Rhizobium hainanense sp. nov., Isolated from Tropical Legumes

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A fast-growing rhizobial group isolated from leguminous plants in Hainan Province, a tropical region of China, is proposed as a new *Rhizobium* species on the basis of 16S rRNA gene sequencing, DNA-DNA hybridization, and phenotypic characterization. This new species belongs to the phylogenetic branch which includes *Rhizobium leguminosarum*. We propose the name *Rhizobium hainanense* sp. nov. for this species. The strain CCBAU 57015 (I66) is the type strain; it has been deposited in the culture collection of Beijing Agricultural University, People's Republic of China.

Diversity in rhizobia nodulating tropical legumes has been revealed by many studies, and these bacteria belong to several bacterial genera and species. Many slow-growing strains isolated from tropical legumes belong to the genus *Bradyrhizobium* (11, 15). A unique group of strains inducing stem and root nodules on *Sesbania rostrata*, a tropical legume of Africa, constitute the genus *Azorhizobium* (8). *Rhizobium tropici* (18) and *Rhizobium etli* (22) were proposed for the rhizobia isolated from the common bean (*Phaseolus vulgaris*) and *Leucaena leucocephala* in the tropical region. Recently, *Sinorhizobium saheli* and *Sinorhizobium teranga* were proposed for some isolates from *Acacia* and *Sesbania* (6). Some other groups nodulating plants of the genera *Acacia* and *Leucaena* were identified by numerical taxonomy (33) and genetic analysis (13). In our previous research, some rhizobia were isolated from nodules of various species of leguminous plants, including trees, herbs, and vines growing in Hainan province, a tropical region of China, and were characterized by using numerical taxonomy and DNA-DNA hybridization (11). All slow-growing rhizobia among them were classified as *Bradyrhizobium japonicum* strains, while the fast-growing rhizobia from Hainan were diverse both in phenotypic and genetic aspects. Some strains belonged to previously described *Rhizobium* species; the others formed unique subgroups. Among them subgroup IV was distinguished from all previously described *Rhizobium* species by numerical taxonomy and by an analysis of DNA hybridization data. The 13 strains in this subgroup were isolated from 12 leguminous species classified in nine different genera (11). A partial 16S rRNA gene sequence from strain I66, the reference strain of subgroup IV, showed a close relationship with those of *Rhizobium* species but could not be used to separate subgroup IV from *R. tropici* B, because the partial 16S rRNA gene sequence was identical to that of *R. tropici* B CIAT 899^T (25). In order to determine the exact taxonomic position of subgroup IV, some further studies were performed, including full 16S rRNA gene (ribosomal DNA [rDNA]) sequencing, analysis of symbiotic performance with hosts for this subgroup and with selected hosts according to the guidance in the "Proposed Minimal Standards for the Description of New Genera and Species of Root- and Stem-Nodulating Bacteria" (12), and additional research on DNA relatedness between this group and *R. tropici*, *R. etli*, and *Agrobacterium rhizogenes*. The results of these studies led to a proposal for a new species, *Rhizobium*

hainanense, for this fast-growing *Rhizobium* group, which we describe here.

MATERIALS AND METHODS

Bacterial strains. Strains used in this study are listed in Table 1. The *R. hainanense* (subgroup IV) strains are fast-growing rhizobia isolated from the Hainan Province of China (11). The yeast mannitol agar (YMA) (24) medium was used for strain maintenance, and tryptone-yeast extract medium (1) was used for cell production. All strains were incubated at 28°C.

DNA-DNA hybridization. Cells were grown and harvested by centrifugation. The pellets were washed twice with 10 mM Tris-HCl (pH 7.0). DNA was extracted by the method of Marmur (16). The initial-reassociation-rate method (7) was used for determining the percentage of DNA-DNA hybridization.

