Rhizobium vallis sp. nov., isolated from nodules of three leguminous species

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Four bacterial strains isolated from root nodules of *Phaseolus vulgaris*, *Mimosa pudica* and *Indigofera spicata* plants grown in the Yunnan province of China were identified as a lineage within the genus *Rhizobium* according to the analysis of 16S rRNA gene sequences, sharing most similarity with *Rhizobium lusitanum* P1-7^T (99.1 % sequence similarity) and *Rhizobium rhizogenes* IAM 13570^T (99.0 %). These strains also formed a distinctive group from the reference strains for defined species of the genus *Rhizobium* in a polyphasic approach, including the phylogenetic analyses of the 16S rRNA gene and housekeeping genes (*recA*, *atpD*, *glnII*), DNA–DNA hybridization, BOX-PCR fingerprinting, phenotypic characterization, SDS-PAGE of whole-cell proteins, and cellular fatty acid profiles. All the data obtained in this study suggested that these strains represent a novel species of the genus *Rhizobium*, for which the name *Rhizobium vallis* sp. nov. is proposed. The DNA G+C content (mol%) of this species varied between 60.9 and 61.2 (T_m). The type strain of *R. vallis* sp. nov. is CCBAU 65647^T (=LMG 25295^T =HAMBI 3073^T), which has a DNA G+C content of 60.9 mol% and forms effective nodules on *Phaseolus vulgaris*.

Rhizobia are symbiotic nitrogen-fixing bacteria that reduce N₂ to ammonia after forming root and/or stem nodules with leguminous plants. To date, about 80 rhizobial species have been reported within the four main genera *Rhizobium*, *Ensifer* (*Sinorhizobium*), *Mesorhizobium* and *Bradyrhizobium*, as well as in several other genera in the classes *Alphaproteobacteria* and *Betaproteobacteria* (Wang *et al.*, 2006). The genus *Rhizobium* (Frank, 1889) was the first and only rhizobial genus described for a century, before the genus *Bradyrhizobium* was reported (Jordan, 1982). Since then, a series of taxonomic changes have been made in the genus *Rhizobium* and at the time of writing it contains 47 species, including the recently described species *Rhizobium soli* (Yoon *et al.*, 2010), *Rhizobium borbori* (Zhang *et al.*, 2011b), *Rhizobium vignae* (Ren *et al.*, 2011) and *Rhizobium tubonense* (Zhang *et al.*, 2011a).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *atpD*, *recA* and *glnII* gene sequences of strains CCBAU 65647^T, CCBAU 65648, CCBAU 65643 and CCBAU 65751 are FJ839677, GU211765, GU211766 and EU618035 (16S rRNA gene), GU211768, HM453324, HM453323 and EU617993 (*atpD*), GU211770, HM453320, HM453319 and EU622122 (*recA*), and GU211771, HM453322, HM453321 and EU618013 (*glnII*), respectively.

Two supplementary tables and two supplementary figures are available with the online version of this paper.

In a survey of rhizobial resources in Yunnan, a tropical province of China, four strains isolated from root nodules of *Phaseolus vulgaris*, *Mimosa pudica* and *Indigofera spicata* were identified as a distinct lineage in the genus *Rhizobium* by sequence analysis of the 16S rRNA gene, which was used to screen all the isolates. In order to clarify the taxonomic position of the four strains, a polyphasic study including phylogenetic analyses of the 16S rRNA gene and housekeeping genes (*recA*, *atpD*, *glnII*), BOX-PCR fingerprinting, DNA–DNA hybridization, phenotypic characterization, SDS-PAGE of whole-cell proteins, and cellular fatty acid composition was performed in comparison with reference strains of defined species of the genus *Rhizobium*. Based on the results, these four isolates represent a different group from all defined species and, therefore, a novel species is proposed.

The four novel bacterial strains and the reference strains used in this study are presented in Table 1. All the bacterial strains were maintained on yeast mannitol agar (YMA; Vincent, 1970) at 4 °C for short-term storage and in YM broth (Vincent, 1970) supplemented with 20 % (w/v) glycerol at –80 °C for long-term storage.

