NOTE

Identification of isolates from soybean nodules in Xinjiang Region as *Sinorhizobium xinjiangense* and genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*

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Eight fast-growing rhizobial isolates from Xinjiang soils were identified as *Sinorhizobium xinjiangense* by analyses of 16S rRNA gene sequences, SDS-PAGE of proteins, intergenic spacer sequences and DNA–DNA hybridization. Based on all of the results, these isolates and the reference strains for *S. xinjiangense* were a distinct genomic species, although the 16S rRNA genes were closely related to that of *Sinorhizobium fredii*.

Keywords: phylogeny, DNA–DNA relatedness, *Sinorhizobium xinjiangense*, soybean, Xinjiang region

Soybean (*Glycine max*) is an important legume crop cultivated worldwide. Nodulation of this plant is well known and different rhizobia, including *Bradyrhizobium japonicum* (Jordan, 1982), *Bradyrhizobium elkanii* (Kuykendall *et al.*, 1992), *Bradyrhizobium liaoningense* (Xu *et al.*, 1995), *Mesorhizobium tianshanense* (Chen *et al.*, 1995), *Sinorhizobium fredii* (Chen *et al.*, 1988; Scholla & Elkan, 1984) and *Sinorhizobium xinjiangense* (Chen *et al.*, 1988) have been isolated from soybean nodules in different geographic regions. Among these species, *S. fredii* and *S. xinjiangense* are fast-growing, acid-producing rhizobia isolated from Chinese soils. These two species were distinct phena in numerical taxonomy (Chen *et al.*, 1988) and they have identical full-length 16S rDNA sequences (de Lajudie *et al.*, 1994; Yanagi & Yamasato, 1993). Since there was no genetic evidence to differentiate these two species, the taxonomic position of *S. xinjiangense* has been questioned (de Lajudie *et al.*, 1994; Jarvis *et al.*, 1992). To answer this question, new *S. xinjiangense* isolates were identified and compared with *S. fredii* by genetic and phylogenetic methods.

Abbreviation: IGS region; intergenic spacer region.

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The GenBank accession numbers for the 16S rRNA gene sequences of strains CCBAU 110^T, CCBAU 83804, CCBAU 83844 and Rx22 are AF250354, AF250355, AF250377 and AF250353, respectively. The GenBank accession numbers for the IGS sequences of strains CCBAU 110^T, USDA 205^T, USDA 1037 and LMG 7834 are AF284450, AF284451, AF325824 and AF325825, respectively.

Soils cultivated with soybean in Shihezi, Dongwan and Wulanwusu, three different geographic areas in the Xinjiang Region (China), were used to inoculate soybean seedlings grown in Leonard jars (Leonard, 1943). Root nodules were collected from plants after 30 d growth in a greenhouse and isolated by a routine method (Vincent, 1970). The symbiotic ability of each isolate was verified by a nodulation test (Vincent, 1970) and acetylene reduction. Eight fast-growing isolates were further characterized, together with reference strains (Table 1).

The reference strains and isolates USDA 205^T, CCBAU 110^T, CCBAU 83804, CCBAU 83844 and Rx22 were chosen for sequencing. The PCR-amplified 16S rRNA genes were sequenced directly using the universal primers 25f [5'-AAC T(G/T)A AGA GTT TGA TCC TGG CTC-3'] and 1492r [5'-TAC GG(C/T) TAC CTT GTT ACG ACT T-3'] according to the procedures of Hurek *et al.* (1997). The obtained sequences and related sequences obtained from GenBank were aligned using the program of CLUSTAL W (version 1.81). Distances between each pair of the aligned sequences were calculated using the method of Jukes & Cantor (1969). Tree topology was inferred by the neighbour-joining method (Saitou & Nei, 1987) and the phylogenetic tree was reconstructed using the TREECON software package (Van de Peer & De Wachter, 1994). The sequence obtained for USDA 205^T (not shown) was identical with sequence X67231, reported in GenBank. In comparative analysis, more than 99% similarity was observed among the 16S

