Sinorhizobium medicae sp. nov., Isolated from Annual *Medicago* spp.

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The taxonomic position of isolates of a new genomic species (designated genomic species 2) obtained from several annual *Medicago* species and originating from different geographical locations was established through the results of phenotypic tests (including the results of auxanographic and biochemical tests and symbiotic properties) and 16S rRNA phylogenetic inferences. A comparison of the complete 16S rRNA sequence of a representative of genomic species 2 (strain A 321^T [T = type strain]) with the 16S rRNA sequences of other members of the *Rhizobiaceae* and closely related taxa showed that genomic species 2 was phylogenetically related to *Sinorhizobium meliloti*, *Sinorhizobium fredii*, *Sinorhizobium saheli*, and *Sinorhizobium teranga*. The levels of sequence similarity and observed numbers of nucleotide substitutions in *Sinorhizobium* strains indicated that A 321^T and *S. meliloti* exhibited the highest level of sequence similarity (99.7%), with four nucleotide substitutions and one deletion. The results of a numerical analysis based on data from 63 auxanographic and biochemical tests clearly separated genomic species 2 isolates from *S. meliloti*. Genomic species 2 isolates nodulated and fixed nitrogen with *Medicago polymorpha*, whereas *S. meliloti* isolates were ineffective and formed rudimentary nodules on this host plant. On the basis of phenotypic and 16S sequence analysis data, genomic species 2 isolates cannot be assigned to a previously described species. We propose that these isolates belong to a new species, *Sinorhizobium medicae*.

The genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Sinorhizobium* consist of soil bacteria that are capable of forming nitrogen-fixing nodules on leguminous plants (and on members of the nonlegume genus *Parasponia*). The genus *Sinorhizobium* was first created for the fast-growing nodule-forming soybean isolates which previously had been included in *Rhizobium fredii* (4). The low levels of similarity of this species with the other *Rhizobium* spp., as deduced from numerical taxonomy analyses, led to the proposal of the new genus (4). However, when a partial 16S rRNA sequence analysis was performed, *Rhizobium meliloti* and *R. fredii* were found to be close neighbors since they have identical partial 16S sequences, and Jarvis et al. (15) suggested that these organisms should be reclassified in the genus *Rhizobium*.

More recently, new isolates obtained from *Sesbania* and *Acacia* species were found to constitute two new species belonging to the *R. fredii*-*R. meliloti* taxonomic group (5). In this study, which was based on phenotypic criteria, DNA-DNA and DNA-rRNA hybridization results, and 16S rRNA sequence comparisons, the authors found high levels of divergence between this group and the other *Rhizobium* species, which led them to place four species in the genus *Sinorhizobium* and to present an emended genus description. The emended genus *Sinorhizobium* contains four species, *Sinorhizobium meliloti*, *Sinorhizobium fredii*, *Sinorhizobium teranga*, and *Sinorhizobium saheli*, and *S. fredii* is the type species. This genus is now well-described, but the status of some species needs further refinement. In this paper, we present a revision of the species *S. meliloti*.

S. meliloti is classically described as an organism which forms

symbiotic relationships with members of three genera of legumes, the genera *Medicago*, *Melilotus*, and *Trigonella* (10). Since the perennial species *Medicago sativa* is the most widely cultivated species of lucerne in the world, symbiotic strains obtained from this legume have been studied intensively and are included in many genetic and taxonomic studies of the *Rhizobiaceae*. In contrast, the taxonomy of sinorhizobia obtained from annual *Medicago* species is less well understood. Cross-inoculation data have shown that annual *Medicago* species exhibit various degrees of specificity in their symbioses (2, 3, 21); the most promiscuous plant species (e.g., *Medicago minima*, *Medicago rigidula*, and *Medicago truncatula*) fix nitrogen with most annual *Medicago*-infective isolates, while other *Medicago* species (e.g., *Medicago laciniata*, *Medicago polymorpha*, *Medicago sativae*, and *Medicago rugosa*) are more specific and form effective symbioses with only a few isolates. These findings suggest that there is a subspecific sinorhizobial structure. A multilocus electrophoresis and restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene yielded two genetically distinct divisions, divisions A and B, for isolates obtained from various *Medicago* species (7), which supported the hypothesis that the species should be divided. In a previous study in which the genetic diversity of sinorhizobia that nodulate spontaneous annual *Medicago* species in the south of France was examined, 73 *M. truncatula*-infective sinorhizobia were characterized by using the DNA polymorphism of four amplified DNA regions localized on the symbiotic plasmid or on the chromosome. These isolates fell into two genotypic groups (22). DNA-DNA hybridization data showed that these groups represented two different genomic species (22). The first species corresponded to *S. meliloti*, whereas the members of the second group exhibited low levels of DNA homology with several *S. meliloti* strains and belonged to another genomic species. These results corroborated those of Eardly et al. (7), which strongly suggested that rhizobia that

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TABLE 1. *Sinorhizobium* strains used in this study

Strain	Species ^a	PCR-RFLP group ^a	Host plant	Geographical origin	Reference(s)
A 17	<i>S. meliloti</i>	1	<i>Medicago truncatula</i>	France (Aude)	22
A 110B	<i>S. meliloti</i>	1	<i>Medicago truncatula</i>	France (Aude)	22
V 11A	<i>S. meliloti</i>	1	<i>Medicago truncatula</i>	France (Var)	22
V 127B	<i>S. meliloti</i>	1	<i>Medicago truncatula</i>	France (Var)	22
A 27	Genomic species 2	2	<i>Medicago truncatula</i>	France (Aude)	22
A 34	Genomic species 2	2	<i>Medicago truncatula</i>	France (Aude)	22
A 321 ^T	Genomic species 2	2	<i>Medicago truncatula</i>	France (Aude)	22
V 113	Genomic species 2	2	<i>Medicago truncatula</i>	France (Var)	22
V 22	Genomic species 2	2	<i>Medicago truncatula</i>	France (Var)	22
V 25	Genomic species 2	2	<i>Medicago truncatula</i>	France (Var)	22
V 214	Genomic species 2	2	<i>Medicago truncatula</i>	France (Var)	22
Reference strains					
M3	Genomic species 2	2	<i>Medicago orbicularis</i>	Syria	7
M104	Genomic species 2	2	<i>Medicago truncatula</i>	Syria	7
RCR 2011 (= SU 47 = NZP4009 = LMG 6130)	<i>S. meliloti</i>	1	<i>Medicago sativa</i>	Australia	5, 18
USDA 205 ^T	<i>S. fredii</i>	ND ^b	<i>Glycine max</i>	People's Republic of China	5, 18

^a PCR-RFLP groups 1 and 2 and the genomic species have been described previously (22).

