Rhizobium vignae sp. nov., a symbiotic bacterium isolated from multiple legume species

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A group of rhizobial strains isolated from nodules of multiple legume species grown in different geographical regions of China had identical 16S rRNA genes. Phylogenetic analysis based on the 16S rRNA gene sequences showed that the novel strains formed a subclade in the genus Rhizobium together with Rhizobium galegae, Rhizobium huautlense and Rhizobium alkalisoli, with 99.8% gene sequence similarity between the strains. The DNA–DNA relatedness values between the representative strain CCBAU 05176T and R. galegae ATCC 43677T, R. huautlense S02T and R. alkalisoli CCBAU 01393T were 22.6%, 8.9% and 15.9%, respectively. The novel strains were distinguished from recognized species of the genus Rhizobium by using a polyphasic approach, including PCR-based restriction fragment length polymorphism analysis (RFLP) of the 16S–23S intergenic spacer (IGS), phenotypic and physiological tests, sequence comparisons of housekeeping genes and cellular fatty acid profiles. Therefore, it is suggested that this group of strains represents a novel species for which the name Rhizobium vignae sp. nov. is proposed. The type strain is CCBAU 05176T (=HAMBI 3039T=LMG 25447T).

In previous studies, seven strains of root-nodule bacteria isolated from Vigna radiata (Zhang et al., 2006), Astragalus dahuricus, Astragalus oxyglistis (Zhao et al., 2008) and Desmodium microphyllum (Gu et al., 2007) grown in different regions of China were classified into the genus Rhizobium and were demonstrated to be closely related to Rhizobium galegae by using amplified rDNA restriction analysis (ARDRA). The nodulation ability of the strains on their hosts of origin was confirmed in each of the previous studies. In the present study, the taxonomic status of these strains was clarified by analysis of the 16S–23S rRNA intergenic spacer (IGS-RFLP) and housekeeping gene (atpD, recA and glnII) sequences, cellular fatty acid profiles, DNA–DNA relatedness and the determination of phenotypic characteristics.

Seven novel strains (Table 1) and the reference strains were purified and cultured using a standard procedure on yeast mannitol agar (YMA) medium (Vincent, 1970). They were maintained on YMA at 4 °C for temporary storage and in 20% (w/v) glycerol at −80 °C for long-term storage.

Total DNA from each strain was prepared as described by Terefework et al. (2001) and used as the template in the amplification of the 16S rRNA gene, 16S–23S intergenic spacer and atpD (ATP synthase subunit beta), recA (recombinase A protein) and glnII (glutamine synthetase II) housekeeping genes.
The 16S rRNA genes of all seven strains were amplified and sequenced using the primers P1 and P6 according to the procedure of Tan et al. (1997). The sequences were aligned with those held in the GenBank database using the CLUSTAL W program in the MEGA 4.0 software suite (Tamura et al., 2007). Aligned sequences were analysed using the same 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) and maximum-parsimony methods. The robustness of the tree topology was calculated from bootstrap analysis using 1000 replications (Felsenstein, 1985). As no significant topological differences were found between the phylogenetic trees constructed using the two methods, only the tree constructed by using the NJ method after distance analysis of aligned sequences is shown (Fig. 1). The 16S rRNA gene sequences of all seven novel strains were identical and were closely related to R. galegae ATCC 43677T, R. alkalisoli CCBAU 01393T and R. huaulense S02T with 99.8% gene sequence similarities (Table 2).

It is generally considered that the high sequence variation of the 16S–23S IGS region allows discrimination between closely related strains (Kwon et al., 2005). This fragment was amplified from the seven novel strains and reference strains by PCR with primers FGPS6 (5'-GGAGAGTTT-AGATCTTGGCTCA-3') and 23S-38 (5'-CCGGGTTTC-CCCATTCCG-3') (Rasolomampionana et al., 2005). The procedure described by Kwon et al. (2005) was applied for PCR amplification. Aliquots (5–8 µl) of PCR products were digested separately with the restriction endonucleases MspI, HaeIII and HhaI during 4–6 h at 37 °C. The digested fragments were separated by electrophoresis at 100 V for 4 h in 3% (w/v) agarose gels (20 cm x 8 cm, 5 V cm⁻¹) and were photographed under UV light after ethidium bromide staining. The restriction patterns were normalized, combined and clustered using the GeLCompar II version 3.0 software package (Applied Maths). The Dice coefficient and the UPGMA method were used to obtain the restriction pattern based dendrogram (see Supplementary Fig. S1 in IJSEM Online). The data indicated that the seven novel strains were divided into two RFLP patterns sharing 92% similarity, which clearly differed from phylogenetically related species of the genus Rhizobium (≤70% similarity).