Fig. 1. Phylogenetic tree of 16S rRNA gene sequences showing phylogenetic relationships among the isolates, *Sinorhizobium* spp. and other rhizobia. Bootstrap values of more than 50% are given at the branching points (100 replications). GenBank accession numbers are shown in parentheses. Bar, 5% nucleotide difference. Sequences were aligned using the RDP program (Maidak *et al.*, 1999). The distances of aligned sequences were calculated by the method of Jukes & Cantor (1969). Tree topology was inferred by the neighbour-joining method (Saitou & Nei, 1987) and the phylogenetic tree was reconstructed using the TREECON software package (Van de Peer & De Wachter, 1994).

rDNA sequences from the isolates, *S. xinjiangense* and *S. fredii*. They formed a subcluster within the *Sinorhizobium* group in the phylogenetic tree reconstructed using the obtained sequences (Fig. 1). Relationships

among the defined rhizobial genera and species in this tree were similar to those in other reported trees (de Lajudie *et al.*, 1994; Nick *et al.*, 1999).

Whole-cell proteins from eight isolates were compared

Table 1. Isolates and reference strains used in this study

USDA, Beltsville *Rhizobium* Culture Collection, Beltsville Agricultural Research Centre, Beltsville, MD, USA; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China.

Isolate or strain	Host	Geographical origin*
Isolate (CCBAU number):		
83801, 83804	<i>Glycine max</i>	Dongwan
83835, 83808, 83803, 83833	<i>Glycine max</i>	Wulanwusu
83844, 83816	<i>Glycine max</i>	Shihezi
<i>Sinorhizobium xinjiangense</i> CCBAU 110 ^T , CCBAU 108, Rx22	<i>Glycine max</i>	Xinjiang
<i>Sinorhizobium meliloti</i> USDA 1002 ^T	<i>Medicago sativa</i>	USDA
<i>Sinorhizobium terangae</i> USDA 4101 ^T	<i>Acacia laeta</i>	Senegal
<i>Sinorhizobium saheli</i> USDA 4102 ^T	<i>Sesbania cannabine</i>	USDA
<i>Sinorhizobium medicae</i> USDA 1037 ^T	<i>Medicago</i> spp.	USDA
<i>Sinorhizobium fredii</i> 2048	<i>Glycine soja</i>	Liaoning
<i>Sinorhizobium fredii</i> USDA 205 ^T , USDA 194	<i>Glycine soja</i>	Henan

* Xinjiang, Henan and Liaoning are provinces of China; Dongwan, Wulanwusu and Shihezi are cities or counties in Xinjiang.

Fig. 2. Cluster results of SDS-PAGE of whole-cell protein patterns of the isolates and reference strains for *Sinorhizobium* species using the unweighted pair group method with arithmetic means (3.0% tolerance). Similarities (%) between the various branches are given at the branching points.

5 ml VM ethanol medium (Reinhold-Hurek *et al.*, 1993a, b) at 28 °C for 48 h. The precultures were centrifuged at 5000 *g* for 10 min at room temperature and suspended in 6 ml sterile PBS buffer (NaCl, 8 g; KCl, 0.2 g; Na₂HPO₄·2H₂O, 2.9 g; KH₂PO₄, 0.2 g; distilled water 1 l; pH 7.2). Petri dishes (diameter 14 cm) containing VM medium were inoculated with the suspended solution and incubated at 28 °C for 48 h. Harvesting of the cells, preparation of whole-cell proteins and SDS-PAGE were performed as described by Kiredjian *et al.* (1986). Normalization of the protein profiles and data analysis were performed using the Universal Software 1D Advanced from AABI Fullerton California. Tolerance, defined as the percentage of band distance that another band must be to be called the same band, is 3.0%. The formula $S = 100 \times 2c / (a + b)$ was used to calculate the similarity between two strains (samples), where *S* is percentage similarity, *c* is the number of bands shared by both samples, *a* and *b* are the band numbers recognized in each of the two samples. The unweighted pair group method with arithmetic means was used for clustering. A similarity of 95.4% was obtained between two independent protein extractions of strain CCBAU 110^T that were used to check the reproducibility. Identical or very similar protein patterns were observed among the isolates and the *S. xinjiangense* type strain CCBAU 110^T and they formed a distinctive group at a similarity level of 83.0% (Fig. 2). Two strains of *S. fredii* formed another group at a similarity level of 85.5% and reference strains for *Sinorhizobium meliloti*, *Sinorhizobium saheli*, *Sinorhizobium terangae* and *Sinorhizobium medicae* were single branches.