Genomic DNA was extracted from each of the four novel strains by the method of Terefework *et al.* (2001) and used as template for 16S rRNA gene amplification. The almost complete 16S rRNA gene was amplified with the primers P1

Table 1. Isolates and reference strains used in this study

Strain*	Host plant	Geographical origin
<i>Rhizobium vallis</i> sp. nov.		
CCBAU 65647 ^T	<i>Phaseolus vulgaris</i>	Yunnan, China
CCBAU 65648	<i>Phaseolus vulgaris</i>	Yunnan, China
CCBAU 65643	<i>Indigofera spicata</i>	Yunnan, China
CCBAU 65751	<i>Mimosa pudica</i>	Yunnan, China
Reference strains		
<i>R. etli</i> CFN 42 ^T	<i>Phaseolus vulgaris</i>	Mexico
<i>R. leguminosarum</i> USDA 2370 ^T	<i>Pisum sativum</i>	USA
<i>R. rhizogenes</i> IAM 13570 ^T	<i>Phaseolus vulgaris</i>	
<i>R. tropici</i> IIA LMG 9517	<i>Phaseolus vulgaris</i>	Brazil
<i>R. tropici</i> IIB LMG 9503 ^T	<i>Leucaena leucocephala</i>	Colombia
<i>R. lusitanum</i> LMG 22705 ^T	<i>Phaseolus vulgaris</i>	Portugal
<i>R. miluonense</i> CCBAU 41251 ^T	<i>Lespedeza chinensis</i>	Hunan, China
<i>R. multihospitium</i> CCBAU 83401 ^T	<i>Halimodendron halodendron</i>	Xinjiang, China

*CCBAU, Culture Collection of Beijing Agricultural University, Beijing, PR China; CFN, Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Mexico; USDA, US Department of Agriculture Agricultural Research Service National *Rhizobium* Culture Collection; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; LMG, Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Gent, Belgium.

and P6 (Chen *et al.*, 1995) and sequenced directly as described by Hurek *et al.* (1997). All sequences were aligned using the CLUSTAL W program in the MEGA 4.0 software (Tamura *et al.*, 2007). Distances were calculated according to the models of Jukes & Cantor (1969), Tajima & Nei (1984), Tamura & Nei (1993) and Kimura's two-parameter (Kimura, 1983). Phylogenetic trees based upon 16S rRNA gene sequences were reconstructed with the neighbour-joining, maximum-parsimony and maximum-likelihood methods and were bootstrapped with 1000 replications. Regardless of tree reconstruction method, similar topology was obtained in all phylogenetic trees, and only the neighbour-joining tree with the model of Jukes & Cantor (1969) is shown in Fig. 1. The four novel strains had almost identical 16S rRNA gene sequences (99.9 % to 100 % similarities) and formed a unique phylogenetic lineage in the genus *Rhizobium* (Fig. 1). The representative strain, CCBAU 65647^T, showed 99.1 % similarity with *Rhizobium lusitanum* P1-7^T, 99.0 % similarity with *Rhizobium rhizogenes* IAM 13570^T and 98.8, 98.7, 98.5, 98.5, 98.2 and 97.7 % similarity, respectively, with *Rhizobium miluonense* CCBAU 41251^T, *Rhizobium multihospitium* CCBAU 83401^T, *Rhizobium etli* CFN 42^T, *Rhizobium tropici* IIA LMG 9517, *Rhizobium tropici* IIB CIAT 899^T and *Rhizobium leguminosarum* bv. *viciae* USDA 2370^T.

DNA–DNA hybridization is considered to be a standard method for species definition and the recommended minimum value for strains of the same species is 70 % (Graham *et al.*, 1991; Wayne *et al.*, 1987). To determine DNA–DNA relatedness and DNA base composition, total DNA was extracted from each strain using the method of Marmur (1961). DNA–DNA hybridization was performed

using the initial renaturation rate method (De Ley *et al.*, 1970). DNA–DNA relatedness values between strain CCBAU 65647^T and the other three strains were 97.3, 96 and 82.2 %, respectively, which is above the species threshold of 70 %, indicating that they were the same genomic species. Strain CCBAU 65647^T showed 39.3 and 39.4 % DNA–DNA relatedness, respectively, with the most related strains, *R. rhizogenes* IAM 13570^T and *R. lusitanum* P1-7^T (Supplementary Table S1 available in IJSEM Online), which was much lower than the species threshold. These results suggested that the genomic species represented by strain CCBAU 65647^T was different from defined species.

The DNA G+C content was 60.9 mol% (T_m) for strain CCBAU 65647^T and 61.2 mol% for strain CCBAU 65751, measured using the thermal denaturation method of Marmur & Doty (1962), with *Escherichia coli* K-12 as a standard. These values were within the range for members of the genus *Rhizobium* (Jordan, 1982).

