i>Coronilla varia</i>	<i>Glycyrrhiza uralensis</i>	<i>Ononis arvensis</i>	<i>Oxytropis campanulata</i>	<i>Hedysarum alpinum</i>	<i>Astragalus cicer</i>
<i>Astragalus</i>											
A11	0	0	0	I	I	0	E	I	E	E	E
A23	0	0	0	0	0	I	I	E	E	I	E
CIAM 0212	0	I	I	0	I	0	I	E	E	E	E
CIAM 0213	E	I	0	I	I	0	E	I	E	E	E
CIAM 0214	E	I	0	0	0	0	E	I	E	E	I
CIAM 0219	0	I	0	I	I	0	I	E	E	I	I
<i>Oxytropis</i>											
CIAM 2209	E	I	0	0	0	0	E	E	E	E	E
CIAM 2210	E	I	I	0	I	0	E	I	E	E	E
256	E	0	I	I	I	0	E	0	E	E	E
<i>Coronilla</i>											
CIAM 3401	0	0	0	E	0	E	E	0	0	0	I
CIAM 3402	0	0	0	0	0	E	I	0	0	0	I
NZP 5462	E	E	E	E	0	E	E	I	E	I	E

^a E, effective symbiosis; I, ineffective symbiosis; 0, no nodules.

ACKNOWLEDGMENTS

We are grateful to Kristina Lindstrom and En-Tao Wang for kindly providing rhizobia strains, Yury Soskov for legume seeds, Esperanza Martinez for encouraging discussion, and Michael Dunn for reading the manuscript.

This work was supported by the Research Program "Interbioazot-2000."

REFERENCES

- Allen, O. N., and E. K. Allen. 1981. The Leguminosae: a source book of characteristics, uses and nodulation. Wisconsin University Press, Madison.
- Bouzar, H., and L. W. Moore. 1987. Isolation of different *Agrobacterium* biovars from a natural oak savanna and tallgrass prairie. *Appl. Environ. Microbiol.* **53**:717-721.
- Chen, W. X., G. S. Li, Y. L. Qi, E. T. Wang, H. L. Yuan, and J. L. Li. 1991. *Rhizobium huakuii* sp. nov. isolated from root nodules of *Astragalus sinicus*. *Int. J. Syst. Bacteriol.* **41**:275-280.
- Crow, V. L., B. D. W. Jarvis, and R. M. Greenwood. 1981. Deoxyribonucleic acid homologies among acid-producing strains of *Rhizobium*. *Int. J. Syst. Bacteriol.* **31**:152-172.
- Eardly, B. D., J. P. W. Young, and R. S. Selander. 1992. Phylogenetic position of *Rhizobium* sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *nifH* genes. *Appl. Environ. Microbiol.* **58**:1809-1815.
- Elkan, G. H., and C. R. Bunn. 1992. The rhizobia, p. 2197-2213. In A. Balows, H.-G. Truper, M. Dworkin, H. Harder, and K.-H. Schleifer (ed.), The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd ed., vol. III. Springer-Verlag, New York.
- Jarvis, B. D. W., H. L. Downer, and J. P. W. Young. 1992. Phylogeny of fast-growing soybean-nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. *Int. J. Syst. Bacteriol.* **42**:93-96.
- Jarvis, B. D. W., M. Gillis, and J. De Ley. 1986. Intra- and intergeneric similarities between the ribosomal ribonucleic acid cistrons of *Rhizobium* and *Bradyrhizobium* species and some related bacteria. *Int. J. Syst. Bacteriol.* **36**:129-138.
- Jarvis, B. D. W., C. E. Pankhurst, and J. J. Patel. 1982. *Rhizobium loti*, a new species of legume root nodule bacteria. *Int. J. Syst. Bacteriol.* **32**:378-380.
- Jordan, D. C. 1984. Family III: *Rhizobiaceae* Conn 1938, p. 234-244. In N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology. vol. 1. Williams & Wilkins, Baltimore.
- Krieg, N. R. 1981. Systematics V., p. 407-472. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
- Laguerre, G., M. P. Fernandez, V. Edel, P. Normand, and N. Amarger. 1993. Genomic heterogeneity among French *Rhizobium* strains isolated from *Phaseolus vulgaris* L. *Int. J. Syst. Bacteriol.* **43**:761-767.
- Lindström, K. 1989. *Rhizobium galegae*, a new species of legume root nodule bacteria. *Int. J. Syst. Bacteriol.* **39**:365-367.
- Martínez, E. 1994. Recent developments in *Rhizobium* taxonomy. *Plant Soil* **161**:11-20.
- Martínez, E., R. Palacios, and F. Sanchez. 1987. Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. *J. Bacteriol.* **169**:2828-2834.
- Martínez-Romero, E., L. Segovia, F. M. Mercante, A. A. Franco, P. Graham, and M. A. Pardo. 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int. J. Syst. Bacteriol.* **41**:417-426.
- Novikova, N. I., E. A. Pavlova, and E. V. Limeshchenko. 1993. Phage sensitivity and host range of *Rhizobium* strains isolated from root nodules of temperate legumes. *Plant Soil* **151**:45-53.
- Novikova, N. I., and V. I. Safronova. 1992. Transconjugants of *Agrobacterium radiobacter* harbouring *sym* genes of *Rhizobium galegae* can form an effective symbiosis with *Medicago sativa*. *FEMS Microbiol. Lett.* **93**:261-268.
- Orso, S., M. Gouy, E. Navarro, and P. Normand. 1994. Molecular phylogenetic analysis of *Nitrobacter* spp. *Int. J. Syst. Bacteriol.* **44**:83-86.
- Otten, L., J. Canaday, J.-G. Gérard, P. Fournier, P. Crouzet, and F. Paulus. 1992. Evolution of agrobacteria and their Ti plasmids. *Mol. Plant Microbe Interact.* **5**:279-287.
- Prevost, D., L. M. Bordeleau, S. Caudry-Reznick, H. M. Schulman, and H. Antoun. 1987. Characteristics of rhizobia isolated from

