Rhizobium mesosinicum sp. nov., isolated from root nodules of three different legumes

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Thirteen novel strains were isolated from root nodules of three different leguminous plants of the genera *Albizia*, *Kummerowia* and *Dalbergia* grown in China. Cells of these strains were Gram-negative, strictly aerobic, non-spore-forming, motile rods. Phylogenetic analysis of 16S rRNA gene sequences revealed that they belong to the genus *Rhizobium*. A representative strain, CCBAU 25010^T, showed 98.8% 16S rRNA gene sequence similarity to its closest phylogenetic relative, *Rhizobium sulae* IS123^T. The 16S–23S intergenic spacer (ITS) sequence of CCBAU 25010^T shared 91.5 and 87.2% sequence similarity, respectively, with those of *Rhizobium etli* CFN 42^T and *Rhizobium leguminosarum* LMG 14904^T. Analysis of the sequences of the housekeeping genes *atpD* and *recA* was in agreement with the results of ITS sequence analysis. The *nodC* gene sequence of CCBAU 25010^T was identical to that of *Rhizobium tropici* CFN 299. DNA–DNA hybridization values for strain CCBAU 25010^T ranged from 20.7% (with *Rhizobium mongolense* USDA 1844^T) to 34.4% (with *R. leguminosarum* USDA 2370^T). Cell protein SDS-PAGE, BOX-PCR and several phenotypic characteristics, such as use of sole carbon sources and antibiotic resistance, could differentiate the novel strains from defined *Rhizobium* species. We therefore propose that the novel strains reported in this study form a novel species, *Rhizobium mesosinicum* sp. nov., with the type strain CCBAU 25010^T (=LMG 24135^T =JCM 14777^T).

Species of the legume genus *Albizia* are commonly grown in temperate zones of Asia and Africa. There are about 150 species in the genus, and 17 of them can be found in southern China (Wu *et al.*, 1988). The diversity of their microsymbionts has been characterized, revealing that a wide range of rhizobial species can establish effective symbioses with *Albizia* species (Wang *et al.*, 2006).

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Abbreviation: ITS, intergenic spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, 16S–23S ITS, *atpD* and *recA* sequences of strains CCBAU 25010^T, CCBAU 25217 and CCBAU 41044 are respectively DQ100063, EF070130 and AY395697 (16S rRNA gene), EU120729, EU120730 and DQ352862 (16S–23S ITS), EU120726, EU120727 and DQ310808 (*atpD*) and EU120732, EU120731 and EU034028 (*recA*). The accession number for the *nodC* sequence of strain CCBAU 25010^T is EU120728.

Neighbour-joining trees based on ITS and *atpD*–*recA* sequences, SDS-PAGE and BOX-PCR profiles of representative novel and reference strains and detailed DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

Moreover, the novel species *Mesorhizobium albiziae* has been described (Wang *et al.*, 2007), which was found to nodulate *Albizia kalkora* in a subtropical region of China. *Kummerowia* species are leguminous plants of medicinal value that originate from East Asia, and some of them have been introduced to other areas. Our previous data indicated that *Kummerowia* species were non-selective hosts for rhizobia and that they might nodulate with various bacteria in different regions (Lin *et al.*, 2007, 2008). Rosewoods (species of *Dalbergia* L.), belonging to the subfamily Papilionoideae of the Leguminosae, are slow-growing ornamental trees, some of which have medicinal value (Ito *et al.*, 2003), and are usually used as hard timber in furniture production. They produce pharmaceutical substances that have anti-giardial, anti-allergic, antimicrobial and anticancer activities. There are about 150 species in the genus, which are distributed in tropical and subtropical areas of the world, and most of them form nodules (Allen & Allen, 1981). It has been reported that bacterial strains isolated from nodules of *Dalbergia* species endemic to Madagascar belong to the *Alphaproteobacteria* and

Betaproteobacteria (Rasolomampianina *et al.*, 2005). A rhizobial strain isolated from *Dalbergia lanceolaria* was reported to produce indole acetic acid (Ghosh & Basu, 2002). In China, 28 rosewood species have been recorded, mainly in the subtropical regions. The microsymbionts associated with *Dalbergia* species in Chinese soils have not been studied. In this study, a group of *Dalbergia* rhizobia are reported that showed close phylogenetic relationships with some strains isolated from *Albizia* (Wang *et al.*, 2006) and *Kummerowia* (Lin *et al.*, 2007), also grown in subtropical regions. Based upon a comparative study with multiple analyses, these strains were classified into a single group distinct from defined *Rhizobium* species.

