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 05176^T and *R. galegae* ATCC 43677^T, *R. huautlense* S02^T and *R. alkalisoli* CCBAU 01393^T 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 05176^T (=HAMBI 3039^T=LMG 25447^T).

Rhizobia are Gram-negative rod-shaped bacteria that induce the formation of nodules on the roots or stems of leguminous plants. Within the nodules, they convert atmospheric nitrogen to ammonia and then provide organic nitrogenous compounds such as glutamine or ureides to the plant. In the past two decades, the description of novel taxa of root- and stem-nodule bacteria has been accelerated by the adoption of a polyphasic taxonomic approach (Vandamme *et al.*, 1996) to characterize new isolates from various leguminous plants. The genus *Rhizobium*, proposed for a group of fast-growing, nodule-forming rhizobia, was firstly described by Frank (1889) and at the time of writing contained 34 species, including the recently described species *Rhizobium alkalisoli* (Lu *et al.*, 2009), *Rhizobium tibeticum* (Hou *et al.*, 2009) and *Rhizobium mesosinicum* (Lin *et al.*, 2009).

Abbreviation: NJ, neighbour joining.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, *atpD*, *recA*, and *glnII* gene sequences of *Rhizobium vignae* sp. nov. CCBAU 05176^T are GU128881, GU128888, GU128902 and GU128895, respectively. The 16S rRNA, *recA* and *glnII* gene sequence accession numbers for the six additional novel strains are GU128882–GU128887, GU128903–GU128908 and GU128896–GU12901, respectively.

Supplementary figures and a supplementary table are available with the online version of this paper.

In previous studies, seven strains of root-nodule bacteria isolated from *Vigna radiata* (Zhang *et al.*, 2006), *Astragalus dahuricus*, *Astragalus oxyglottis* (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.

Table 1. Bacterial strains used in this study and their relevant characteristics

Strain	Host plant	Geographical origin	Reference
<i>R. vignae</i> sp. nov.			
CCBAU 05176 ^T	<i>Astragalus dahuricus</i>	Hebei province, China	Zhao <i>et al.</i> (2008)
CCBAU 45252	<i>Vigna radiata</i>	Zhengzhou, Henan province, China	Zhang <i>et al.</i> (2006)
CCBAU 45248	<i>Vigna radiata</i>	Luoyang, Henan province, China	Zhang <i>et al.</i> (2006)
CCBAU 45111	<i>Vigna radiata</i>	Xixia, Henan province, China	Zhang <i>et al.</i> (2006)
CCBAU 45071	<i>Vigna radiata</i>	Lankao, Henan province, China	Zhang <i>et al.</i> (2006)
CCBAU 23084	<i>Desmodium microphyllum</i>	Anhui province, China	Gu <i>et al.</i> (2007)
CCBAU 83006	<i>Astragalus oxyglottis</i>	Xinjiang Autonomous Region	Zhao <i>et al.</i> (2008)
<i>R. galegae</i> ATCC 43677 ^T	<i>Galega orientalis</i>	Finland	Lindström (1989)
<i>R. huautlense</i> S02 ^T	<i>Sesbania herbacea</i>	Mexico	Wang <i>et al.</i> (1998)
<i>R. alkalisoli</i> CCBAU 01393 ^T	<i>Caragana intermedia</i>	Inner Mongolia, China	Lu <i>et al.</i> (2009)
<i>R. cellulosilyticum</i> ALA10B2 ^T	<i>Populus alba</i>	Spain	García-Fraile <i>et al.</i> (2007)

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 43677^T, *R. alkalisoli* CCBAU 01393^T and *R. huautlense* S02^T 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'-GGAGAGTT-AGATCTTGGCTCA-3') and 23S-38 (5'-CCGGGTTTC-CCCATTTCGG-3') (Rasolomampianina *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 *Msp*I, *Hae*III and *Hha*I 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 × 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*, *glnI*, *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 05176^T shared 95.6, 96.0 and 94.4 % sequence similarities with *R. galegae* ATCC 43677^T 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

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 76^T was used as outgroup. Bar, 0.01 expected changes per site.

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 (T_m). 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% (T_m), 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

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.