The concatenation of housekeeping gene sequences has been suggested as an easy tool to assess interorganismal relationships (Martens *et al.*, 2008). In the present study, using the same template DNA used for 16S rRNA gene amplification and the PCR protocols of Vinuesa *et al.* (2005a), the housekeeping genes *atpD*, *recA* and *glnII* were amplified separately with the primer pairs *atpD*255F and *atpD*782R, *recA*41F and *recA*640R, and *glnII*12F and *glnII*689R, respectively (Gaunt *et al.*, 2001; Turner & Young, 2000; Valverde *et al.*, 2006; Vinuesa *et al.*, 2005a, b). The PCR products were sequenced directly and a phylogenetic tree (Supplementary Fig. S1) was reconstructed based on the concatenated gene sequences (*atpD*, *recA* and *glnII*) by using MEGA 4.0 software. In the concatenated tree, the novel strains

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Rhizobium vallis* sp. nov. strains (in bold face) and closely related species within the genus *Rhizobium*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 1 % nucleotide substitutions.

formed two lineages in a subclade together with reference strains for *Rhizobium leguminosarum* and *Rhizobium etli*, demonstrating that these four strains represent diverse populations and that their housekeeping genes have evolved divergently.

The symbiotic genes (*nod*) are required for the successful establishment of highly specific symbiosis between rhizobia and legumes, and are important determinants of rhizobial host specificity (Guan *et al.*, 2008). Therefore, comparisons of symbiotic genes may reveal the host ranges of rhizobia and allow estimation of their symbiotic properties (Kalita *et al.*, 2006; Laranjo *et al.*, 2008; van Berkum *et al.*, 2007). In this study, partial *nodC* (*N*-acetylglucosaminyltransferase) fragments were amplified with the previously described primers NodC540 and NodC1160 (Sarita *et al.*, 2005) from strains CCBau 65647^T and CCBau 65648. The amplified fragments were directly sequenced with the same primers. No *nodC* fragment was amplified from strains CCBau 65643 and CCBau 65751 with these primers and *nodCF* and *nodCI* (Laguerre *et al.*, 2001). In the phylogenetic

analysis of *nodC* (Fig. 2), strain CCBau 65647^T had 100 % sequence similarity with strain CCBau 65648 and the three reference strains *R. leguminosarum* bv. *phaseoli* H132, *Rhizobium giardinii* bv. *phaseoli* H251 and *Rhizobium gallicum* bv. *phaseoli* PhD12, which was independent from the phylogeny of the 16S rRNA and housekeeping genes. All of these reference strains are symbionts of *Phaseolus vulgaris*. The *nodC* sequence similarities were ≤ 71.2 % between CCBau 65647^T and other reference strains belonging to *R. tropici*, *R. leguminosarum*, *R. giardinii*, *R. etli* and others.

BOX-PCR fingerprinting is widely applied for characterization and identification of bacterial strains (de Bruijn, 1992; Huber & Selenska-Pobell, 1994; Judd *et al.*, 1993; Nick & Lindstrom, 1994; Nick *et al.*, 1999). BOX-PCR was performed by using the primer BOXAIR (5'-CTACGGCA-AGGCGACGCTGACG-3') and procedure of Versalovic *et al.* (1994). The amplified products were separated by electrophoresis in 1.5 % (w/v) agarose gels stained with ethidium bromide. The fingerprints were analysed by the Gelcompar II program (Applied Maths) and an unweighted

Fig. 2. Neighbour-joining dendrogram based on *nodC* gene sequences showing the relationships among representative strains of *Rhizobium vallis* sp. nov. and related species. Bootstrap confidence levels greater than 50 % are indicated at nodes. Bar, 5 % nucleotide substitutions.

pair group method with arithmetic mean (UPGMA) dendrogram was reconstructed using the BOX-PCR profile. All the test strains had unique BOX-PCR fingerprints, indicating that they were not clones, and these strains formed a cluster at a similarity level of 94 % (Fig. 3), which was greater than the similarities obtained among the recognized species of the genus *Rhizobium*.

SDS-PAGE of whole-cell proteins has been used widely in rhizobial taxonomy (Diouf *et al.*, 2000; Tan *et al.*, 1997; Wang *et al.*, 2006) and is suitable to group strains at species level. Methods described previously (Tan *et al.*, 1997; Wang *et al.*, 2006) were applied to extract whole-cell proteins from the test strains and to perform SDS-PAGE analysis. Digitization, normalization and numerical analyses of the protein profiles were performed with the GelCompar II version 4.5 software (Applied Maths). Similarities between pairs of protein patterns were expressed by the Pearson's coefficient and an UPGMA dendrogram was reconstructed (Vauterin & Vauterin, 1992). The four novel strains formed a cluster at a similarity level of 73 % (Supplementary Fig. S2), which was quite different from recognized species of the genus *Rhizobium* and was greater than the similarities obtained among the reference strains of *R. tropici*, *R. multihospitium*, *R. rhizogenes* and *R. lusitanum*. These data showed that the four strains were different strains with similar genomic backgrounds.