Housekeeping genes (atpD, glnII, recA, dnaK etc.) throughout the genome are being used increasingly to investigate the phylogeny of bacteria. Most recently, multilocus sequence typing (MLST) of ten housekeeping genes was performed and this method was demonstrated to be superior to multilocus enzyme electrophoresis (MLEE) (van Berkum et al., 2006) and DNA–DNA hybridization (Martens et al., 2008). In the present study, PCR amplification and sequencing of partial atpD, recA and glnII genes was performed according to Gaunt et al. (2001) and Turner & Young (2000). The sequence similarities of the atpD, recA and glnII genes were 98.6–100% between the seven strains of the novel group and were less than 96% between members of the novel group and the type strains of recognized species (Table 2). Phylogenetic trees were constructed based on these sequences by using the NJ method after distance analysis of aligned sequences according to the Kimura two-parameter (Kimura, 1983) model. The phylogenetic position of the seven strains was similar in the analyses of the three housekeeping genes and they formed a lineage most related to R. galegae as shown in the atpD gene sequence phylogenetic tree (Fig. 2) (recA- and glnII-based phylogenetic trees are available as Supplementary Figs S2 and S3 in IJSEM Online). The seven novel strains showed high gene sequence similarities with each other for the three housekeeping genes (Table 2), but they were not clones of the same strain because they were isolated from different regions and different legume species (Table 1). They also showed close relationships with R. galegae, which were consistent with the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1). Strain CCBAU 05176T shared 95.6, 96.0 and 94.4% sequence similarities with R. galegae ATCC 43677T for the atpD, recA and glnII genes, respectively (Table 2).

DNA–DNA hybridization is considered as a standard method to define bacterial species (Graham et al., 1991; Wayne et al., 1987). In this study, all seven novel strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host plant</th>
<th>Geographical origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. vignae sp. nov.</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CCBAU 05176T</td>
<td>Astragalus dahuricus</td>
<td>Hebei province, China</td>
<td>Zhao et al. (2008)</td>
</tr>
<tr>
<td>CCBAU 45252</td>
<td>Vigna radiata</td>
<td>Zhengzhou, Henan province, China</td>
<td>Zhang et al. (2006)</td>
</tr>
<tr>
<td>CCBAU 45248</td>
<td>Vigna radiata</td>
<td>Luoyang, Henan province, China</td>
<td>Zhang et al. (2006)</td>
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<tr>
<td>CCBAU 45111</td>
<td>Vigna radiata</td>
<td>Xixia, Henan province, China</td>
<td>Zhang et al. (2006)</td>
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<tr>
<td>CCBAU 45071</td>
<td>Vigna radiata</td>
<td>Lankao, Henan province, China</td>
<td>Zhang et al. (2006)</td>
</tr>
<tr>
<td>CCBAU 23084</td>
<td>Desmodium microphyllum</td>
<td>Anhui province, China</td>
<td>Gu et al. (2007)</td>
</tr>
<tr>
<td>CCBAU 83006</td>
<td>Astragalus oxyglossis</td>
<td>Xiniang Autonomous Region</td>
<td>Zhao et al. (2008)</td>
</tr>
<tr>
<td>R. galegae ATCC 43677T</td>
<td>Galega orientalis</td>
<td>Finland</td>
<td>Lindström (1989)</td>
</tr>
<tr>
<td>R. huaulense S02T</td>
<td>Sesbania herbacea</td>
<td>Mexico</td>
<td>Wang et al. (1998)</td>
</tr>
<tr>
<td>R. alkalisoli CCBAU 01393T</td>
<td>Caragana intermedia</td>
<td>Inner Mongolia, China</td>
<td>Lu et al. (2009)</td>
</tr>
<tr>
<td>R. cellulosilyticum ALA1082T</td>
<td>Populus alba</td>
<td>Spain</td>
<td>Garcia-Fraile et al. (2007)</td>
</tr>
</tbody>
</table>

Table 1. Bacterial strains used in this study and their relevant characteristics
and the reference strains of related species were used for DNA–DNA hybridization experiments. Total DNA was extracted from each strain by using the method of Marmur (1961) and the DNA G+C mol% was determined using the thermal denaturation method (Tm). DNA from *Escherichia coli* K-12 was used as the standard (Marmur & Doty, 1962). The DNA G+C contents of the novel strains varied between 59.1 and 61.6 mol% (Tm), which was in the range previously determined for members of the genus *Rhizobium* (Jordan, 1984). DNA–DNA relatedness within the novel group and between the novel strains and related species of the genus *Rhizobium* was determined.