- three legumes indigenous to the Canadian high arctic: *Astragalus alpinus*, *Oxytropis maydelliana* and *Oxytropis arctobia*. *Plant Soil* **98**:313–324.
22. **Rinaudo, G., S. Orenge, M. P. Fernandez, H. Meugnier, and R. Bardin.** 1991. DNA homologies among members of the genus *Azorhizobium* and other stem- and root-nodulating bacteria isolated from the tropical legume *Sesbania rostrata*. *Int. J. Syst. Bacteriol.* **41**:114–120.
 23. **Sawada, H., H. Ieki, H. Oyaizu, and S. Matsumoto.** 1993. Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. *Int. J. Syst. Bacteriol.* **43**:694–702.
 24. **Scholla, M. H., and G. H. Elkan.** 1984. *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybeans. *Int. J. Syst. Bacteriol.* **34**:484–486.
 25. **Segovia, L., J. P. W. Young, and E. Martinez-Romero.** 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov. *Int. J. Syst. Bacteriol.* **43**:374–377.
 26. **Sneath, P. H., and R. B. Sokal.** 1973. Numerical taxonomy. The principles and practices of numerical classification. W. H. Freeman & Co., San Francisco.
 27. **Trinick, M. J.** 1980. Relationships amongst the fast growing rhizobia of *Lablab purpureus*, *Luecaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. *J. Appl. Bacteriol.* **49**:39–53.
 28. **van Rysin, J. (ed.).** 1977. Classification and clustering. *Proc. Adv. Semin. Math. Res. Center, University of Wisconsin, Madison.* Academic Press, New York.
 29. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. IBP Handbook no. 15. Blackwell Scientific Publications, Oxford.
 30. **Wedlock, D. N., and B. D. W. Jarvis.** 1986. DNA homologies between *Rhizobium fredii*, rhizobia that nodulate *Galega* sp., and other *Rhizobium* and *Bradyrhizobium* species. *Int. J. Syst. Bacteriol.* **37**:550–558.
 31. **Willems, A., and M. D. Collins.** 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. *Int. J. Syst. Bacteriol.* **43**:305–313.
 32. **Wong, F. Y. K., E. Stackebrandt, J. K. Ladha, D. E. Fleischman, R. A. Date, and J. A. Fruest.** 1994. Phylogenetic analysis of *Bradyrhizobium japonicum* and photosynthetic stem-nodulating bacteria from *Aeschynomene* species grown in separated geographical regions. *Appl. Environ. Microbiol.* **60**:940–946.
 33. **Yanagi, M., and R. Yamasato.** 1993. Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiol. Lett.* **107**:115–120.
 34. **Yelton, M. M., S. S. Yang, S. A. Edie, and S. T. Lim.** 1983. Characterization of an effective salt-tolerant, fast growing strain of *Rhizobium japonicum*. *J. Gen. Microbiol.* **129**:1537–1547.
 35. **Young, J. P. W.** 1992. Phylogenetic classification of nitrogen-fixing organisms, p. 43–86. *In* G. Stacey, R. H. Burris, and H. J. Evans (ed.), *Biological nitrogen fixation*. Chapman & Hall, New York.
 36. **Young, J. P. W.** 1993. Molecular phylogeny of rhizobia and their relatives, p. 587–592. *In* R. Palacios, J. Mora, and W. Newton (ed.), *New horizons in nitrogen fixation*. Kluwer Academic Publishers, Dordrecht.
 37. **Zhang, X., R. Harper, M. Karsisto, and K. Lindström.** 1991. Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. *Int. J. Syst. Bacteriol.* **41**:104–113.