Phylogenetic and Genetic Relationships of *Mesorhizobium tianshanense* and Related Rhizobia

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The genetic and phylogenetic relationships for strains of *Mesorhizobium tianshanense* and its relatives were compared by an analysis of the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins, DNA-DNA hybridization, and full 16S rRNA gene sequencing. The strains of *M. tianshanense* formed a cluster which was distinct from those of other rhizobium species in the clustering analysis of SDS-PAGE. DNA-DNA relatedness between A-1BS (type strain of *M. tianshanense*) and the type or reference strains for *Mesorhizobium loti*, *M. huakuii*, *M. ciceri*, *M. mediterraneum*, and cluster U, an unnamed rhizobial group, ranged from 4.4 to 43.8%. The phylogenetic analysis based on the 16S rRNA gene sequences showed that *M. tianshanense* was closely related to the *Mesorhizobium* phylogenetic branch and could be distinguished from the other four species in this branch. These results further confirmed that these bacteria constitute a distinct rhizobial species.

The genetic approaches now widely applied to the taxonomy of root nodule bacteria have opened the possibility to infer their phylogenies and to correctly define the species and genera of these bacteria. The improved methods for identifying bacteria and a growing interest in characterization of new rhizobial isolates have brought about many changes in the taxonomy of rhizobia since 1984; a revised taxonomic system for these bacteria was proposed in “Bergey’s Manual of Systematic Bacteriology” (16). Several reviews on the development of rhizobial taxonomy and phylogeny have been published recently (20, 33). The main developments include the descriptions of the genera *Azorhizobium* (8), *Sinorhizobium* (4, 6), and *Mesorhizobium* (14, 18), as well as many new species. Up to now, six distinct phylogenetic branches, *Azorhizobium*, *Bradyrhizobium*, the *Rhizobium-Agrobacterium rhizogenes* branch, *Mesorhizobium* (14), the *Rhizobium galegae-Agrobacterium* branch, and *Sinorhizobium*, have been identified, and all of them were located in the alpha subclass of Proteobacteria (6, 31–33). *Mesorhizobium* has been proposed recently by Jarvis et al. (14), and five species, *M. loti* (15), *M. huakuii* (3), *M. ciceri* (23), *M. mediterraneum* (22), and *M. tianshanense* (2) were included on the basis of the data from full sequences of 16S rRNA genes. A group of rhizobia named cluster U has been classified in this genus by 16S ribosomal RNA (rDNA) sequencing (6). Some isolates from nodules of *Amorpha fruticosa* also belong to it, as indicated by the PCR-based restriction fragment length polymorphism patterns of their 16S rDNA (unpublished data). *M. tianshanense* was described in our previous paper dealing with a group of rhizobia isolated from saline and arid soils in the Xinjiang region of China (2). Some of the strains in this species grow as slowly as *Bradyrhizobium* spp. Other strains grow faster than *Bradyrhizobium* but slower than *Rhizobium leguminosarum*. According to the data from the partial 16S rDNA sequence, this species belongs to the *M. loti*-*M. huakuii* branch (2). But its exact taxonomic and phylogenetic positions were controversial since the partial sequence of *M. tianshanense* A-1BS (18) and since the DNA-DNA relatedness between this species and some newly emerged species had not been determined. Also, the phylogenetic relationships based on the partial and the full sequences (31, 32, 34) may not be the same for some species, such as for *Rhizobium galegae*.

Currently, polyphasic taxonomy, including phenotypic and genotypic characterizations, is emphasized for classification of rhizobia (12). In order to confirm the taxonomic and phylogenetic positions of *M. tianshanense*, we performed an analysis of the full-length sequence of the 16S rRNA gene for A-1BS, the type strain of *M. tianshanense*. The DNA-DNA hybridization and whole-cell protein electrophoresis tests were also done for other *M. tianshanense* strains and some reference strains. The electrophoretic patterns of proteins have been widely used in bacterial classification, and different techniques have been developed (17, 21, 25). It has been proved that protein profiles can provide a level of discrimination similar to or slightly higher than that of DNA-DNA hybridization studies.

