Mesorhizobium shangrilense sp. nov., isolated from root nodules of *Caragana* species

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Five strains of bacteria isolated from nodules of *Caragana bicolor* and *Caragana erinacea* in Yunnan Province of China were classified within the genus *Mesorhizobium* in the class *Alphaproteobacteria*. The highest degree of 16S rRNA gene sequence similarity was determined to be to *Mesorhizobium loti* LMG 6125^T (99.7%) and *Mesorhizobium ciceri* UPM-Ca7^T (99.7%). Polyphasic taxonomic methods including SDS-PAGE of whole-cell soluble proteins, comparative housekeeping sequence analysis of *atpD*, *glnII* and *recA*, fatty acid profiles and a series of phenotypic and physiological tests allowed us to cluster the five strains into a coherent group while differentiating them from all previously established *Mesorhizobium* species. The DNA–DNA relatedness between the representative strain CCBAU 65327^T and the type strains of *M. loti* and *M. ciceri* was 26.5 and 23.4 %, respectively, clearly indicating that strain CCBAU 65327^T represents a novel species for which we propose the name *Mesorhizobium shangrilense* sp. nov. Strain CCBAU 65327^T (=LMG 24762^T =HAMB1 3050^T) is designated as the type strain, and could nodulate *Caragana microphylla*, *Caragana intermedia*, *Glycyrrhiza uralensis*, *Astragalus adsurgens*, *Vigna unguiculata*, *Vigna radiata* and *Phaseolus vulgaris* in cross-nodulation tests.

Caragana is a genus of about 100 species of flowering plants in the family Leguminosae, native to north-eastern Europe, Russia, central Asia, the Himalayas and China. Members of this genus have been introduced into many regions in northern latitudes because of their remarkable cold- and drought-resistant properties and nitrogen-fixing ability. Approximately 70 species have been recorded in China and most of them grow on hills and in gullies, thickets, plains and plateaux (Zhang, 2005). They are ecologically important because they serve as forage resources for wild animals and are preferred plants for

use in reforestation against desertification and erosion (Fan *et al.*, 2007; Su *et al.*, 2005). Most of the rhizobia associated with *Caragana* belong to the genus *Mesorhizobium* (Chen *et al.*, 1995; Guan *et al.*, 2008; Lu *et al.*, 2009; Yan *et al.*, 2007) and two species, *Mesorhizobium tianshanense* and *Mesorhizobium caraganae*, have been described so far (Chen *et al.*, 1995; Guan *et al.*, 2008).

During the study of rhizobia nodulating *Caragana* species from three different regions of China, 144 strains (of a total of 174) were characterized and classified in the genus *Mesorhizobium* based on 16S rRNA gene sequence analysis, BOX-PCR fingerprinting and SDS-PAGE profiles of whole-cell soluble proteins (Lu *et al.*, 2009). Five of the isolates from nodules of *Caragana bicolor* and *Caragana erinacea* grown in Yunnan Province of China exhibited distinct characteristics and differed from the known species of *Mesorhizobium*. Therefore, in the present study these five strains were analysed further and compared with the species within the genus *Mesorhizobium* on the basis of phenotypic, genotypic, phylogenetic and symbiotic characteristics to determine their species status.

Abbreviations: MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, *atpD*, *recA*, *glnII*, *nodC* and *nifH* gene sequences of strain CCBAU 65327^T are EU074203, EU672471, EU672501, EU672486, EU687487 and EU877532, respectively.

Fatty acid profiles, sequence similarities, *nodC* and *nifH* gene phylogenetic trees and a phenogram derived from UPGMA/*S*_{SM} analysis and SDS-PAGE analysis of whole-cell soluble proteins of strains from *Mesorhizobium shangrilense* sp. nov. and related species are available as supplementary material with the online version of this paper.

Rhizobia isolated from root nodules were purified by a standard procedure on YMA medium (Vincent, 1970), and isolates were maintained on YMA (Vincent, 1970) at 4 °C for temporary storage and in 20 % glycerol at –70 °C for long-term storage.