^b ND, not determined.

nodulate *Medicago* species should be placed into at least two species.

In this study, nine strains which were obtained from annual *Medicago* species (including two strains from Eardly et al. [7]) and were genomically distinct from *S. meliloti* were compared with reference strains of *S. meliloti* and the closely related species *S. fredii*.

Consistent with the recommendations for *Rhizobiaceae* taxonomy (12), we used cultural characteristics (from auxanographic and biochemical tests), genomic characteristics (16S rRNA sequence data), and symbiotic characteristics to establish the taxonomic status of the organisms studied.

MATERIALS AND METHODS

Bacterial strains. The 15 *Sinorhizobium* strains which we studied are listed in Table 1. Eleven strains, which were identified as either *S. meliloti* or genomic species 2 strains, were isolated from two *M. truncatula* L. ecotypes which are found at two sites in southern France, named Aude and Var and identified as A and V, respectively. Two strains isolated from *M. truncatula* and *Medicago orbicularis* in Syria were also included; these strains are representatives of division B as defined by isoenzyme characteristics (7). *S. fredii* USDA 205 is the type strain of the type species of the genus *Sinorhizobium* (5). In phenotypic tests, we used strain RCR 2011 as the reference strain for *S. meliloti* (5, 18). This strain, which was included in division A of Eardly et al. (7), is closely related to *S. meliloti* ATCC 9930^T (T = type strain) and is currently used as a reference strain for *S. meliloti* (5).

All strains were maintained on yeast extract-mannitol (YEM) medium (27). Unless otherwise stated, cultures were incubated at 28°C.

DNA extraction. Bacterial DNA was extracted from 150-ml stationary-phase TY broth cultures as described by Brenner et al. (1). TY medium contained (per liter of distilled water) 10 g of Bacto Tryptone (Difco) and 6 g of yeast extract (Difco), as well as 6 mM CaCl₂ · 2H₂O.

16S rRNA gene sequencing. The 16S rRNA gene of strain A 321^T was amplified by PCR by using the procedure of Mullis and Faloona (20). The following two prokaryote-specific primers were used: FGPS6-255 (5'-TGGAAAGCTTG ATCCTGGCT-3') and FGPS1509'-153 (5'-AAGGAGGGGATCCAGCCGCA-3') (the underlined sequences are *Hind*III and *Bam*HI restriction sites, respectively). The amplified fragments were purified with a GeneClean kit (Bio 101, La Jolla, Calif.) according to the manufacturer's instructions. The PCR fragments were cleaved with endonucleases *Hind*III and *Bam*HI and ligated into the Bluescript SK⁻ vector (Stratagene, La Jolla, Calif.) digested by the same enzymes. Plasmid purification from host strain DH5αF' was performed with QIAGEN midi-prep columns (Diagen, Düsseldorf, Germany). Five clones were pooled for the sequencing reaction in order to obscure possible errors due to the *Taq* polymerase.

Sequencing was performed by using the dideoxy chain termination method of Sanger et al. (24). A T7 sequencing kit (Pharmacia LKB, Uppsala, Sweden) was used.

DNA sequence analysis. 16S rRNA sequences were aligned by using the

Clustal multiple-alignment program (13). Sites involving gaps were excluded from all analyses. Evolutionary distances corrected for multiple substitutions were computed by using Kimura's two-parameter model (17). A phylogenetic tree was inferred by using the neighbor-joining (NJ) method (23). The NJ method is considered one of the most efficient methods for recovering phylogenetic trees (23). The 16S rRNA sequences of the following organisms belonging to the *Rhizobiaceae* and related bacteria were used for comparison (GenBank accession numbers are in parentheses): *Rhizobium ciceri* UPM-Ca7^T (UO7934), *Rhizobium galegae* LMG 6214^T (X67226), *Rhizobium huakuii* IAM 14158^T (D12797), *Rhizobium leguminosarum* A LMG 9518 (X67233), *Rhizobium loti* A LMG 6125^T (X67229), *Rhizobium loti* B LMG 4284 (X67230), *Rhizobium mediterraneum* UPM-Ca36^T (L38825), *Rhizobium tropici* (IFO15247) (D11344), *Rhizobium tropici* IIB LMG 9517 (X67234), *Rhizobium* ORS 1001-Cluster U (X68389), *Rhizobium* sp. strain LMG 9509 (X67232), *S. meliloti* LMG 6133^T (= ATCC 9930^T) (X67222), *S. fredii* LMG 6217^T (= USDA 205^T) (X67231), *S. teranga* ORS 1009^T (X68387), *S. saheli* ORS 609^T (X68391), *Bradyrhizobium japonicum* NZP 5549^T (X66024), *Azorhizobium caulinodans* LMG 6465 (X67221), *Agrobacterium rhizogenes* LMG 150^T (X67224), *Agrobacterium rubi* ATCC 13335^T (D14503 and D01259), *Agrobacterium tumefaciens* LMG 198^T (X67223), *Agrobacterium vitis* LMG 257 (D14502 and D1258), *Brucella abortus* (X13695), *Mycoplana dimorpha* NCIB 9439^T (D12786), *Ochrobactrum anthropi* CIP 14970^T (D12794), *Phyllobacterium myrsinacearum* NCIB 12127 (D12789), and *Rochalimaea vinsonii* (M73230).