**MATERIALS AND METHODS**

**Bacterial strains.** The tested strains are listed in Table 1. All strains of *M. tianshanense* were characterized in our previous research (2) and maintained in 20% (vol/vol) glycerol-water solution at −20°C. The bacteria were grown on yeast mannitol agar (28) medium at 28°C.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins.** Bacterial strains were grown at 28°C for 2 days in flasks on tryptone-yeast extract medium, which contained (per liter) 5 g of tryptone (Oxoid), 3 g of yeast extract (Oxoid), 0.7 g of CaCl2·2H2O (pH 6.8–7.0). Then each culture was centrifuged at 12,000 × g for 3 min. The pellet was washed once in 10 mM Tris-HCl, pH 7.6, and the cells were suspended in 0.5 ml of 10 mM Tris-HCl, pH 7.6 (21). The concentration of protein was adjusted to achieve an optical density at 280 nm of 1.0 in an ultraviolet spectrophotometer. After ultrasonic disruption on ice for 30 s with a microtip probe at 40 W, 2× treatment buffer (0.5 g of SDS, 3 ml of glycerol, 1 ml of 2-mercaptoethanol, 4 ml of bromophenol blue, 2 ml of 1 M Tris-hydrochloride, and distilled water for a final volume of 10 ml at pH 6.8) was added. The samples were stored at −20°C for at least 1 week before being analyzed by electrophoresis. The SDS-polyacrylamide gel (200 by 200 mm and 1 mm thick) and a shark’s tooth comb were used for electrophoresis. The samples were incubated at 100°C for 2 min before the gel was run. Twenty-five samples per gel were subjected to discontinuous slab gel electrophoresis in an SDS-Tris-glycine buffer system, as described by Laemmli (17). The protein patterns were visualized by silver staining (25). The bands of protein for each strain were scanned with a Densitometer Extra-Scanner (LKB...
BarnHI was purified from 0.8% low-melting-point agarose gel by using the method of blue-white screening procedure (24).

Coli Wieslander (30). Purified rDNA and plasmid pUC18 vector were cut with bond-N+; Amersham). The protocols specified by the manufacturer were used.

B. japonicum SEMIA 5061
B. japonicum SEMIA 5079
M. tianshanense strains
A-1BS
A-6
032B
060A
016Bm
91x10
005B
098B
91x101
91x07
91x72
91x11
91x09
91x13
Glycyrrhiza pallidiflora
Glycyrrhiza uralensis
Caragana plorensis
Swainsomondro holodendron
Halimodendron holodendron
Glycine max
Glycine max
Glycine max
Caragana plorensis
Sophora alopecuroides
Sophora alopecuroides
Sophora alopecuroides
Sophora alopecuroides
Sophora alopecuroides
Glycyrrhiza sp.

A photograph of the gel was taken after staining with ethidium bromide, and then the DNAs were Southern blotted onto nitrocellulose (Hybond-N+; Amersham). The protocols specified by the manufacturer were used. Amplified 16s rDNA was used. A photograph of the gel was taken after staining with ethidium bromide, and then the DNAs were Southern blotted onto nitrocellulose (Hybond-N+; Amersham). The protocols specified by the manufacturer were used. Amplified 16s rDNA was used.

A-1BS by following the instructions of the manufacturer. After exposure of an X-ray film, the nitrocellulose with DNAs was cut into strips according to the lanes on the film. Then the strips were placed in 19-ml scintillation vials, and liquid scintillation counting was performed. The DNA-DNA homology was expressed as the percentage calculated by dividing the count for each lane by the count for the homology lane and multiplying by 100.

Co.) and a clustering analysis was performed by using the Sj coefficient and the unweighted-average linkage method (26).

DNA-DNA hybridization. The DNAs were extracted and purified according to the methods of Tiesman and Rizzino (27). 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 Yamagi and Yamamoto (32) and Willems and Collins (31).

Amplification and cloning of 16s rRNA gene. The total DNAs were digested with EcoRI restriction endonuclease and were subjected to electrophoresis in 1% agarose gels (11). For each lane, 3 μg of DNA was used. A photograph of the gel was taken after staining with ethidium bromide, and then the DNAs were Southern blotted onto nitrocellulose (Hybond-N+; Amersham). The protocols specified by the manufacturer were used for Southern blotting, fixation by UV cross-linking, and hybridization at 65°C. The random primer labeling kit (Rediprime; Amersham Life Science) and PFG/CTP were used for labeling the total DNA probe from M. tianshanense A-1BS1 by following the instructions of the manufacturer. After exposure of an X-ray film, the nitrocellulose with DNAs was cut into strips according to the lanes on the film. Then the strips were placed in 19-ml scintillation vials, and liquid scintillation counting was performed. The DNA-DNA homology was expressed as the percentage calculated by dividing the count for each lane by the count for the homology lane and multiplying by 100.