Approximately 1350 bp of the 16S rRNA gene was amplified and sequenced for each of the five strains using primers P1 and P6 according to the procedure of Tan *et al.* (1997). The sequences were aligned with those of related *Mesorhizobium* species using the CLUSTAL W program in the MEGA 4.0 software package (Tamura *et al.*, 2007). Aligned sequences were analysed by using the MEGA software to produce a Jukes–Cantor distance (Jukes & Cantor, 1969) and to construct an optimal unrooted tree using the neighbour-joining (NJ) (Saitou & Nei, 1987) method and maximum-parsimony (MP). The robustness of the tree topology was calculated from bootstrap analysis using 1000 replications of the sequences (Felsenstein, 1985). All five strains had identical 16S rRNA gene sequences that were closely related to those of *M. loti* LMG 6125^T and *M. ciceri* UPM-Ca7^T, with 99.7 % similarity (Supplementary Table S1 available in IJSEM Online). Percentages of 16S rRNA gene sequence similarity between the new isolates and the type strains of species of the genus *Mesorhizobium*, where values are higher than 97 %, are shown in Supplementary Table S1. Relationships revealed by two cluster methods (NJ and MP) were similar and the NJ tree is shown in Fig. 1a.

All five strains and the closely related reference strains of *Mesorhizobium* species were subjected to SDS-PAGE analysis of whole-cell soluble proteins, as described previously (Tan *et al.*, 1997). The normalized densitometric traces of the electrophoretic protein patterns were grouped using the Gelcompar II software package (Applied Maths). The similarity between each pair of samples (strains) was expressed by using the Dice coefficient and a UPGMA dendrogram was reconstructed (Vauterin & Vauterin, 1992). The strikingly similar protein patterns observed among the five novel strains indicated their considerable homogeneity (Supplementary Fig. S1). However, they were not clones, because they were isolated from different sampling sites and different species of *Caragana* (Table 1). Comparison of the protein patterns with those of other related *Mesorhizobium* species showed that these were not similar to the new group (Supplementary Fig. S1).

Sequence analyses included housekeeping genes *atpD* (encoding ATP synthase beta subunit, approx. 510 bp) and *recA* (homologous recombination protein A, approx. 530 bp), amplified according to Gaunt *et al.* (2001), and a 660 bp intragenic fragment of *glnII* amplified according to Turner & Young (2000). NJ trees of these genes (Fig. 1b–d) showed that the groupings and precise branching patterns in these trees were similar, but not entirely consistent with those based on the 16S rRNA gene. The five novel strains have highly similar or identical *atpD*, *recA* and *glnII* sequences, and sequence similarities with the most related

Mesorhizobium species were clearly lower than those found among other accepted *Mesorhizobium* species (Supplementary Table S1).

The symbiotic genes (*nod* and *nif*) of rhizobia are adaptive genes that have, in many cases, an evolutionary history independent of the housekeeping genes (Wang *et al.*, 2007). Comparison of their phylogenies with those derived from housekeeping genes may reveal events of lateral gene transfer among rhizobial symbiotic genes (Chen *et al.*, 2008; Haukka *et al.*, 1998; Liu *et al.*, 2005). In this study, partial sequences of the symbiotic genes *nodC* (approx. 900 bp) and *nifH* (approx. 760 bp) from the five novel strains were amplified by using primers (*nodCF* and *nodCI*, *nifHF* and *nifHI*) and PCR conditions described by Laguerre *et al.* (2001). In the phylogenetic trees based on *nodC* and *nifH* genes (Supplementary Fig. S2a, b), the four novel strains isolated from *C. bicolor* had identical sequences, while strain CCBAU 65336 that was isolated from *C. erinacea* was a little different. Comparison of the phylogenetic trees reconstructed on the basis of housekeeping and symbiotic genes (Fig. 1 and Fig. S2) demonstrated the different evolutionary histories of these genes and the close relationship between the symbiotic genes and their host plants suggested by Ueda *et al.* (1995).

High similarities (>96.2 %; Supplementary Table S1) of symbiotic genes among the *Caragana* mesorhizobia and the type strains of *Mesorhizobium temperatum*, *Mesorhizobium septentrionale* and *Mesorhizobium tianshanense* indicate that they may have common host ranges, as suggested by Laguerre *et al.* (2001) and Chen *et al.* (2008). To test whether the *Caragana* mesorhizobia share the same hosts as these three species, cross-inoculation tests between the above *Mesorhizobium* species and their original hosts were performed. Seed treatment and inoculation of *Caragana* species, *Astragalus adsurgens* and other common legumes were performed using the standard method of Vincent (1970), except that seeds of *Glycyrrhiza uralensis* were firstly immersed in concentrated sulfuric acid for 4 h before surface-sterilization. Seedlings inoculated with different strains were grown in a greenhouse under natural daylight for 6 weeks. Non-inoculated control seedlings were cultured under the same conditions. The results confirmed that each of the five strains from *Caragana* and the three type strains (*M. tianshanense*, *M. temperatum* and *M. septentrionale*) could form effective nodules on the hosts *Glycyrrhiza uralensis*, *Caragana microphylla*, *Caragana intermedia* and *Astragalus adsurgens*. The representative strain CCBAU 65327^T of the novel *Mesorhizobium* group was further used for cross-nodulation tests with another 12 legume species. Results showed that *Phaseolus vulgaris*, *Vigna unguiculata* and *Vigna radiata* could nodulate with CCBAU 65327^T, while no nodules were found on the CCBAU 65327^T-inoculated seedlings of *Glycine max*, *Trifolium pratense*, *Medicago sativa*, *Pisum sativum*, *Melilotus albus*, *Amorpha fruticosa*, *Lespedeza cuneata*, *Dunbaria rotundifolia* or *Vicia sepium*.