Phenotypic tests (i) Growth characteristics. In order to determine the capacity of the organisms to grow at different pH values and temperatures and their tolerance to sodium chloride, starter cultures were grown at 28°C to the log phase (10⁹ bacteria per ml) in YEM broth, and 5-ml portions of YEM broth were inoculated in triplicate with 50-μl portions of the starter cultures. The presence or absence of growth was scored in inoculated tubes after 3 days. The pH tolerance of strains was determined by using YEM broth preparations in which the pH values were adjusted to 4.0, 5.0, 6.0, 9.0, and 10 (± 0.1 pH unit) by adding HCl or NaOH. Growth was also examined at 37 and 42°C. Sodium chloride tolerance was determined by using YEM broth preparations in which the concentrations of NaCl were 2 and 3% (wt/vol). Control cultures were grown under standard conditions in YEM medium (0.1% [wt/vol] NaCl, pH 7, 28°C).

(ii) Antibiotic susceptibility. Triplicate antibiotic resistance tests were performed by measuring the diameters of inhibition zones on YEM agar plates containing the following antibiotic discs: polymyxin (300 mg), streptomycin (10 mg), tetracycline (30 mg), neomycin (30 mg), chloramphenicol (30 mg), nalidixic acid (30 mg), vancomycin (30 mg), and penicillin G (6 mg) (SANOFI Diagnostic Pasteur, Marnes-la-Coquette, France). The susceptibilities of the strains were deduced from the antibiograms (SANOFI Diagnostic Pasteur) on the basis of the diameters of the inhibition zones.

(iii) Biochemical and auxanographic tests. A total of 49 carbohydrates were used as sole carbon sources in API 50CH microtube system rapid procedures (BioMérieux, La Balme Les Grottes, France). The preparations were inoculated as recommended by the manufacturer, and the test strips were incubated at 28°C. Results were scored every day for 5 days.

(iv) Data analysis. The genetic distance between each possible pair of isolates tested for auxanographic and biochemical characteristics was estimated by comparing the phenotypic variables, using the Jaccard similarity coefficient (26). In this procedure the sum of effective matches is computed and divided by the total

	1	11	21	31	41	51
<i>S. teranga</i>	-----	-----	-----	-----	-----	AC-----
<i>S. saheli</i>	-----	-----	-----	-----	---GTG---T	AC-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
CC 169	-----	-----	-----	-----	-----	-----
A 321	AACGAACGCT	GGCGGCAGGC	TTAACACATG	CAAGTCGAGC	GCCCCGCAAG	GGGAGCGGCA
	61	71	81	91	101	111
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. saheli</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
CC 169	-----	-----	-----	-----	-----	-----
A 321	GACGGGTGAG	TAACGCGTGG	GAATCTACCC	TTTTCTACGG	AATAACGCAG	GGAAACTTGT
	121	131	141	151	161	171
<i>S. teranga</i>	-----	---GC----	-TT-----	-----	AG-----	-----
<i>S. saheli</i>	-----	---G-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	---G-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	---G-----	-----	-----	-----	-----
CC 169	-----	---A-----	-----	-----	-----	-----
A 321	GCTAATACCG	TATAAGCCCT	TCGGGGGAAA	GATTTATCGG	GAAAGGATGA	GCCCGCGTTG
	181	191	201	211	221	231
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. saheli</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
CC 169	-----	-----	-----	-----	-----	-----
A 321	GATTAGCTAG	TTGGTGGGGT	AAAGGCCTAC	CAAGGCGACG	ATCCATAGCT	GGTCTGAGAG
	241	251	261	271	281	291
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. saheli</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
CC 169	-----	-----	-----	-----	-----	-----
A 321	GATGATCAGC	CACATTGGGA	CTGAGACACG	GCCCCAAACTC	CTACGGGAGG	CAGCAGTGGG
	301	311	321	331	341	351
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. saheli</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	GAATATTGGA	CAATGGGCGC	AAGCCTGATC	CAGCCATGCC	GCGTGAGTGA	TGAAGGCCCT
	361	371	381	391	401	411
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. saheli</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	AGGGTTGTAA	AGCTCTTTCA	CCGGTGAAGA	TAATGACGGT	AACCGGAGAA	GAAGCCCCGG
	421	431	441	451	461	471
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. saheli</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	CTAACTTCGT	GCCAGCAGCC	GCGGTAATAC	GAAGGGGGCT	AGCGTTGTTC	GGAATTACTG
	481	491	501	511	521	531
<i>S. teranga</i>	-----	-----	---CAT---	---C-----	-----G--	-----C
<i>S. saheli</i>	-----	-----	---CAT---	---C-----	-----G--	-----C
<i>S. fredii</i>	-----	-----	---CAT---	---C-----	-----G--	-----C
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	GGCGTAAAGC	GCACGTAGGC	GGATTGTTAA	GTGAGGGGTG	AAATCCCAGG	GCTCAACCCCT
	541	551	561	571	581	591
<i>S. teranga</i>	-----	---G-----	GTG-----	---G-----	-----	-----
<i>S. saheli</i>	-----	---G-----	GTG-----	---G-----	-----	-----
<i>S. fredii</i>	-----	---G-----	GTG-----	---G-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	GGAAGTGCCT	TTCATACTGG	CAATCTAGAG	TCCAGAAGAG	GTGAGTGGAA	TTCCGAGTGT
	601	611	621	631	641	651
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----C-
<i>S. saheli</i>	-----	-----	-----	-----	-----	-----C-
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----C-
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	AGAGGTGAAA	TTCGTAGATA	TTCGGAGGAA	CACCACTGGC	GAAGGCGGCT	CACTGGTCTG
	661	671	681	691	701	711
<i>S. teranga</i>	---A-----	-----	-----	-----	-----	-----C---
<i>S. saheli</i>	---A-----	-----	-----	-----	-----	-----C---
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	GTACTGACGC	TGAGGTGCGA	AAGCGTGGGG	AGCAAACAGG	ATTAGATACC	CTGGTAGTCC