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DNA-DNA hybridization. The DNA-DNA hybridization is an important criterion for definition of bacterial species (29), and different methods have been recommended for determining these values among rhizobia (12). Usually, the DNA-DNA relatedness among distinct species should be lower than 70%, and the conservation in the ribosomal gene sequences should be lower than 95% between distinct genera, among other criteria (20). In our previous research, the spectrophotometer method described by De Ley et al. (7) was used for measuring the DNA-DNA hybridizations among the strains of *M. tianshanense* and among other described rhizobial species (2). The *M. tianshanense* strains formed a homologous-DNA group at the species level (DNA-DNA relatedness higher than 70%). The DNA-DNA relatedness between A-lBST and other species in the family *Rhizobiaceae* and related bacteria was calculated. The similarity values are shown in Table 2.

On the basis of the K$_{auc}$ values, an unrooted phylogenetic tree (Fig. 2) was generated by the DRAWTREE program. The relationships among the described species are similar to those described in references 6 and 31 to 33. The *Bradyrhizobium* species formed one branch; *Azorhizobium* occupied the second branch; the species of *Sinoirhizobium* constituted the third branch; the fourth branch included *Rhizobium* species and *Agrobacterium* rhizogenes; *R. galegae* and *Agrobacterium* species formed the fifth branch; and the *Mesorhizobium* branch is the sixth one. *M. tianshanense*, represented by A-lBST, fell in the *Mesorhizobium* branch. The similarities between *M. tianshanense* and *M. loti*, *M. huakuii*, *M. ciceri*, *M. mediterraneum* and cluster U are 97.4, 97.9, 96.4, 96.4, and 97.4%, respectively. Based on these data, it is clear that *M. tianshanense* is a member of the *Mesorhizobium* branch and is distinct from all of the related species. In this study, it was found that 3.6% of the base pairs of the full sequences of 16S rDNAs for *M. tianshanense* and *M. ciceri* are different (Table 2), although the two species have identical sequences for a 260-bp fragment between primers Y1 and Y2 (14, 18). A similar situation exists for *R. galegae* and *Sinoirhizobium* *frendii-Sinoirhizobium* *meilii*. Within a 260-bp fragment, *R. galegae* is only 1.2% (3 bp) different from *M. loti* (31). But the difference is 6.3% (about 90 bp) when the full-length sequences are compared (32, 34). Eardley and Biever (9) reported that cluster analysis of polyorphic nucleotide sequence positions in full and partial segments of the 16S rRNA genes of *R. galegae*, *M. loti*, and *Agrobacterium* *tumefaciens* revealed marked disagreement in phylogenetic tree topology depending on the portions of the genes included in the analysis. In another case, phylogenetically related rhizobial strains, such as *Sinoirhizobium* strains, were found to have a large number of similar-but-distinct 16S rDNA sequences (13). Also, some rhizobial strains, such as the type strain for *Sinoirhizobium* *saheli*, have two different sequences for 16S genes (13). In this case, the importance of DNA-DNA hybridization experiments in drawing the species boundaries was emphasized by Haukka et al. (13). Up to now, five species have been distinguished in the *Mesorhizobium* branch.
<table>
<thead>
<tr>
<th>No. and Name</th>
<th>Similarity to bacterial species (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>100% B. elkanii saheli</td>
</tr>
<tr>
<td>2</td>
<td>96.5% B. denitrificans</td>
</tr>
<tr>
<td>3</td>
<td>94.9% M. tianshanense huakuii</td>
</tr>
<tr>
<td>4</td>
<td>94.6% A. turnefaciens</td>
</tr>
<tr>
<td>5</td>
<td>94.4% S. vitis</td>
</tr>
<tr>
<td>6</td>
<td>94.3% A. rhizogenes</td>
</tr>
<tr>
<td>7</td>
<td>93.8% S. fredii</td>
</tr>
<tr>
<td>8</td>
<td>93.7% M. galegae</td>
</tr>
<tr>
<td>9</td>
<td>93.7% R. tropici</td>
</tr>
<tr>
<td>10</td>
<td>93.7% R. leguminosam</td>
</tr>
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</table>
The results in this paper and those in our previous paper (2) clearly proved that M. tianshanense is a distinct species in the Mesorhizobium branch.

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REFERENCES


12. Gillis, M. Personal communication.