Fig. 1. Comparison of 16S rRNA (a), *atpD* (b), *recA* (c) and *glnII* (d) gene phylogenies, showing the relationships among the novel group (strain CCBAU 65327^T) and defined species of the genus *Mesorhizobium*. Trees were reconstructed by using the neighbour-joining method with a Jukes–Cantor distance matrix. Bootstrap values (%) are based on 1000 replicates and are shown at each node. Bars represent 0.005 or 0.01 expected changes per site.

Table 1. Isolates and reference strains used in this study

Strain	Host plant	Geographical origin/Altitude*	Reference
<i>Mesorhizobium shangrilense</i> sp. nov.			
CCBAU 65327 ^T	<i>Caragana bicolor</i>	Deqin, Yunnan, China/3653 m	This study
CCBAU 65321	<i>Caragana bicolor</i>	Zhongdian, Yunnan, China/3267 m	This study
CCBAU 65323	<i>Caragana bicolor</i>	Zhongdian, Yunnan, China/3267 m	This study
CCBAU 65324	<i>Caragana bicolor</i>	Zhongdian, Yunnan, China/3267 m	This study
CCBAU 65336	<i>Caragana erinacea</i>	Zhongdian, Yunnan, China/3399 m	This study
Reference strains			
<i>M. loti</i> NZP 2213 ^T	<i>Lotus corniculatus</i>	New Zealand	Jarvis <i>et al.</i> (1997)
<i>M. ciceri</i> USDA 3383 ^T	<i>Cicer arietinum</i>	Spain	Nour <i>et al.</i> (1994)
<i>M. tianshanense</i> CCBAU 3306 ^T	<i>Glycyrrhiza pallidiflora</i>	Xinjiang, China	Chen <i>et al.</i> (1995)
<i>M. septentrionale</i> SDW 014 ^T	<i>Astragalus adsurgens</i>	Liaoning, China	Gao <i>et al.</i> (2004)
<i>M. temperatum</i> SDW 018 ^T	<i>Astragalus adsurgens</i>	Liaoning, China	Gao <i>et al.</i> (2004)
<i>M. plurifarum</i> LMG 11892 ^T	<i>Acacia senegal</i>	Senegal	de Lajudie <i>et al.</i> (1998)
<i>M. amorphae</i> ACCC 19665 ^T	<i>Amorpha fruticosa</i>	Beijing, China	Wang <i>et al.</i> (1999)
<i>M. chacoense</i> PR5	<i>Prosopis alba</i>	Argentina	Velazquez <i>et al.</i> (2001)
<i>M. mediterraneum</i> UPM-Ca36 ^T	<i>Cicer arietinum</i>	Spain	Jarvis <i>et al.</i> (1997)
<i>M. huakuui</i> CCBAU 2609 ^T	<i>Astragalus sinicus</i>	Nanjing, China	Jarvis <i>et al.</i> (1997)
<i>M. caraganae</i> CCBAU 11299 ^T	<i>Caragana microphylla</i>	Liaoning, China	Guan <i>et al.</i> (2008)
<i>M. albiziae</i> CCBAU 61158 ^T	<i>Albizia kalkora</i>	Sichuan, China	Wang <i>et al.</i> (2007)
<i>M. gobiense</i> CCBAU 83330 ^T	<i>Oxytropis glabra</i>	Xinjiang, China	Han <i>et al.</i> (2008)
<i>M. tarimense</i> CCBAU 83306 ^T	<i>Lotus frondosus</i>	Xinjiang, China	Han <i>et al.</i> (2008)

*Deqin and Zhongdian are counties of Yunnan Province.