	721	731	741	751	761	771
<i>S. teranga</i>	-----	-----	-----	-----C-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	ACGCCGTAAA	CGATGAATGT	TAGCCGTCGG	GCAGTTTACT	GTTCCGGTGGC	GCAGCTAACG
	781	791	801	811	821	831
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	CATTAAACAT	TCCGCCTGGG	GAGTACGGTC	GCAAGATTAA	AACTCAAAGG	AATTGACGGG
	841 *	851	861	871	881	891
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	GGCCCGCACA	AGCGGTGGAG	CATGTGGTTT	AATTCGAAGC	AACGCGCAGA	ACCTTACCAG
	901	911	921	931	941	951
<i>S. teranga</i>	-----	-----G-----	-----TAC-----	-----GT-T-----	-----	-----C-----
<i>S. sahelii</i>	-----	-----G-----	-----C-----	-----G-----T-----	-----	-----C-----
<i>S. fredii</i>	-----	-----	-----C-----	-----G-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----C-----	-----G-----	-----	-----
A 321	CCCTTGACAT	CCCGATCGCG	GATAGGAGAG	ATCCTATCCT	TCAGTTCCGC	TGGATCGGAG
	961	971	981	991	1001	1011
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	ACAGGTGCTG	CATGGCTGTC	GTCAGCTCGT	GTCGTGAGAT	GTTGGGTAA	GTCCCGCAAC
	1021	*	1041	1051	1061	1071
<i>S. teranga</i>	-----	-----	-----	-----TG-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----TG-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----T-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	GAGCGCAACC	CTCGCCCTTA	GTTGCCAGCA	TTCAGTTGGG	CACTCTAAGG	GGACTGCCGG
	1081	1091	1101	1111	1121	1131
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	TGATAAGCCG	AGAGGAAGGT	GGGGATGACG	TCAAGTCCTC	ATGGCCCTTA	CGGCCTGGGC
	1141	1151	1161	1171	1181	1191
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	TACACACGTG	CTACAATGGT	GGTGACAGTG	GGCAGCGAGA	CCGCGAGGTC	GAGCTAATCT
	1201	1211	1221	1231	1241	1251
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	CCAAAAGCCA	TCTCAGTTCG	GATTGCACCTC	TGCAACTCGA	GTGCATGAAG	TTGGAATCGC
	1261	1271	1281	1291	1301	1311
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	TAGTAATCGC	AGATCAGCAT	GCTGCGGTGA	ATACGTTCCC	GGGCCTTGTA	CACACCGCCC
	1321	1331	1341	1351	1361	1371
<i>S. teranga</i>	-----	-----	-----	-----	-----	-----
<i>S. sahelii</i>	-----	-----	-----	-----	-----	-----
<i>S. fredii</i>	-----	-----	-----	-----	-----	-----
<i>S. meliloti</i>	-----	-----	-----	-----	-----	-----
A 321	GTCACACCAT	GGGAGTTGGT	TCTACCCGAA	GGTAGTGC GC	TAACCGCAAG	GAGGCAGCTA
	1381	1391	1401	1411	1421	
<i>S. teranga</i>	-----	-----	-----	-----	-----	
<i>S. sahelii</i>	-----	-----	-----	-----	-----	
<i>S. fredii</i>	-----	-----	-----	-----	-----	
<i>S. meliloti</i>	-----	-----	-----	-----	-----	
A 321	ACCACGGTAG	GGTCAGCGAC	TGGGGTGAAG	TCGTAACAAG	GTA	

FIG. 1. Alignment of the total 16S rRNA gene sequence of strain A 321^T (genomic species 2) with sequences of *S. fredii* USDA 205^T, *S. meliloti* ATCC 9930^T, *S. sahelii* ORS 609^T, and *S. teranga* ORS 1009^T. The partial sequence of isolate CC169 (8) extends from position 17 to position 276. Gaps are indicated by asterisks.

TABLE 2. Homology matrix for complete 16S rRNA sequences

Organism	No. of differences or % homology ^a				
	<i>S. saheli</i>	<i>S. teranga</i>	<i>S. fredii</i>	<i>S. meliloti</i>	Strain A 321 ^T
<i>S. saheli</i>		15	8	20	24
<i>S. teranga</i>	98.9		19	31	31
<i>S. fredii</i>	99.4	98.6		14	16
<i>S. meliloti</i>	98.5	97.7	99.0		4
Strain A 321 ^T	98.2	97.7	98.8	99.7	

^a The values on the upper right are the numbers of observed differences (nucleotides plus gaps), and the values on the lower left are the percentages of similarity corrected for multiple base changes by the method of Kimura (17) (gaps were excluded from the analysis).

possible number of matches for each pairwise calculation. Clustering from a matrix of pairwise genetic distances was performed by the unweighted pair group method with arithmetic averages (26).

(v) **Symbiotic properties.** Strains obtained from *M. truncatula*, strain RCR 2011 obtained from *M. sativa*, and strain M3 obtained from *M. orbicularis* were tested for the ability to nodulate and fix nitrogen on *M. truncatula* (ecotype Aude) and *M. polymorpha* (ecotype Combaillaux). Seeds were surface sterilized in 1% (wt/vol) calcium hypochlorite for 2 min, rinsed with sterile water, and then scarified. These seeds were germinated for 72 h on 0.7% (wt/vol) agar medium in a growth chamber at 28°C, and germinated seeds were placed aseptically in Gibson tubes (11) supplemented with a nitrogen-free plant nutrient solution (3). Each tube was inoculated with a rhizobial suspension from an early-stationary-phase culture. Uninoculated plants and plants to which nitrogen [10 mM Ca(NO₃)₂] was added were used as controls. Fifteen replicates were prepared for each treatment. After 50 days, the plants were harvested, the number of nodules was counted, and the nodule dry weight was determined after the nodules were dried for 48 h at 70°C.

Nucleotide sequence accession number. The 16S rRNA sequence of strain A 321^T which we determined has been deposited in the GenBank data library under accession number L39882.