Amplification and cloning of 16S rDNA. The 16S rRNA genes were amplified in a 100- μ l reaction mixture by using universal forward primer P1 (5'-CGggatc cAGAGTTTGATCCTGGCTCAGAACGAACGCT-3'; corresponding to positions 8 to 37 in *Escherichia coli* rDNA [2]) and universal reverse primer P6 (5'-CGggatccTACGGTACCTTGTTCAGACTTACCCCC-3'; corresponding to positions 1479 to 1506). The lowercase letters in the primer indicate the restriction site of *Bam*HI. The protocol described by Chun and Goodfellow (5) and a PCR kit purchased from Promega Co. (Madison, Wis.) were used. Amplified 16S rDNA was purified from 0.8% low-melting-point agarose gel by using the method of Wieslander (27). Purified rDNA and plasmid pUC18 vector were cut with *Bam*HI and ligated at 16°C for 16 h. Ligated plasmids were transformed into *E. coli* DH5 α , and transformants were selected on the basis of the results of the blue-white screening procedure (21, 27).

DNA sequencing. Plasmids containing 16S rDNA were extracted and purified according to the methods of Tiesman and Rizzino (23). Purified plasmids were sequenced by using the fmol DNA sequencing system (Promega Co.) according to the manufacturer's instructions. Six primers for sequencing were selected based on the work of Yanagi and Yamasato (30) and Willems and Collins (28). They were P1 and P6 and an additional four primers, P2 through P5, with, respectively, the following sequences (*E. coli* numbering system): 5'-GCTAGT TGGTGGGGTAA-3' (positions 247 to 263), 5'-CGTGCCAGCAGCCGCGGT -3' (positions 514 to 531), 5'-TAGATACCCTGGTAGTCC-3' (positions 799 to 806), and 5'-CCGCAACGAGCGCAACCC-3' (positions 1097 to 1115).

Phylogenetic analysis. The generated rDNA sequences and the sequences for reference strains obtained from the EMBL, GenBank, and DDBJ data libraries were resampled 1,000 times for a bootstrap analysis (9) using the SEQBOOT program (PHYLIP, version 3.572c [10]). Deletions and insertions of more than one base length were counted as one change. Pairwise similarity values were calculated and converted to a distance matrix with the Jukes-Cantor coefficient in the DNADIST program. Phylogenetic trees were produced by the neighbor-joining method in the NEIGHBOR program, and a consensus tree was generated by the majority-rule and strict-consensus tree program (PHYLIP, version 3.572c).

Symbiotic performance test. Glass tubes (40 by 400 mm) half filled with vermiculite were used as seedling containers, and the inoculating methods described by Vincent (24) were used. The temperature in the greenhouse was kept at 28°C during the day and at 12°C during the night, with illumination of 10,000 to 20,000 lx for 14 h a day.

Nucleotide sequence accession number. The 16S rDNA sequence determined in this study has been deposited in the EMBL and GenBank nucleotide sequence databases under accession number U71078.

RESULTS AND DISCUSSION

Analysis of DNA hybridization. DNA-DNA hybridization is the standard arbiter for the designation of species, and the

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TABLE 1. List of bacterial strains examined

Strain	Host	Origin	Source ^a
<i>M. loti</i> NZP 2213 ^T	<i>L. corniculatus</i>	New Zealand	NZP
<i>S. meliloti</i> USDA 1002 ^T	<i>M. sativa</i>	United States	USDA
<i>R. leguminosarum</i> USDA 2370 ^T		United States	USDA
<i>R. tropici</i> B BR 853	<i>L. leucocephala</i>		CNPS
<i>R. tropici</i> B BR 847	<i>L. leucocephala</i>		CNPS
<i>R. tropici</i> B CIAT 899 ^T	<i>P. vulgaris</i>		CNPBS
<i>R. tropici</i> A CFN 299	<i>P. vulgaris</i>		CNPBS
<i>R. tropici</i> A C-05-I	<i>P. vulgaris</i>		CNPBS
<i>R. etli</i> CFN 42 ^T	<i>P. vulgaris</i>		CNPBS
<i>R. galegae</i> HAMBI 540 ^T	<i>Galega orientalis</i>	Finland	HAMBI
<i>M. huakuii</i> CCBAU 2609 ^T	<i>A. sinicus</i>	Nanjing, China	CCBAU
<i>S. fredii</i> USDA 205 ^T	<i>Glycine soja</i>	Henan, China	USDA
<i>M. tianshanense</i> A-1BS ^T	<i>Glycyrrhiza pallidiflora</i>	Xinjiang, China	CCBAU
<i>B. japonicum</i> USDA 6 ^T	<i>G. max</i>	United States	USDA
<i>R. hainanense</i> I66 ^T (CCBAU 57015)	<i>D. sinuatum</i>	Hainan, China	CCBAU
<i>A. rhizogenes</i> IAM 13570 ^T		Japan	IAM