Strains CCBAU 25010^T and CCBAU 25116 were isolated from *Albizia julibrissin* grown in a temperate region of Shandong province on the east coast of China. Strains CCBAU 25217 and CCBAU 33126 were isolated from plants of two *Kummerowia* species. Strains from *Dalbergia* species were isolated near to the Changjiang river in Hunan province (Table 1). Bacterial strains were isolated from host root nodules following standard procedures (Vincent, 1970) and white, mucoid, translucent, convex colonies were observed on YMA medium (Vincent, 1970) at 28 °C after 3 days of incubation. Comparative analysis of 16S rRNA gene sequences revealed that these strains belong to the genus *Rhizobium*. Cells of the strains were Gram-negative, non-spore-forming, short rods, similar to those of other *Rhizobium* species.

Comparison of 16S rRNA gene sequences is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria (Weisburg *et al.*, 1991). Almost-complete 16S rRNA gene sequences of three strains (CCBAU 25010^T, CCBAU 25217 and CCBAU 41044) were obtained

following PCR amplification, as described by van Berkum *et al.* (1996). The sequences were aligned using CLUSTAL_X software (Thompson *et al.*, 1997) with sequences held in GenBank. A 16S rRNA gene sequence-based phylogenetic tree (Fig. 1) was generated using the neighbour-joining method with programs of the software package MEGA 3.1 (Kumar *et al.*, 2004) and bootstrapped with 1000 replications of each sequence. The strains from this study formed a distinct cluster within the genus *Rhizobium* (Fig. 1). The sequences of CCBAU 25010^T and CCBAU 25217 were nearly identical, and they shared 99.5 % sequence similarity with that of CCBAU 41044. The most similar sequence (98.8 %) from a defined species was that of *Rhizobium sullae* IS123^T. 16S rRNA gene sequence similarities among the novel strains were greater than 99.5 % and lower than 98.8 % with other *Rhizobium* species. These data clearly demonstrated that these strains were members of a genomic species that differed from defined *Rhizobium* species.

All 13 novel strains could form nodules on their original hosts. Strains CCBAU 25010^T, CCBAU 25217 and CCBAU 41044, representing the novel *Rhizobium* group, were used for cross-nodulation tests. Seeds were treated with standard procedures for surface sterilization, germination and inoculation as described by Vincent (1970). Strains CCBAU 25010^T and CCBAU 25217 were inoculated onto seeds of *Medicago sativa*, *Glycine max* and *Phaseolus vulgaris*. Strain CCBAU 41044 was inoculated onto seedlings of *Medicago sativa*, *Robinia* sp., *Albizia* sp., *Glycine max*, *Phaseolus vulgaris* and *Glycyrrhiza* sp., common leguminous plants grown in the regions where the rhizobia studied in this work were collected. Strain CCBAU 41044 was able to form nodules on *Medicago sativa*, but did not nodulate *Robinia* sp., *Albizia* sp., *Glycine*