RESULTS

DNA analysis. The total 16S rRNA sequence of genomic species 2 isolate A 321^T was determined. This sequence was aligned and compared with the 16S rRNA sequences of other members of the alpha 2 subclass of the *Proteobacteria*. From the results of this comparison of aligned sequences we deduced that on the basis of its 16S ribosomal DNA (rDNA) sequence strain A 321^T clusters with the *Sinorhizobium* lineage (data not shown). Figure 1 shows the 16S rDNA sequence of isolate A 321^T aligned with the 16S rDNA sequences of *S. meliloti* ATCC 9930^T, *S. fredii* USDA 205^T, *S. teranga* ORS 1009^T, and *S. saheli* ORS 609^T. Table 2 shows similarity values and observed numbers of nucleotide differences for the sequences of *Sinorhizobium* species. The 16S rDNA of A 321^T was most similar to the 16S rDNA of *S. meliloti* (level of similarity, 99.7%, corresponding to four nucleotide substitutions and one deletion). The four nucleotide substitutions occurred in two variable regions of the gene (Fig. 1), at position 133 and from position 923 to position 933; the deletion was located at position 845.

Evolutionary distances between members of the *Rhizobiaceae* and some related genera were computed, and a phylogenetic tree was constructed by using the NJ algorithm and the pairwise evolutionary distances (Fig. 2). Bootstrap confidence intervals of the various clusters on the NJ tree were estimated from 1,000 replications (9). Bootstrap values greater than 95% are shown in Fig. 2. The results of this analysis clearly placed the *Sinorhizobium* species in a monophyletic cluster for 99% of the bootstrap replicates. Isolate A 321^T and *S. meliloti* were clustered on a monophyletic lineage within the genus *Sinorhizobium* in 100% of the bootstrap replicates.

Phenotypic tests. All of the isolates studied were gram-negative, motile, non-spore-forming, rod-shaped bacteria. On

YEM medium, they were mucous, and they acidified the medium, changing the pH from 7 to 5. The generations time were between 3 and 5 h. All of the isolates grew at 28°C at pH 5 to 10 and at 37°C but not at 42°C. Strain RCR 2011 and the *S. meliloti* isolates grew at pH 4, whereas all of the genomic species 2 isolates except M104 and *S. fredii* did not grow at this pH. All of the isolates tolerated 2% (wt/vol) NaCl. *S. meliloti* isolates grew in the presence of 3% (wt/vol) NaCl, while the growth of genomic species 2 isolates and *S. fredii* was variable under these conditions.

The results of tests to determine resistance to eight antibiotics showed that all of the isolates were resistant to nalidixic acid and penicillin G and susceptible to polymyxin. Strain RCR 2011 (*S. meliloti*) and strain USDA 205^T (*S. fredii*) were susceptible to chloramphenicol. Strain USDA 205^T was the only strain that was susceptible to streptomycin. Genomic species 2 strains could be distinguished by their susceptibility to neomycin. In contrast, chloramphenicol susceptibility, tetracycline susceptibility, and vancomycin susceptibility were variable depending on the isolate and could not be used to separate group 1 and 2 rhizobia.

A total of 45 carbon sources were tested, and the following

FIG. 2. Phylogenetic relatedness of species belonging to the alpha 2 subclass of the *Proteobacteria* as determined with the NJ method and nucleic acid pairwise distances. Horizontal distances are proportional to levels of evolutionary divergence expressed as numbers of substitutions per site; vertical separations are for clarity only. Bootstrap probability values greater than 95% are indicated at the branch points. Bar = 0.0071 substitution per site.

TABLE 3. Carbon sources that can be used to distinguish taxa

Taxon	Utilization of:										
	Glycerol	Melibiose	Lyxose	Xylitol	Tagatose	2-Keto-gluconate	Dulcitol	Melezi-tose	Sorbose and D-fucose	D-Arabinose	D-Xylose, salicin, and ribose
Reference strains											
<i>S. fredii</i> USDA 205 ^T	+	+	—	—	—	—	—	—	—	+	+
<i>S. meliloti</i> RCR 2011	+	+	—	+	+	+	+	—	+	—	+
Strains from annual <i>Medicago</i> spp. (genomic species 2) ^b											
PCR-RFLP group 1 ^c	+	+	v	—	+	+	v	v	+	—	+
PCR-RFLP group 2 ^c	v	v	v	+	+	+	v	v	—	—	—

^a +, all strains are positive; —, all strains are negative; v, variable.

^b A total of 13 isolates obtained from *M. truncatula* and *M. orbicularis* (Table 1).

^c See Table 1.

10 were not assimilated by any isolate: L-arabinose, amygdalin, inulin, gluconate, 5-keto-gluconate, α -methyl-D-glucoside, glycogen, α -methyl-D-mannoside, starch, and L-xylose. The following 25 compounds were used as sole carbon sources by all of the isolates tested: adonitol, D-arabitol, L-arabitol, arbutin, D-cellobiose, erythriol, esculin, fructose, L-fucose, galactose, β -gentiobiose, *N*-acetylglucosamine, glucose, inositol, lactose, maltose, mannitol, mannose, D-raffinose, rhamnose, sorbitol, sucrose, trehalose, D-turanose, and β -methyl-D-xyloside. The results of distinctive auxanographic tests are shown in Table 3.

Numerical analysis of phenotypic data. The numerical analysis of phenotypic data included data from 49 auxanographic tests, as well as data for tolerance to NaCl, pH, and antibiotics; a total of 63 characteristics were used in this analysis. The resulting dendrogram is presented in Fig. 3, and this dendrogram shows that the *Medicago* isolates were separated into two major groups. The first group contained *S. meliloti* RCR 2011 and four isolates from *M. truncatula* received as *S. meliloti*. The second group contained eight isolates from *M. truncatula* and the isolate from *M. orbicularis* (isolate M3), all of which were previously identified as members of genomic group 2. The two groups diverged at a genetic distance of approximately 0.13. The *S. fredii* strain branched far from all of the *Medicago* isolates.

Symbiotic properties on *M. polymorpha* and *M. truncatula*. Isolates obtained from *M. truncatula* and reference isolates RCR 2011 and M3 were tested for the ability to form nodules

on *M. polymorpha* and *M. truncatula*. Plant growth (shoot dry weight) on mineral medium without combined nitrogen was used to estimate nitrogen fixation efficiency (Table 4).