![Fig. 1. Phylogenetic tree constructed from 16S rRNA gene sequences with the Kimura two-parameter and NJ methods showing the relationships among the seven novel strains (bold type) and recognized species of the genus *Rhizobium*. Bootstrap values >70% are indicated at the nodes. *Bradyrhizobium elkanii* USDA 76T was used as outgroup. Bar, 0.01 expected changes per site.](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Similarity (%) with strain CCBAU 05176T</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>atpD</td>
</tr>
<tr>
<td>Other six strains in the group</td>
<td>100</td>
</tr>
<tr>
<td><em>R. galegae</em> ATCC 43677T</td>
<td>99.8</td>
</tr>
<tr>
<td><em>R. huautlense</em> S02T</td>
<td>99.8</td>
</tr>
<tr>
<td><em>R. alkalisoli</em> CCBAU 01393T</td>
<td>99.8</td>
</tr>
<tr>
<td><em>R. cellulosilyticus</em> ALA10B2T</td>
<td>98.3</td>
</tr>
</tbody>
</table>

Table 2. Sequence similarities (%) for 16S rRNA, *atpD*, *recA* and *glnII* genes and DNA–DNA relatedness (%) between strains of the novel group and reference strains.

NA, not available.
using the spectrophotometric method (De Ley et al., 1970). The representative strain CCBAU 05176\textsuperscript{T} had DNA–DNA relatedness values of 88–94\% with the other six strains in the novel group and had similarities of 8.9–22.6\% with the type strains of recognized species (Table 2). Therefore, the seven novel strains formed a single genomic species which differed from recognized species based on the recommendation of a threshold value of 70\% DNA–DNA relatedness for the delineation of separate species (Graham et al., 1991; Wayne et al., 1987).

To clarify whether the seven novel strains could be defined as a separate species, phenotypic features were determined in comparison with the related species according to the methods described by Gao et al. (1994). The features tested included the utilization of sole carbon and nitrogen sources, resistance to antibiotics, tolerance of NaCl, the pH and temperature ranges for growth (Gao et al., 1994) and other physiological and biochemical tests. The phenotypic characterization showed that a combination of various features (Table 3) could differentiate the novel group of strains from closely related species of the genus \textit{Rhizobium}. Members of the novel group could not use melibiose as the sole carbon source but the reference strains of the related species could. Some differences were also observed between the strains within the novel group. The four strains that originated from \textit{V. radiata} grown in Henan province had identical features and differed from the other three strains by an inability to use dulcitol, DL-asparagine and L-proline as sole carbon sources, sensitivity to erythromycin (50 \mu g \text{ml}^{-1}) and an inability to grow at NaCl (2\%, w/v) or pH 10. These differences could relate to the geographical or host origins as was reported previously for \textit{Sinorhizobium meliloti} (Yan et al., 2000) and \textit{Sinorhizobium fredii} (Peng et al., 2002) populations in Xinjiang, China.