Table 1. Strains used in this study

Strain	Host plant	Geographical origin	Reference
<i>Rhizobium mesosinicum</i> sp. nov.			
CCBAU 25010 ^T , CCBAU 25116	<i>Albizia julibrissin</i>	Shandong province, China	Wang <i>et al.</i> (2006)
CCBAU 25217	<i>Kummerowia stipulacea</i>	As above	Lin <i>et al.</i> (2007)
CCBAU 33126	<i>Kummerowia striata</i>	Jiangxi province, China	Lin <i>et al.</i> (2007)
CCBAU 41044, CCBAU 41135	<i>Dalbergia balansae</i>	Hunan province, China	
CCBAU 41015, CCBAU 41026, CCBAU 41227	<i>Dalbergia hupeana</i>	As above	
CCBAU 41131	<i>Dalbergia dyeriana</i>	As above	
CCBAU 41176, CCBAU 41228	<i>Dalbergia mimosoides</i>	As above	
CCBAU 41226	<i>Dalbergia hancei</i>	As above	
Reference strains			
<i>R. sullae</i> IS123 ^T	<i>Hedysarum coronarium</i>	Spain	Squartini <i>et al.</i> (2002)
<i>R. etli</i> CFN 42 ^T	<i>Phaseolus vulgaris</i>	Mexico	Segovia <i>et al.</i> (1993)
<i>R. leguminosarum</i> bv. <i>viciae</i> USDA 2370 ^T	<i>Pisum sativum</i>	USA	Jordan (1984)
<i>R. tropici</i> CFN 299 ^T	<i>Phaseolus vulgaris</i>	Brazil	Martínez-Romero <i>et al.</i> (1991)
<i>R. gallicum</i> USDA 2918 ^T	<i>Phaseolus vulgaris</i>	France	Amarger <i>et al.</i> (1997)
<i>R. yanglingense</i> CCBAU 71623 ^T	<i>Gueldenstaedtia multiflora</i>	Gansu, China	Tan <i>et al.</i> (2001)
<i>R. mongolense</i> USDA 1844 ^T	<i>Meliloti luthenica</i>	Inner Mongolia	van Berkum <i>et al.</i> (1998)
<i>R. indigoferae</i> CCBAU 71042 ^T	<i>Indigofera amblyantha</i>	Shaanxi, China	Wei <i>et al.</i> (2002)

Fig. 1. Neighbour-joining tree of nearly full-length 16S rRNA gene sequences showing the phylogenetic relationships of *Rhizobium mesosinicum* sp. nov. strains CCBAU 25010^T, CCBAU 25217 and CCBAU 41044 and related species in the genus. Bootstrap values obtained from 100 pseudoreplicates are provided at the corresponding nodes. Bar, 0.5% nucleotide substitution. Strain CCBAU 41044 represents another eight rhizobial strains from *Dalbergia* species that had almost identical 16S rRNA gene sequences (GenBank accession numbers DQ310796–DQ310798, DQ310800, DQ310802, DQ310803, DQ485277 and DQ485278).

max, *Phaseolus vulgaris* or *Glycyrrhiza* sp. Neither strain CCBAU 25010^T nor strain CCBAU 25217 could nodulate *Medicago sativa*, *Glycine max* or *Phaseolus vulgaris* within 1 month of inoculation. To study the presence of symbiotic genes, a partial *nodC* fragment (800 bp) was amplified from strain CCBAU 25010^T with primers CF (5'-ATAGCAGCGATACGCCGTTCCCTC-3') and CV (5'-TGCGACCATCAGTTATGTCCACCAG-3') based on the *nodC* sequence of *Rhizobium tropici*. PCR conditions were as follows: preheating at 95 °C for 5 min followed by 30 cycles of denaturing at 91 °C for 1 min, annealing at 55 °C for 45 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 6 min. Attempts to amplify *nodC* or *nifH* gene fragments from other novel strains failed, despite using several primer pairs. The same primers used for amplification were used for *nodC* gene sequencing. The sequence of the *nodC* gene fragment amplified from CCBAU 25010^T was 100% identical to that of *R. tropici* CFN 299 (Martínez-Romero *et al.*, 1991).