On *M. truncatula*, all of the bacterial isolates tested induced pink well-developed nodules, and the shoot dry weights of inoculated plants were significantly greater than the shoot dry weights of uninoculated controls, except for plants inoculated with isolate V 11A. There was no significant difference between plants inoculated with *S. meliloti* and plants inoculated with genomic species 2. On *M. polymorpha*, all of the isolates tested formed nodules. However, the genomic species 2 isolates formed pink, efficient nodules, and all of the *S. meliloti* isolates, including RCR 2011, formed rudimentary, inefficient, white nodules. The shoot dry weights for the *M. polymorpha*-*S. meliloti* associations were not significantly different from the shoot dry weights of uninoculated controls.

DISCUSSION

The genus *Medicago* comprises more than 60 different species, including annuals and perennials. The primary center of diversity of the genus is in the Mediterranean basin (19). The biodiversity of sinorhizobia which naturally form symbiotic associations with annual *Medicago* species was investigated previously by workers who used phenotypic and genomic approaches (3, 7, 22). These studies revealed that there are two groups of *Medicago*-infective *Sinorhizobium* isolates. The first group (group A of Eardly et al. [7], group I of Brunel et al. [3], and group 1 of Rome et al. [22]) corresponds to *S. meliloti*. The second group (group B of Eardly et al. [7], group II of Brunel et al. [3], and group 2 of Rome et al. [22]) represents a separate genomic species. The purpose of this study was to establish the taxonomic position of this genomic species by using phenotypic tests and 16S rRNA sequencing.

16S rRNA sequence analysis data are currently used to measure phylogenetic relationships. Relatively well-conserved regions of the 16S rRNA sequence can be used to infer natural relationships between distantly related species, whereas variable regions can be used to analyze closely related species (14). Comparisons of the 16S rRNA sequences of species belonging to the *Rhizobiaceae* have demonstrated that this approach can be used to classify isolates at the species and higher levels (18, 28–30). In the present study, the total 16S rRNA sequence of a genomic species 2 isolate was determined to estimate the relationship between this genomic species and the other members of the genus *Sinorhizobium*. After alignment and comparison of sequences we found that genomic species 2 strain A 321^T and the type strain of *S. meliloti* had distinct sequences

FIG. 3. Dendrogram showing the genetic distances between isolates obtained from annual *Medicago* species and two reference strains, *S. meliloti* RCR 2011 and *S. fredii* USDA 205^T, based on a numerical unweighted pair group with arithmetic average analysis of the results of 63 phenotypic tests.

TABLE 4. Symbiotic properties of isolates on *M. truncatula* and *M. polymorpha*

Strain or prepn	<i>M. polymorpha</i>		<i>M. truncatula</i>	
	Plant dry wt (10 ⁻³ , g)	No. of nodules	Plant dry wt (10 ⁻³ , g)	No. of nodules
Control with N (10 mM)	34 ± 11.3 ^a	0	146 ± 47.6	0
Uninoculated control	1.4 ± 0.64	0	9 ± 8.8	0
<i>S. meliloti</i> isolates (PCR-RFLP group 1)				
RCR 2011	2.3 ± 0.94	4.5 ± 2.5 ^a	38 ± 17.5	3 ± 1.3
A 13	3.0 ± 1.0	11 ± 5.87	18 ± 17.4	3 ± 1.5
A 17	2.3 ± 0.86	6.2 ± 3.46	53 ± 24.5	3 ± 1.45
A 110B	1.4 ± 0.92	7.3 ± 4.8	58 ± 27.5	3 ± 1
V 11A	2.7 ± 0.94	7.7 ± 4.4	5 ± 2.25	3 ± 1.2
V 127B	2.2 ± 0.72	4.6 ± 4.7	69 ± 17.6	4 ± 1.5
Genomic species 2 isolates (PCR-RFLP group 2)				
A 27	27.75 ± 4.6	5.6 ± 3.6	70 ± 23.8	4 ± 1.7
A 321 ^T	26 ± 3.5	7.9 ± 3.8	74 ± 24.5	4 ± 1.7
V 22	27 ± 2.1	8.4 ± 1.55	49 ± 22.5	3 ± 1.4
V 25	25 ± 5.2	6 ± 1.6	67 ± 23.9	3 ± 1.6
V 214	15.1 ± 6.2	9.54 ± 3.4	53 ± 17.5	4 ± 2
V 113	26.2 ± 4.5	6.5 ± 2.7	37 ± 14.7	4 ± 2
M3	24 ± 5.35	6.25 ± 2	64 ± 12.4	3 ± 1.65

^a Average ± standard deviation.

(Fig. 2). The difference observed is consistent with DNA-DNA hybridization results since *S. meliloti* and genomic species 2 were found to exhibit 42 to 60% DNA homology (22). Thus, it can be deduced from these results that *S. meliloti* and genomic species 2 have diverged sufficiently to be considered two distinct species. Isolate A 321^T and isolate CC169 belonging to group B of Eardly et al. (8) have identical 16S rRNA sequences between position 17 and position 276 (Fig. 1), a variable region in the ribosomal gene which allows *S. meliloti* and genomic species 2 to be distinguished. This finding confirmed that genomic species 2 and the group B strains of Eardly et al. (7) are equivalent, as noted previously (3, 22). A phylogenetic tree was constructed by transforming the 16S rDNA sequence variations into evolutionary distance values (Fig. 2). *Bradyrhizobium japonicum* and *Azorhizobium caulinodans* were used as the outgroups. This analysis revealed the relationship between genomic species 2 and the *Sinorhizobium* species, as well as the relationship between the genus *Sinorhizobium* and the other genera of the *Rhizobiaceae*. The results of this phylogenetic analysis showed that genomic species 2 and *S. meliloti* are closely related taxa in the *Sinorhizobium* group and suggested that these two species diverged later than the other species of the genus. The genetic divergence is supported by the results of the numerical analysis of 63 auxanographic and biochemical characteristics (Fig. 3), which showed that *S. meliloti* and genomic species 2 have distinct phenotypic characteristics (Table 5).