^a NZP, Division of Scientific and Industrial Research, Palmerston North, New Zealand; USDA, Beltsville *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, Md.; CNPBS, Centro Nacional de Pesquisa em Biologia do solo, Iseropedica 23851, Rio de Janeiro, Brazil; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan.

criterion for a species is $\geq 70\%$ DNA-DNA relatedness (26). The values of DNA-DNA relatedness within subgroup IV were found to be $>70\%$ and those between strain I66 and type strains of other rhizobial species were found to be very low in our previous studies (11). Since the same phylogenetic branch contained *R. tropici* A, *R. tropici* B, *R. etli*, and *A. rhizogenes*, we studied DNA-DNA relatedness between strain I66^T and type strains for these three groups. The results show that the DNA-DNA relatedness between strain I66^T and *R. tropici* A CFN 299, *R. tropici* B CIAT 899^T, *R. etli* CFN 42^T, and *A. rhizogenes* IAM 13570^T were 23.4, 24.8, 19.2, and 12.7%, respectively. This showed that subgroup IV was distinct from *R. tropici* A, *R. tropici* B, *R. etli*, and *A. rhizogenes*.

16S rRNA gene sequence analysis. The variable fragment of 16S rDNA amplified by using primers Y1 and Y2 (32) on DNA from strain I66 was sequenced in our previous research (25). The sequence of that fragment in the full sequence obtained in this research is identical with the partial sequencing result (25). An unrooted phylogenetic tree (Fig. 1) was generated by the neighbor-joining method (10) based on a comparison of the 16S rRNA gene sequences of all available rhizobial species and related bacteria. The rhizobial species formed six clusters (Fig. 1), which were similar to those reported by previous workers (17, 19, 20, 31). These included *Sinorhizobium*, *Mesorhizobium* (14), *Rhizobium galegae*-*Agrobacterium tumefaciens* and *Agrobacterium vitis*, *Rhizobium*-*A. rhizogenes*, *Bradyrhizobium*, and *Azorhizobium*. The phylogenetic relatedness in each cluster and among the clusters had been discussed elsewhere (6, 8, 17, 19, 20, 29, 31). Strain I66^T falls in the *Rhizobium*-*A. rhizogenes* cluster (Fig. 1), which consisted of *R. etli*, *R. leguminosarum*, *R. tropici* A, *R. tropici* B, and *A. rhizogenes*, and was most closely related to *R. leguminosarum* (the similarity value was 96.9%). This clearly showed that subgroup IV belonged phylogenetically to the genus *Rhizobium*. Although the phylogenetic position of subgroup IV was not found to be distinct from that of *R. tropici* B by analysis of the partial 16S rRNA sequences (25),

it was distinguished clearly from the *R. tropici* B position by the full sequence of its 16S rRNA genes. Similar situations occurred with *R. galegae* (29, 30). So it seems that the partial sequencing of 16S rDNA has limited value in determining phylogenetic relationships among rhizobial species.

Diagnostic tests. We selected the differential features between subgroup IV and other species from the same phylogenetic branch from published data and confirmed them by testing the type strains. The results are shown in Table 2. These results show that phenotypic features can be used to distinguish subgroup IV from phylogenetically related bacteria.