Whole-cell soluble-protein SDS-PAGE (Tan *et al.*, 1999) and BOX-PCR genomic fingerprinting (Vandamme *et al.*, 2002; Nick *et al.*, 1999) were utilized to identify differences between the novel strains and *Rhizobium* species and to investigate the diversity amongst the novel strains. Of the strains isolated from *Dalbergia* species, we used strain CCBAU 41044 only, since the profiles of these strains were identical. SDS-PAGE profiles showed similar protein profiles (Supplementary Fig. S1, available in IJSEM Online; compare lanes 1–4 and 12), with a few differences in the region covering 24–36 kDa, but the profiles of the novel strains were clearly different from those of *R. sullae* IS123^T (lane 5) and *Rhizobium leguminosarum* USDA 2370^T (lane 6). The diversity within the species was

reflected by BOX-PCR (Supplementary Fig. S2). Three fingerprint types were apparent (compare lanes 1–4). The fingerprints of strains CCBAU 25010^T (lane 1) and CCBAU 25116 (lane 2) were nearly identical, suggesting that they probably represent the same bacterial clone, and they resemble the fingerprint type of CCBAU 41044 (lane 8).

A 1 kb fragment of the 16S–23S rRNA gene intergenic spacer (ITS) was amplified from strains CCBAU 25010^T, CCBAU 25217 and CCBAU 41044; they showed close relationships in a phylogenetic tree (Supplementary Fig. S3a). The ITS sequence of strain CCBAU 25010^T was similar to that of CCBAU 25217 (98% similarity), and they showed 93.1 and 93.4% identity to that of CCBAU 41044. The ITS sequences of these three strains had 91.5% similarity with that of *R. etli* CFN 42^T and 87.2% with that of *R. leguminosarum* LMG 14904^T. The ITS sequence similarity between these three strains and other *Rhizobium* species was lower than 88.6%. Phylogenetic relationships estimated from ITS sequences (Supplementary Fig. S3a) differed little from those revealed by the sequence of the 16S rRNA gene. Previously work has shown that ITS sequences are more diverse than 16S rRNA gene sequences among *Bradyrhizobium* isolates (Willems *et al.*, 2001). According to Kwon *et al.* (2005), phylogenetic analysis based on 16S rRNA gene and ITS region sequences showed remarkable heterogeneity amongst rhizobia. It had been reported that phylogenies based on the *atpD* and *recA* genes were in good agreement with those based on the 16S rRNA gene (Gaunt *et al.*, 2001). However, in our study, the phylogeny based on concatenated *atpD* and *recA* sequences was not congruent with that of the 16S rRNA gene (Supplementary Fig. S3b), but was congruent with the ITS phylogeny (Supplementary Fig. S3a). The concatenated

atpD-recA sequence of CCBAU 25010^T was related to those of CCBAU 25217 and CCBAU 41044 (95.6 and 92.4% similarity, respectively). Sequence identities were lower than 91% with other *Rhizobium* species, and the novel strains formed a distinct branch close to *R. tropici*.

Physiological and biochemical characters were tested using conventional methods (Sneath & Sokal, 1962). Utilization of 45 carbon and 13 nitrogen sources was determined as described by Chen *et al.* (1988). Resistance to antibiotics was tested by using final concentrations of 5, 50 and 100 µg ml⁻¹ of kanamycin, ampicillin, erythromycin, streptomycin, gentamicin, chloromycin and neomycin on YMA medium (Vincent, 1970). Tolerance of NaCl was measured on TY (tryptone-yeast extract) medium containing NaCl at concentrations of 1, 2, 3, 4 and 5% (w/v). The pH range for growth was studied between pH 4.0 and 12.0 in the same medium. Growth at 37 and 60 °C on YMA medium and at 28 °C on Luria-Bertani (LB) medium was also tested with reference strains. Some phenotypic characteristics of the novel strains in comparison with several *Rhizobium* type strains are shown in Table 2. It should be noted that few features differentiate these strains from other *Rhizobium* species; strain CCBAU 25010^T assimilated gluconate, malonate and D-ribose as carbon sources, was resistant to several of the tested antibiotics and grew at pH 4.0.