Since annual *Medicago* species exhibited various sinorhizobial specificities (2, 3, 21), we wondered if the symbiotic properties could be used to discriminate between *S. meliloti* and genomic species 2. To examine this possibility, we chose a promiscuous nodulating plant species and a more selective nodulating plant species for inoculations (*M. truncatula* and *M. polymorpha*, respectively). All isolates of *S. meliloti* and genomic species 2 were able to nodulate and fix nitrogen with the promiscuous plant species, *M. truncatula*. Conversely, only genomic species 2 isolates could nodulate effectively and fix nitrogen with *M. polymorpha*, *S. meliloti* isolates formed ineffective rudimentary and white nodules on this plant. Similar inefficient nodules were also observed and described by Brockwell and Hely (2) and by Brunel et al. (3) with the same host plant species. In the present study we demonstrated that the formation of such nodules is triggered by isolates belonging to *S. meliloti*. Consequently, *S. meliloti* forms effective nodules with *M. truncatula* (3, 22), *M. sativa*, *M. minima*, *M. orbicularis*, and *M. rigidula* (3), whereas strains belonging to genomic species 2 form effective nodules with *M. polymorpha* in addition to the other *Medicago* species listed above.

It has been recognized previously that the phylogenetic relationship of the *nod* genes (*nodD1*, *nodD2*, *nodD3*, *nodA*, *nodB*) matches the host spectrum of rhizobia and reflects the taxonomic divisions within the Leguminosae (6, 31). On the basis of PCR-RFLP results, it was demonstrated that genomic species 2 and *S. meliloti* strains have different *nodD* gene pat-

TABLE 5. Phenotypic characteristics that distinguish *S. meliloti* and genomic species 2 isolates

Taxon	Utilization of the following compounds as sole carbon sources:		Growth in the presence of 3% (wt/vol) NaCl	Growth at pH 4	Intrinsic antibiotic resistance		Effective nitrogen fixation on <i>M. polymorpha</i>
	Sorbose and D-fucose	D-Xylose, salicin, and ribose			Neomycin	Chloramphenicol	
<i>S. meliloti</i> ^a	+	+	+	+	R	V ^c	-
Genomic species 2 ^d	-	-	v	v	S	R	+

^a *S. meliloti* RCR 2011 and sinorhizobia isolated from annual *Medicago* spp. (Table 1).^b +, positive; -, negative; v, variable; R, resistant; S, susceptible.^c The isolates obtained from annual *Medicago* spp. are resistant, and strain RCR 2011 is susceptible.^d See Table 1.

TABLE 6. Summary of data from this study and four recent studies that included extensive characterization of sinorhizobia obtained from annual *Medicago* species

Authors or study	Reference	No. of isolates used	Method used	Group or results	
				Genomic species 1	Genomic species 2
Eardley et al.	7	232	Isoenzymes	A1	B
Eardley et al.	8	2	Partial 16S rRNA sequencing	Sequence identical to <i>S. meliloti</i> sequence	New sequence (strain CC169)
Brunel et al.	3	43	PCR-RFLP: 16S rRNA + intergenic spacer	AI	AII
Rome et al.	22	73	PCR-RFLP: <i>nifD-K</i>	BI	BII
			PCR-RFLP: 16S rRNA + intergenic spacer	1	2
			PCR-RFLP: <i>nifD-K</i>	1	2
			PCR-RFLP: <i>nodD1</i> and <i>nodD2</i>	1	2
This study			DNA-DNA hybridization	Genomic species 1 (<i>S. meliloti</i>)	Genomic species 2
			Phenotypic tests	1	2
			Total 16S rRNA sequencing	Sequence identical to <i>S. meliloti</i> sequence	New sequence (strain A 321 ^T)

terns (22). In this study we found that genomic species 2 strains do not have the same host spectrum as *S. meliloti* strains. Consequently, it is possible that differences in *nodD* patterns may be related to an adaptation between genomic species 2 isolates and certain *Medicago* host plant species, such as *M. polymorpha*.

A summary of data from four recent studies, including the results of an extensive characterization of annual *Medicago* sinorhizobia, is shown in Table 6. Common reference strains were included in all of these studies, which allowed comparisons to be made. The large number of strains studied, together with the wide range of geographical origins, allowed us to conclude that *Sinorhizobium* strains that nodulate *Medicago* species can be separated into two genetic groups on the basis of isoenzyme patterns (7), chromosomal and plasmid typing data (22), DNA-DNA hybridization data (22), phenotypic test data, symbiotic properties, and 16S rRNA sequence data and that these groups correspond to two species. Genomic species 1 corresponds to *S. meliloti* since strain RCR 2011, which belongs to this group, exhibited 73% DNA-DNA homology with the *S. meliloti* type strain (strain ATCC 9930) (25). Moreover, strains RCR 2011 and ATCC 9930^T had identical PCR-RFLP 16S rDNA genotypes (18) and isoenzyme electrophoretic types (7). Since strain RCR 2011 is closely related to strain ATCC 9930^T, it is currently used as an *S. meliloti* reference strain (3, 5, 7, 18, 22, 25).

Genomic species 2, which was clearly identified by all of the approaches described above (Table 6), is a new *Sinorhizobium* species, for which we propose the name *Sinorhizobium medicae*.