Strain	Similarity (%) with strain CCBAU 05176 ^T				
	16S rRNA	<i>atpD</i>	<i>recA</i>	<i>glnII</i>	DNA–DNA relatedness
Other six strains in the group	100	98.6–100	100	99.6–100	88.0–94.0
<i>R. galegae</i> ATCC 43677 ^T	99.8	95.6	96.0	94.4	22.6
<i>R. huautlense</i> S02 ^T	99.8	93.0	89.5	NA	8.9
<i>R. alikisoli</i> CCBAU 01393 ^T	99.8	95.8	89.5	89.7	15.9
<i>R. cellulosilyticum</i> ALA10B2 ^T	98.3	90.8	85.1	NA	18.5

Fig. 2. Phylogenetic tree constructed from *atpD* gene sequences showing the relationships between the novel group and closely related bacteria. The tree was constructed by the NJ method with a Kimura two-parameter distance matrix. Bootstrap values (%) are based on 1000 replicates and values >70 % are indicated at the nodes. Bar, 0.02 expected changes per site.

using the spectrophotometric method (De Ley *et al.*, 1970). The representative strain CCBAU 05176^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 *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 *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 µg 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 *Sinorhizobium meliloti* (Yan *et al.*, 2000) and *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^T and 30 fatty acids (including those found for strain CCBAU 05176^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_{16:0}, C_{16:0} N alcohol, C_{19:0} cyclo ω8c and summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c) were present as the main compounds (1.31–46.97 %) in strains CCBAU 05176^T, CCBAU 45252, *R. galegae* HAMBI 540^T and *R. huautlense* S02^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^T, CCBAU

Table 3. Distinctive features of *Rhizobium vignae* sp. nov. and the most closely related species of the genus *Rhizobium*

Taxa: 1, *Rhizobium vignae* sp. nov. ($n=7$); 2, *R. galegae* ATCC 43677^T; 3, *R. huaulense* S02^T; 4, *R. alkanisoli* CCBAU 01393^T. +, All strains positive; –, all strains negative. The number of positive strains is indicated where the characteristic was variable.

Characteristic	1	2	3	4
Substrates used as sole carbon source				
D-Amygdalin	2	+	+	+
L-Arginine	2	+	+	+
DL-Asparagine	3	–	–	–
Dextrin	5	–	+	–
Dulcitol	3	–	+	–
Glycine	2	–	–	–
Melibiose	–	+	+	+
L-Proline	3	–	+	–
Salicin	+	–	+	–
Sodium formate	2	–	+	–
L-Threonine	2	–	+	–
Vanillic acid	2	–	–	–
Substrates used as sole nitrogen source				
DL-Threonine	3	–	–	–
Resistance to ($\mu\text{g ml}^{-1}$):				
Bacitracin (300)	+	+	–	–
Erythromycin (100)	3	–	–	–
Erythromycin (50)	3	+	+	+
Kanamycin sulfate (50)	2	–	–	–
Neomycin sulfate (50)	–	+	–	–
Streptomycin sulfate (5)	+	+	–	+
Tetracycline hydrochloride (5)	1	–	–	–
Erythrosine blue (0.1 %)	–	+	–	–
Methyl green (0.1 %)	–	+	–	–
Sodium nitrite (0.1 %)	+	–	+	–
NaCl (2 %, w/v)	3	–	–	–
pH 10.0	3	–	+	–
Nitrate reduction	+	–	+	+

45252, *R. galegae* HAMBI 540^T and *R. huaulense* S02^T, respectively. Fatty acids C_{12:0} 3-OH, C_{15:0} iso 3-OH, C_{15:0} anteiso, C_{16:0} iso, C_{16:0} iso 3-OH, C_{16:0} anteiso, C_{16:1} ω 11c, C_{17:0} iso 3-OH, C_{18:1} ω 7c 11-methyl and C_{18:3} ω 6c 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 *glnII* 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 $\times$ 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 reduction of methyl blue and Nile blue are observed. Acid production and reduction are observed in litmus milk. Resistant to ($\mu\text{g ml}^{-1}$): 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, D-mannose, D-ribose, D-sorbitol, D-amydalin, D-xylose, sodium DL-malate, glucose, calcium gluconate, dulcitol, inositol, L-rhamnose, L-proline, L-threonine, raffinose pentahydrate, salicin, sodium citrate, sodium D-gluconate, maltose, sodium pyruvate, sodium succinate, sodium acetate, 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, L-alanine, DL-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, sorbose, soluble starch, sodium tartrate, sodium hippurate, L-methionine or L-arginine. The common and/or main fatty acid compounds are C_{16:0}, C_{16:0} N alcohol, C_{19:0} cyclo ω 8c, summed feature 4 (C_{17:1} iso I/anteiso B and/or C_{17:1} anteiso B/iso I) and summed feature 8 (C_{18:1} ω 7c and/or C_{18:1} ω 6c).

The type strain, CCBAU 05176^T (=HAMBI 3039^T=LMG 25447^T), 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% (T_m). Additional strains were isolated from *Astragalus dahuricus*, *A. oxyglottis*, *Vigna radiata* and *Desmodium microphyllum* grown in different regions of China.

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