DNAs extracted by the method of Marmur (1961) were used for determination of DNA base composition by the spectrophotometric method (De Ley *et al.*,

with those from the reference strains for *Sinorhizobium* species by SDS-PAGE. Strains were precultured in

Table 2. DNA base composition (T_m method) and DNA–DNA relatedness (%) among the reference strains and other isolates or strains

DNA–DNA relatedness was determined by the spectrophotometric method (De Ley *et al.*, 1970). The values were means from two independent tests.

Isolate or strain	G + C (mol %)	Similarity (%) with:	
		CCBAU 110 ^T	USDA 205 ^T
CCBAU 83801	60.7	74.2	36.7
CCBAU 83803	60.1	88.8	27.5
CCBAU 83804	60.8	80.1	38.4
CCBAU 83808	60.5	89.7	25.2
CCBAU 83816	60.8	89.2	20.8
CCBAU 83833	60.2	82.1	25.4
CCBAU 83835	60.9	77.8	27.0
CCBAU 83844	60.3	85.4	31.0
<i>S. xinjiangense</i> CCBAU 110 ^T	60.5	100.0	39.0
<i>S. xinjiangense</i> Rx22	60.6	80.7	32.4
<i>S. xinjiangense</i> CCBAU 108	60.9	85.5	22.2
<i>S. fredii</i> USDA 205 ^T		39.0	
<i>S. fredii</i> USDA 194		31.5	
<i>S. fredii</i> 2048		28.7	
<i>S. meliloti</i> USDA 1002 ^T		21.2	
<i>S. medicae</i> USDA 1037 ^T		27.7	
<i>S. teranga</i> USDA 4101 ^T		28.6	
<i>S. saheli</i> USDA 4102 ^T		21.0	
<i>S. kostiense</i> HAMBI 1489 ^T		3.0	
<i>S. arboris</i> HAMBI 1552 ^T		0.0	

1970; Marmur, 1961) using DNA from *Escherichia coli* K-12 as the standard. The levels of DNA relatedness were determined by the initial renaturation rate method (De Ley *et al.*, 1970; de Lajudie *et al.*, 1994). The optimal reannealing temperature was 78 °C in 2 × SSC (10 × SSC: 1.5 M NaCl; 0.15 M sodium citrate; pH 7.0). The DNA G + C content was 60.1–60.9 mol% among the eight isolates and the three *S. xinjiangense* strains (Table 2); this was in the range for *Sinorhizobium* spp. DNA–DNA relatedness among the new isolates and *S. xinjiangense* strains was 74.2–89.7% (mean value of 83.3%) (Table 2). DNA–DNA relatedness among the *S. fredii* type strain and the new isolates was 20.8–38.4% (mean value 29.0%). *S. xinjiangense* and other *Sinorhizobium* species shared DNA–DNA relatedness of 21.0–39.0% (Table 2).