Table 2. Characteristics of representative novel strains and type strains of related *Rhizobium* species

Strains: 1, CCBAU 25010^T; 2, CCBAU 25217; 3, CCBAU 41044; 4, *R. leguminosarum* USDA 2370^T (data from Drouin *et al.*, 1996); 5, *R. sultae* IS123^T (Squartini *et al.*, 2002); 6, *R. tropici* CFN 299^T (this study); 7, *R. mongolense* USDA 1844^T (van Berkum *et al.*, 1998); 8, *R. yanglingense* CCBAU 71623^T (Tan *et al.*, 2001); 9, *R. gallicum* R602sp^T (Amarger *et al.*, 1997). +, Positive; (+), weakly positive; -, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Assimilation of:									
Gluconate	+	(+)	-	-	-	-	-	-	-
Malonate	+	-	-	-	-	-	-	-	-
Inulin	+	+	+	-	+	-	-	(+)	-
Xylose	+	+	+	+	-	+	-	-	-
D-Ribose	+	+	+	-	-	+	-	-	-
Tartrate	(+)	+	-	+	+	-	-	+	-
Growth at/in:									
3% NaCl	-	-	-	-	-	-	+	-	-
pH 4.0	+	+	-	-	-	-	(+)	-	-
pH 10.0	+	+	+	+	+	-	+	-	-
37 °C	+	+	+	-	-	+	+	+	+
LB medium	+	+	+	+	+	-	+	-	-
Resistance to:									
Kanamycin	+	+	-	-	-	-	-	-	-
Ampicillin	+	+	+	+	+	+	-	ND	ND
Erythromycin	+	+	+	-	-	-	ND	(+)	ND
Streptomycin	+	-	-	-	ND	-	-	ND	+

DNA base composition (G+C content) was determined spectrophotometrically as described by Marmur & Doty (1962). The G+C content for strain CCBAU 25010^T was 61.8 mol%, which falls within the range of values reported for recognized *Rhizobium* species (59–64 mol%; Jordan, 1984), supporting the suggestion that they belong to this genus. To estimate DNA–DNA relatedness, an initial renaturation rate method was used (De Ley *et al.*, 1970). In view of the extremely high DNA–DNA hybridization values of the nine strains isolated from *Dalbergia* species, strain CCBAU 41044 was selected to perform DNA hybridization with CCBAU 25010^T and CCBAU 25217. The level of DNA–DNA relatedness varied from 61.7 to 98.9% among these three strains. Strain CCBAU 25010^T displayed 20.7–34.4% DNA–DNA relatedness with the type strains of a number of *Rhizobium* species (Supplementary Table S1).

Based on the genotypic, phenotypic and chemotaxonomic data presented in this study, we propose the classification of the 13 novel strains within a novel species of *Rhizobium*, for which the name *Rhizobium mesosinicum* sp. nov. is proposed.

Description of *Rhizobium mesosinicum* sp. nov.

Rhizobium mesosinicum (me.so.si'ni.cum. Gr. pref. *meso* central; N.L. neut. adj. *sinicum* belonging to China; N.L. neut. adj. *mesosinicum* of central China, referring to the location of isolation of the first strains).

Gram-negative, aerobic, non-spore-forming, motile rods (0.5–0.7 × 1.5–2.1 µm). Colonies are circular, cream-coloured, semi-translucent and 2–4 mm in diameter after 3 days of incubation at 28 °C on YMA medium. Tests for catalase, oxidase and urease activity are positive. No reduction of nitrate to nitrite. Optimum growth at 25–30 °C; growth is observed at 37 °C. Optimum growth at pH 6–8; can grow at pH 4–10. Can grow at 2% NaCl and on LB medium. No growth in medium containing adipate, malonate, inulin, melibiose, acetate, hippurate, sorbitol, starch, syringate, tartrate, vanillate, DL-arginine, DL-asparagine, L-glycine, methionine, L-threonine or D-glutamine. Does not hydrolyse starch, gelatin, casein, Tween 80 or DNA. Growth on Voges-Proskauer, methyl red test and indole production are negative. Cells are resistant to (µg ml⁻¹) ampicillin (100), erythromycin (100), kanamycin (50), streptomycin (100) and bacitracin (300), but susceptible to chloromycin (25), neomycin (10) and gentamicin (25). The G+C content of the type strain is 61.8 mol%.

The type strain, CCBAU 25010^T (=LMG 24135^T =JCM 14777^T), was isolated from root nodules of *Albizia julibrissin*, growing in Shandong province, China.

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