Description of *Sinorhizobium medicae* sp. nov. *Sinorhizobium medicae* (me'di.cae. N.L. gen. N. *medicae*, from *medica*, the Latin name for plants belonging to the genus *Medicago*). Gram-negative, aerobic, non-spore-forming rods. Colonies on YEM agar are circular, mucous, and semitranslucent and spread over an entire plate within 3 to 5 days at 28°C. The mean generation times range from 3 to 5 h in YEM medium. The maximum temperature for growth is 40°C for most strains. Growth at 28°C on YEM agar is inhibited by 3% (wt/vol) NaCl and by pH values of less than 5.0. The strains are resistant to penicillin G, nalidixic acid, chloramphenicol, and streptomycin and are able to use 25 of 49 carbohydrates tested. Strains belonging to this species have been isolated from *M. truncatula* (22), *M. orbicularis* (7), *M. polymorpha* (3, 7), and *M. rugosa* (8) from different geographical sites (south of France [3, 22] and

eastern Mediterranean basin [7]) which are located in the center of origin of the genus *Medicago*. The strains are able to nodulate the *Medicago* species from which they were isolated. *S. medicae* can be distinguished from *S. meliloti* on the basis of its host spectrum since *S. medicae* strains fix nitrogen on *M. polymorpha*, whereas *S. meliloti* strains form ineffective nodules on *M. polymorpha*. *S. medicae* can be differentiated from the other members of the genus *Sinorhizobium* by phenotypic and genomic characteristics and particularly by its unique 16S rRNA sequence. The type strain of *S. medicae* is strain A 321, which has the characteristics given above for the species.

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REFERENCES

- Brenner, D. J., A. C. McWhorter, J. K. Leete Knutson, and A. G. Steigerwalt. 1982. *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. *J. Clin. Microbiol.* **15**:1133-1140.
- Brockwell, J., and F. W. Hely. 1966. Symbiotic characteristics of *Rhizobium meliloti*: an appraisal of systematic treatment of nodulation and nitrogen fixation interaction between hosts and rhizobia of diverse origins. *Aust. J. Agric. Res.* **17**:885-899.
- Brunel, B., S. Rome, R. Ziani, and J. C. Cleyet-Marel. 1996. Comparison of nucleotide diversity and symbiotic properties of *Rhizobium meliloti* populations from annual species of *Medicago*. *FEMS Microbiol. Ecol.* **19**:71-82.
- Chen, W. X., G. H. Yan, and J. L. Li. 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int. J. Syst. Bacteriol.* **38**:392-397.
- De Lajudie, P., A. Willems, B. Pot, D. Dewettinck, G. Maestrojuan, M. Neyra, M. D. Collins, B. Dreyfus, K. Kersters, and M. Gillis. 1994. Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int. J. Syst. Bacteriol.* **44**:715-733.
- Doherty, R. C., B. T. Breil, and E. W. Triplett. 1994. DNA sequence of the common nodulation genes of *Bradyrhizobium elkanii* and their phylogenetic relationships to those of other nodulating bacteria. *Mol. Plant-Microbe Interact.* **5**:564-572.
- Eardly, B. D., L. A. Materon, N. H. Smith, D. A. Johnson, M. D. Rumbaugh, and R. K. Selander. 1990. Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. *Appl. Environ. Microbiol.* **56**:187-194.
- Eardly, B. D., J. P. W. Young, and R. K. Selander. 1992. Phylogenetic position of *Rhizobium* sp. strain Or191, 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.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using

- the bootstrap. *Evolution* **39**:783–791.
10. **Fred, E. B., I. L. Baldwin, and E. McCoy.** 1932. Root nodule bacteria and leguminous plants. University of Wisconsin Press, Madison.
 11. **Gibson, A. H.** 1980. Methods for legumes in glasshouse and controlled environment cabinets, p. 139–184. *In* F. J. Bergersen (ed.), *Methods for evaluating biological nitrogen fixation*. John Wiley and Sons, New York.
 12. **Graham, P. H., M. J. Sadowsky, H. H. Keyser, Y. M. Barnett, R. S. Bradley, J. E. Cooper, D. J. De Ley, B. D. W. Jarvis, E. B. Roslycky, B. W. Strijdom, and J. P. W. Young.** 1991. Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *Int. J. Syst. Bacteriol.* **41**:582–587.
 13. **Higgins, D. G., and P. M. Sharp.** 1988. Clustal: a package for performing multiple alignment on a microcomputer. *Gene* **73**:237–244.
 14. **Hillis, D. M., and M. T. Dixon.** 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* **4**:411–453.
 15. **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.
 16. **Jordan, D. C.** 1984. *Rhizobiaceae* Conn 1938, 321^{AL}, p. 234–256. *In* N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.
 17. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**:111–120.
 18. **Laguerre, G., M. R. Allard, F. Revoy, and N. Amarger.** 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl. Environ. Microbiol.* **60**:56–63.
 19. **Lesins, K., and I. Lesins.** 1979. *Genus Medicago (Leguminosae)*. A taxogenic study. Junk, The Hague, The Netherlands.
 20. **Mullis, K. B., and F. A. Faloona.** 1987. Specific synthesis of DNA in vitro via a polymerase catalysed chain reaction. *Methods Enzymol.* **153**:335–350.
 21. **Provorov, N. A.** 1994. The interdependence between taxonomy of legumes and specificity of their interaction with rhizobia in relation to evolution of the symbiosis. *Symbiosis* **24**:183–200.
 22. **Rome, S., B. Brunel, M. P. Fernandez, P. Normand, and J. C. Cleyet-Marel.** 1996. Evidence of two genomic species of *Rhizobium* associated with *Medicago truncatula* revealed by PCR/RFLP and DNA/DNA hybridizations. *Arch. Microbiol.* **165**:285–288.
 23. **Saitou, N., and M. Nei.** 1987. A neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **44**:406–425.
 24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 25. **Scholla, M. H., J. A. Moorefield, and G. H. Elkan.** 1990. DNA homology between species of rhizobia. *Syst. Appl. Microbiol.* **13**:288–294.
 26. **Sneath, P. H. A., and R. R. Sokal.** 1973. *The principles and practice of numerical classification*, p. 223–234. W. H. Freeman and Sons, San Francisco.
 27. **Vincent, J. M.** 1970. A manual for the practical study of root nodule bacteria. IBP (Int. Biol. Programme) Handb. **15**:3–4.
 28. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**:697–703.
 29. **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.
 30. **Yanagi, M., and K. 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.
 31. **Young, J. P. W., and A. W. B. Johnston.** 1989. The evolution of specificity in the legume-*Rhizobium* symbiosis. *Trends Evol. Ecol.* **4**:341–348.