Comparative analysis of 16S–23S intergenic spacer (IGS) sequences was carried out. PCR amplifications were performed in a 50 µl reaction volume. Forward primer 926f [5′-GGT TAA AAC T(C/T)A AA(G/T) GAA TTG ACG G-3′, corresponding to a conserved region of the 3′ end of 16S rDNA sequence in *E. coli*, position 901–926] and reverse primer 115r/23S [5′-CCG GGT T(T/G/C)C CCC ATT CGG-3′, corresponding to a conserved region of the 5′ end of 23S rDNA sequence in *E. coli*, position 97–115], were used to amplify the 16S–23S rRNA IGS sequence. Amplifications were carried out as follows: initial denatura-

tion at 94 °C for 3 min, 30 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 120 s, and then extension at 72 °C for 10 min. The PCR products of IGS were dialysed and sequenced directly using Cy-5 end-labelled primer 1492fc [5′-AAG TCG TAA CAA GGT A(A/G)C CGT-3′, corresponding to *E. coli* 16S sequence position 1471–1492] and reverse primer 85rc/23S [5′-CCC CAC GGC TT(A/T) TCG CA(A/G) CGT ATC AC-3′, corresponding to the *E. coli* 23S rRNA gene]. Methods for sequence alignment and phylogenetic tree reconstruction were the same as in the 16S rRNA gene analysis. The IGS sequence of CCBAU 110^T was 11 bp longer than that of USDA 205^T. Sequences obtained from *S. xinjiangense* CCBAU 110^T and *S. fredii* showed 90.6% similarity. This level of similarity was similar to those between *B. japonicum* USDA 6 (U69638) and *B. liaoningense* LMG 18231 (AJ279302) (90.4% similarity) and between *Rhizobium leguminosarum* CCT 4179 (AF091791) and *Rhizobium tropici* CCT 4164 (AF091799) (92.0% similarity) (sequences from GenBank database). It has been reported that IGS sequences are more variable than 16S rRNA gene sequences and have been used to identify closely related species (Chun *et al.*, 1999; Graham *et al.*, 1997).

In current rhizobial taxonomy, the definition of genus is mainly based upon phylogenetic relationships of 16S

rRNA genes. For the description of species, a polyphasic approach has been emphasized. DNA–DNA relatedness above 70 % has been used as a criterion to define species (Graham *et al.*, 1991), although it has been criticized as arbitrary (Ward, 1998). Phylogenetic clusters may correlate with species (Embley & Stackebrandt, 1997), but it is also difficult to draw a line to differentiate species in terms of their sequence similarity. Meanwhile, although the 16S rRNA gene is a very conserved region, it may not be sufficient to guarantee species identity (Fox *et al.*, 1992). In this study, species were differentiated using phylogenetic, genetic and phenotypic data. Based on the results of 16S rRNA gene analyses, it is concluded that all the isolates are *Sinorhizobium* and they are more closely related to *S. fredii* and *S. xinjiangense* than to other species. The isolates were identified as *S. xinjiangense* because they had great similarity in protein SDS-PAGE analysis and high DNA–DNA relatedness with the reference strains for this species. Although the criterion of 70 % was criticized, the big gap between the values of DNA–DNA relatedness (74.2–89.7 %) among the *S. xinjiangense* strains or the new isolates and those (20.8–39.0 %) among *S. xinjiangense* and *S. fredii* was clear (Table 2). The comparison of protein patterns in SDS-PAGE also offered evidence to differentiate the *S. xinjiangense* from *S. fredii* at a specific level. Referring to the division of *S. meliloti* and *S. medicae* (Rome *et al.*, 1996), it is proposed that *S. xinjiangense* should be a distinctive species having a close phylogenetic relationship with *S. fredii* based on the results of phenotypic characterization (Chen *et al.*, 1988) and results in this study. The geographic isolation may explain why the soybean-nodulating, fast-growing rhizobia in Xinjiang are different from those in other parts of China. Since the variation of IGS sequences within each of the species was not investigated, the IGS sequence data obtained in this work are not helpful for differentiating *S. xinjiangense* and *S. fredii*. However, these sequences could be used to identify strains or species when more data are accumulated.

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