As a standard method for species definition (Graham *et al.*, 1991; Wayne *et al.*, 1987), DNA–DNA hybridization was performed between the representative strain CCBAU 65327^T and other strains in the group and reference strains with 16S rRNA gene sequence similarities higher than 97 %, using Marmur's method for total DNA isolation (Marmur, 1961) and the renaturation-rate technology described previously (De Ley *et al.*, 1970). All experiments were performed three times and mean DNA relatedness values are reported. In this experiment, the standard deviation varied from 2.31 to 7.16 % with a mean of 4.05 %. The results summarized in Supplementary Table S1 showed that the five *Caragana* rhizobial strains formed a genomic species sharing DNA relatedness greater than 88.5 %, while the DNA relatedness ranged from 9.5 to 27.5 % (Supplementary Table S1) between CCBAU 65327^T and reference strains for defined *Mesorhizobium* species, suggesting that the novel group was a distinct genomic species in the genus *Mesorhizobium*. The G + C content of DNA was measured using the thermal denaturation method of De Ley (1970) using *Escherichia coli* K-12 as standard. The G + C content range of the five strains was 60.82–61.59 mol%, and that of CCBAU 65327^T was found to be 61.37 mol% (T_m), which is within the range reported for *Mesorhizobium* (59–64 mol%) (Jarvis *et al.*, 1997).

Colony morphology of the isolates as well as morphology of Hucker's Gram- and Schaeffer–Fulton's spore-stained smears were examined. Phenotypic features of the novel group were determined in comparison with related *Mesorhizobium* species according to the method described

by Gao *et al.* (1994). Tested features included the utilization of sole carbon and nitrogen sources, resistance to antibiotics and chemical dyes, tolerance of NaCl and pH and temperature ranges for growth (Gao *et al.*, 1994). Biochemical tests including catalase and oxidase production, methyl red and Voges–Proskauer reactions and hydrolysis of casein, gelatin, starch, DNA and Tween 80 were performed according to Smibert & Krieg (1994). Production of hydrogen sulfide from cysteine and production of indole from tryptophan were examined as described by Barrow & Feltham (2003). Distinctive features of the novel group are shown in Table 2 and more features are presented in the subsequent description of the novel species.

Analysis of cellular fatty acids has become a useful method to characterize and identify microbes, especially in the study of *Mesorhizobium* species (Schutter & Dick, 2000; Tighe *et al.*, 2000). In this study, the strains were grown on YMA medium (pH 7.0) for 72 h at 28 °C and then approximately 40 mg well-grown cells was harvested. The extraction, preparation and separation of fatty acid methyl esters for strain CCBAU 65327^T were performed according to methods described by Sasser (1990). Identification of these fatty acid methyl esters was completed using the MIDI Sherlock Microbial Identification System. The comparison of fatty acid profiles between CCBAU 65327^T and the other defined *Mesorhizobium* species (including the most recently described species *Mesorhizobium caraganae*, *Mesorhizobium gobiense* and *Mesorhizobium tarimense*) is shown in Supplementary Table S2. Strain CCBAU 65327^T

Table 2. Distinctive features of strain CCBAU 65327^T and the closest related *Mesorhizobium* species

Strain: 1, *M. shangrilense* sp. nov. CCBAU 65327^T; 2, *M. ciceri* USDA 3383^T; 3, *M. loti* NZP 2213^T; 4, *M. tianshanense* CCBAU 3306^T; 5, *M. septentrionale* SDW0 14^T. +, Positive; –, negative. All data are from this study. The concentration of carbon and nitrogen sources is 0.1 % (w/v).

Character	1	2	3	4	5
Substrates used as sole carbon source					
meso-Erythritol	–	+	+	–	–
Glucose	+	–	+	+	+
Sodium DL-malate	+	–	+	+	–
D-Mannose	+	+	+	+	–
Sodium pyruvate	+	–	+	+	–
L-Rhamnose	+	–	+	–	+
Sodium citrate	+	+	–	+	–
Sodium succinate	+	+	+	+	–
Trehalose	+	–	+	+	+
Substrates used as sole nitrogen source					
L-Aspartic acid	+	–	+	+	–
Hypoxanthine	+	+	+	+	–
L-Phenylalanine	+	–	+	+	+
L-Valine	+	–	+	+	–
L-Methionine	+	+	+	–	+
Resistance to:					
Ampicillin (50 µg ml ^{–1})	+	+	+	–	+
Kanamycin sulfate (5 µg ml ^{–1})	+	+	+	+	–
Kanamycin sulfate (50 µg ml ^{–1})	–	–	+	–	–
Streptomycin sulfate (5 µg ml ^{–1})	+	+	+	+	+
Streptomycin sulfate (50 µg ml ^{–1})	+	–	+	–	–
Bacitracin (300 µg ml ^{–1})	+	+	–	–	+
Erythromycin (50 µg ml ^{–1})	–	+	–	–	–
Chloramphenicol (5 µg ml ^{–1})	+	–	+	+	+
Bismarck brown (0.1 %)	–	–	+	–	+
Erythrosine bluish (0.1 %)	–	+	–	–	–
Sodium deoxycholate (0.1 %)	–	+	+	+	+
Growth in/at:					
NaCl (2 %)	+	–	+	+	–
NaCl (3 %)	+	–	–	–	–
pH 9.0	+	–	+	+	–
pH 10.0	+	–	+	+	–