Cellular fatty acid profiles are commonly used to discern and describe novel bacterial species (de Lajudie et al., 1998; Quan et al., 2005; Tighe et al., 2000). In this study, the fatty acid composition of cells was determined by the standard method of the Microbial Identification System (MIDI) and the detailed results are available in Supplementary Table S1 (see IJSEM Online). Two strains of the novel group were included in this analysis. Five fatty acids were detected in strain CCBAU 05176\textsuperscript{T} and 30 fatty acids (including those found for strain CCBAU 05176\textsuperscript{T}) were detected in cells of strain CCBAU 45252. The difference of fatty acid patterns between these two strains was consistent with the divergence in their phenotypic characteristics and indicated the existence of variable strains in the novel group. The value of fatty acid content analysis for the taxonomy of the novel group needs to be evaluated further with more strains. According to this analysis, the fatty acids C\textsubscript{16:0}, C\textsubscript{16:0} \text{N-alcohol}, C\textsubscript{19:0 cyclo \omega 8c} and summed feature 8 (C\textsubscript{18:1 \omega 7c} and/or C\textsubscript{18:1 \omega 6c}) were present as the main compounds (1.31–46.97\%) in strains CCBAU 05176\textsuperscript{T}, CCBAU 45252, \textit{R. galegae} HAMBI 540\textsuperscript{T} and \textit{R. huautlense} S02\textsuperscript{T}, but the proportions were variable between the strains. For example, the content of summed feature 8 was 33.06\%, 16.09\%, 18.91\% and 46.97\% for strains CCBAU 05176\textsuperscript{T}, CCBAU...
45252, R. galegae HAMBI 540T and R. huautlense S02T, respectively. Fatty acids C12:0 3-OH, C15:0 iso 3-OH, C15:0 anteiso, C16:0 iso, C16:0 iso 3-OH, C16:0 anteiso, C16:1ω9c, C17:0 iso 3-OH, C18:1ω7c 11-methyl and C18:3ω6c were found only in strain CCBAU 45252 of the novel group with minor amounts (0.09–0.68 %).

Based on all the results obtained above, the seven new strains represent a novel species of the genus Rhizobium according to the current criteria for the description of rhizobial species (Graham et al., 1991; Wayne et al., 1987). This novel species could be differentiated by IGS-RFLP, fatty acid profiles, phenotypic characteristics, DNA–DNA relatedness and sequencing of the atpD, recA and gltII genes. The name Rhizobium vignae sp. nov. is proposed for this novel symbiotic bacterial species. Considering the apparent differences observed during the phenotypic characterization and in the cellular fatty acid determination between strains of R. vignae sp. nov., biovars or ecotypes may exist within this species.

### Description of Rhizobium vignae sp. nov.

*Rhizobium vignae* (vi.gna’e. N.L. gen. n. vignae of Vigna, referring to the fact that the majority of strains were isolated from the mung bean, *Vigna radiata*).

Gram-negative rods (0.5–0.7 × 1.2–2.5 μm), aerobic and non-spore-forming. Colonies on YMA medium are circular, convex, white and opaque and usually have a diameter of 2–3 mm within 2–3 days at 28 °C. Grows on YMA agar supplied with 0–2 % (w/v) NaCl; optimum NaCl for growth is 1 %. Growth occurs at 10–40 °C and at pH 7–10 on YMA plates, the optima are 28 °C and pH 7.0. Catalase, urease and oxidase activities are present, but not phenylalanine 4-monooxygenase. Nitrate is reduced and secretion of methyl blue and Nile blue are observed. Acid production and reduction are observed in litmus milk. Resistant to (μg ml⁻¹): ampicillin (5), kanamycin sulfate (5), neomycin sulfate (5), streptomycin sulfate (5), bacitracin (300), erythromycin (100), chloramphenicol (5) and gentamicin sulfate (5). Grows on YMA supplied with Bismarck brown (0.1 %), Congo red (0.1 %), sodium nitrite (0.1 %) and sodium deoxycholate (0.1 %). The type strain utilizes D-arabinose, D-fructose, D-galactose, sodium formate, sucrose, trehalose, syringic acid, vanillic acid, glycine and DL-asparagine as sole carbon sources for growth. The type strain uses almost all the sole nitrogen sources tested, including L-aspartic acid, (+)-L-glutamic acid, D-glutamic acid, D-alanine, D-threonine, hypoxanthine, L-arginine, L-cystine, L-isoleucine, L-lysine, L-phenylalanine, L-threonine, L-methionine and L-valine. The type strain does not utilize adipic acid, dextrin, inulin, melezitose, melibiose, *meso*-erythritol, calcium malonate, orcinol, soluble starch, sodium tartrate, sodium hippurate, L-methionine or L-valine. The common and/or main fatty acid compounds are C16:0, C18:0, C18:1ω9c, C18:1ω7c, C19:0 cyclo ω8c, summed feature 4 (C17:1ω6c), C17:0, C18:0 N alcohol, C19:0, C20:0 and C20:1ω9c.

The type strain, CCBAU 05176 T (=HAMBI 3039 =LMG 25447T), was isolated from a root nodule of *Astragalus dahuricus* grown in Hebei Province, China. The DNA G+C content of the type strain is 60.8 mol% (Thm). Additional strains were isolated from *Astragalus dahuricus*, *A. oxyglottis*, *Vigna radiata* and *Desmodium microphyllum* grown in different regions of China.

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