FIG. 1. Unrooted phylogenetic tree, showing the position of *R. hainanense* within the genus *Rhizobium*. Abbreviations: My, *Mycoplana*; S, *Sinorhizobium*; Och, *Ochrobactrum*; A, *Agrobacterium*; Az, *Azorhizobium*; Ph, *Phyllobacterium*; B, *Bradyrhizobium*; M, *Mesorhizobium*. The accession numbers of the 16S rRNA gene sequences are as follows: *Agrobacterium rubi* LMG 156, X67228; *A. tumefaciens* LMG 196, X67223; *A. rhizogenes* LMG 152, X67224; *A. vitis* LMG 8750, X67225; *S. fredii* LMG 6217, X67231; *S. meliloti* LMG 6133, X67222; *Sinorhizobium xinjiangensis* IAM 14142, D12796; *S. saheli* LMG 7837, X68390; *S. teranga* LMG 6463, X68387; *R. galegae* LMG 6215, X67226; *R. leguminosarum* LMG 8820, X67227; *M. loti* LMG 6125, X67229; *R. tropici* B LMG 9518, X67234; *R. tropici* A LMG 9517, X67233; *R. etli* ATCC 14483, U47303; *M. huakuii* IAM 14158, D12791; *Mesorhizobium ciceri* UPM-Ca7, U07934; *Mesorhizobium mediterraneum* UPM-Ca36, L38825; *B. japonicum* LMG 6138, X66024; *Phyllobacterium myrsinacearum* IAM 13584, D12789; *Phyllobacterium rubiacearum* IAM 13587, D12790; *Mycoplana dimorpha* IAM 13154, D12786; *Mycoplana bullata* IAM 13153, D12785; *Ochrobactrum anthropi* IAM 14119, D12794; *Azorhizobium caulinodans* LMG 6465, X67221; *Bradyrhizobium elkanii* ATCC 49852, U35000; *M. tianshanense* A-1BS, U71079; *R. hainanense* I66^T, U71078. In the above strain designations, the following abbreviations are used: LMG, Culture Collection Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; ATCC, American Type Culture Collection, Rockville, Md. D, DDBJ accession numbers.

TABLE 2. Distinctive features of *R. hainanense* and related species in the genus *Rhizobium*

Characteristic	Ability of indicated strain to grow ^a				
	<i>R. leguminosarum</i> USDA 2370 ^T	<i>R. etli</i> CFN 42 ^T	<i>R. tropici</i> A CFN 299	<i>R. tropici</i> B CIAT 899 ^T	<i>R. hainanense</i> I66 ^T
Utilization of sole carbon sources					
α-Alanine	+	+	+	—	—
L-Aspartate	+	—	+	+	+
Glucosamine-HCl	—	+	+	+	+
DL-Cystine	—	—	—	+	+
Erythritol	+	—	—	+	+
α-Ketoglutarate	—	—	—	+	+
L-Leucine	—	—	—	—	+
L-Lysine	—	—	—	+	—
L-Ornithine HCl	—	+	+	—	+
L-Phenylalanine	—	—	—	+	—
L-Tryptophan	—	—	—	+	—
Growth in 2% NaCl	—	—	—	+	+
Growth at 40°C	—	—	—	+	+
Growth at pH 5	—	—	+	+	+
Growth at pH 10	—	—	+	+	+
Resistance to antibiotics					
Terramycin (50 µg/ml)	—	—	—	+	—
Kanamycin (5 µg/ml)	—	—	—	—	+
Chloramphenicol (100 µg/ml)	—	—	—	+	—

^a +, strain can grow; —, strain cannot grow.