was clearly placed in the genus *Mesorhizobium* because it lacked C_{20:3}ω6,9,12c and summed feature 3 (same as summed feature 2 in this study, referring to C_{12:0}, unknown ECL 10.928, iso-C_{16:1} 1 and/or C_{14:0} 3-OH; Supplementary Table S2) and possessed iso-C_{17:0} fatty acids (Tighe *et al.*, 2000). Strain CCBAU 65327^T could be distinguished from the other *Mesorhizobium* species because it contained a higher concentration of C_{19:0} cyclo ω8c.

Based on the results obtained in this study, we believe that the five strains represent a novel species in the genus *Mesorhizobium* that could be differentiated by SDS-PAGE of cellular soluble proteins, fatty acid profiles, phenotypic characteristics, DNA relatedness and sequencing of *atpD*, *glnII*, *recA*, *nodC* and *nifH* genes. As a result, we propose

the name *Mesorhizobium shangrilense* sp. nov. for this group with CCBAU 65327^T as type strain.

Description of *Mesorhizobium shangrilense* sp. nov.

Mesorhizobium shangrilense (shan.gri.len'se. N.L. neut. adj. *shangrilense* pertaining to Shangri-La, the name of the area covering the sites where the first strains were isolated).

Gram-stain-negative, aerobic, non-spore-forming rods, 0.3–0.6 µm wide by 1–3 µm long. Colonies on YMA medium are circular, convex, white, opaque and usually 1–2 mm in diameter after 5–7 days at 28 °C. Growth occurs between 10 and 37 °C with an optimum at 28 °C. The pH range is 5–10 with an optimum at pH 7.0. Can grow on YMA in the presence of 3 % NaCl. Produces catalase and oxidase but not L-phenylalaninase or urease. Methyl blue, but not nitrate or Nile blue is reduced. Acid production in litmus milk. No hydrolysis of gelatin, casein, starch, DNA or Tween 80. Indole but not H₂S is produced and the Voges–Proskauer and methyl red reactions are negative. Resistant to (µg ml^{–1}) ampicillin (50), kanamycin sulfate (5), neomycin sulfate (5), streptomycin sulfate (50), bacitracin (300), erythromycin (5) and chloramphenicol (5). Can use D-arabinose, D-fructose, D-mannose, D-sorbitol, D-xylose, glucose, inulin, inositol, maltose, sodium citrate, sodium malate, L-rhamnose, sodium pyruvate, sodium succinate, sucrose and trehalose as sole carbon sources, and DL-α-aminopropionic acid, hypoxanthine, L-aspartic acid, L-glutamic acid, L-arginine, L-hydroxyproline, L-methionine, L-phenylalanine, L-threonine and L-valine as sole nitrogen sources. Cannot use vanillic acid, adipic acid, calcium gluconate, calcium malonate, D-amygdalin, D-galactose, melezitose, melibiose, D-ribose, turanose, dextrin, DL-asparagine, dulcitol, glycine, L-arginine, L-methionine, L-proline, meso-erythritol, raffinose, salicin, sodium D-gluconate, sodium acetate, sodium formate, sodium tartrate, sorbose, starch, sucrose or syringic acid as sole carbon sources, and cannot use D-threonine, DL-alanine, glycine or L-isoleucine as sole nitrogen sources. DNA G + C content of the type strain is 61.37 mol% (T_m).

The type strain, CCBAU 65327^T (=LMG 24762^T =HAMBI 3050^T), was isolated from root nodules of *Caragana bicolor* growing in Deqin city, Yunnan Province, China.

Acknowledgements

This work was financed by the National Natural Science Foundation of China (project no. 30400001), The National Program for Basic S & T Platform Construction (no. 2005DKA21201) and The Natural Science Foundation of Beijing (6053024). We thank Professor Janet I. Sprent for improvement of the manuscript.

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