Symbiotic performance. In accordance with the suggestion of Graham et al. (12), the nodule-forming abilities of strain I66^T were checked on selected leguminous species, including *Astragalus sinicus* and *Glycyrrhiza uralensis*, which were representative hosts for *Mesorhizobium huakuii* (4, 14) and *Mesorhizobium tianshanense* (3, 14), respectively. Hosts of all 13 strains in subgroup IV were also tested. Nodulating abilities of type cultures for other rhizobial species on *Desmodium sinuatum*, the original host of strain I66^T, were also checked. Results showed that strain I66^T could only nodulate on its original host, *D. sinuatum*, and not on the hosts of other members of subgroup IV, e.g., *Stylosanthes guyanensis*, *Centrosema pubescens*, *Desmodium triquetrum*, *Desmodium gyroides*, *Desmodium heterophyllum*, *Tephrosia candida*, *Acacia sinuata*, *Zornia diphylla*, and *Macroptilium lathyroides*. This meant that the hosts of members of subgroup IV were not in a single cross-inoculation group. Maybe they each have a different host range. Strain I66^T could not nodulate *Medicago sativa*, *Pium sativum*, *P. vulgaris*, *Trifolium repens*, *Lotus corniculatus*, *Glycine max*, *L. leucocephala*, *Macroptilium atropurpureum*, *Galega officinalis*, *A. sinicus*, or *G. uralensis*, but it could nodulate *Vigna unguiculata*, which was a universal host for many different rhizobia. The type cultures of strains *R. leguminosarum* USDA 2370, *Sinorhizobium meliloti* USDA 1002, *Mesorhizobium loti* NZP 2213, *R. tropici* CIAT 899, *M. huakuii* CCBAU 2609, *Sinorhizobium fredii* USDA 205, and *B. japonicum* USDA 6 could not nodulate *D. sinuatum*. Based on the above studies and on previous numerical taxonomy and DNA-DNA hybridization (11), we propose that subgroup IV of Hainan fast-growing rhizobia is a new species, *R. hainanense*.

Description of *R. hainanense* sp. nov. *Rhizobium hainanense* (hai.nan.ense¹ M.L. adj. *Hainan*, name of a province of China; rhizobia isolated from Hainan Province). Aerobic, gram-negative, nonsporeforming rods that are 0.25 to 0.5 by 0.5 to 2.0 µm. Motile by means of a single polar flagellum. Optimum temperature 25 to 30°C and can grow at 40°C. Optimum pH 6 to 8 and can grow at pH 5 to 10. Generation time is 2 to 4 h in YMA medium. Colonies in YMA are circular, opaque, and

convex and have a creamy color. They are usually 1.5 to 2.5 mm in diameter after 3 days of incubation on YMA and produce acid. Arabinose, erythritol, fructose, glucose, inositol, lactose, maltose, melibiose, raffinose, rhamnose, D-ribose, sodium citrate, sodium succinate, sucrose, trehalose, turanose, xylose, DL-asparagine, and L-histidine can be used by these bacteria as the sole carbon and energy source for growth. Calcium malonate, ferulic acid, melezitose, sodium acetate, sodium hippurate, galactose, sorbose, vanillic acid, β-alanine, and glycine cannot be used. L-Alanine, DL-arginine, L-asparagine, DL-citrulline, DL-cystine, D-glutamic acid, L-glutamic acid, glycine, L-histidine, L-leucine, DL-ornithine, L-proline, L-serine, L-threonine, L-tryptophan, and L-valine can be used as the sole nitrogen source, but D-valine cannot be used. The other characteristics are as indicated in Table 2. Strains in this species were isolated from nodules of *S. guyanensis*, *C. pubescens*, *D. triquetrum*, *D. gyroides*, *D. sinuatum*, *D. heterophyllum*, *T. candida*, *A. sinuata*, *Arachis hypogaea*, *Z. diphylla*, *Uraia crinita*, and *M. lathyroides*. The G+C moles percent content of the DNA is 59 to 63. The type strain is I66 (CCBAU 57015). Its generation time is 2 h on YMB medium (24). The G+C moles percent content of its DNA is 61 (*T_m*).

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