Phenotypic characteristics of the four novel strains and reference strains of the most closely related species of the genus *Rhizobium* were tested using the methods of Gao *et al.* (1994), including 45 different carbon sources and 13 amino acids as sole nitrogen sources, resistance to various antibiotics (300, 100, 50 and 5 $\mu\text{g ml}^{-1}$), tolerance to salt (0–5 %, w/v), temperature range for growth (4, 10, 28, 37 and 45 °C) and pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0). Production of catalase, urease, L-phenylalaninase and oxidase, nitrate reduction, and reaction in litmus milk were also studied. Some distinctive features are listed in Table 2. The results demonstrated that the four test strains had very similar features and formed a phenon sharing similarities greater than 80 %, a value recommended as a threshold for delineating species of the genus *Rhizobium* (Chen *et al.*, 1988; Gao *et al.*, 1994), and this phenon could be distinguished by several distinctive characteristics from closely related species of the genus *Rhizobium* (Table 2).

Differences in fatty acid profiles, which are in broad agreement with 16S rRNA gene sequence similarities and appear to accurately distinguish most species, have been used to describe novel bacterial species (Quan *et al.*, 2005; Schutter & Dick, 2000; Tighe *et al.*, 2000). In this study, the fatty acids were analysed for strain CCAU 65647^T and the reference strains *R. etli* CFN 42^T, *R. huautlense* S02^T and *R. leguminosarum* USDA 2370^T. After 72 h of incubation at

Fig. 3. Electrophoretic protein patterns showing the relationships between strains of *Rhizobium vallis* sp. nov. (in bold face) and reference strains of phylogenetically related species.

Table 2. Distinctive features of *Rhizobium vallis* sp. nov. and phylogenetically related species

Strains: 1, *R. vallis* sp. nov. ($n=4$); 2, *R. rhizogenes* IAM 13570^T; 3, *R. lusitanum* P1-7^T; 4, *R. multihospitium* CCBAU 83401^T; 5, *R. tropici* IIA CFN 299; 6, *R. tropici* IIB CIAT 899^T; 7, *Rhizobium miluonense* CCBAU 41251^T; 8, *R. leguminosarum* USDA 2370^T; 9, *R. etli* CFN 42^T; +, Positive; –, negative. Numbers represent the percentage of the strains with a positive reaction.

Characteristic	1	2	3	4	5	6	7	8	9
Utilization as sole carbon source									
Amygdalin	25	+	+	+	+	–	+	+	+
Calcium gluconate	–	–	+	+	–	–	–	–	–
Calcium malonate	–	+	–	–	+	+	+	+	–
Dextrin	–	+	+	–	–	–	–	–	–
meso-Erythritol	–	+	+	+	+	+	+	+	+
Inulin	–	–	+	–	–	–	–	+	–
Sodium DL-malate	–	+	+	+	+	+	–	+	+
Sodium pyruvate	–	–	+	+	+	+	+	+	–
D-Ribose	75	+	–	+	+	+	+	+	+
Salicin	+	+	+	+	–	–	+	+	+
Sodium acetate	–	–	+	–	–	–	+	+	+
Sodium citrate	–	–	+	+	+	+	–	–	+
Sodium D-gluconate	–	–	+	+	–	–	–	+	–
Sodium hippurate	–	–	–	–	–	–	–	+	–
Trehalose	+	–	+	+	+	+	+	–	–
L-Arginine	–	+	+	–	+	+	–	–	+
DL-Asparagine	–	–	+	–	+	+	–	–	+
DL-Proline	75	–	+	+	–	–	–	+	+
Resistance to ($\mu\text{g ml}^{-1}$):									
Gentamicin sulfate (5)	–	+	–	+	+	+	–	–	+
Erythromycin (5)	50	+	+	+	+	+	+	+	+
Neomycin (50)	–	+	+	+	+	–	–	+	+
Ampicillin (50)	–	+	+	+	–	+	+	+	+
Ampicillin (100)	–	–	+	+	–	+	+	–	–
Ampicillin (300)	–	–	–	+	–	+	–	–	–
Kanamycin (5)	+	+	+	+	+	+	–	+	+
Kanamycin (50)	–	+	+	+	–	–	–	+	+
Streptomycin sulfate (5)	–	+	+	+	–	+	+	+	+
Streptomycin sulfate (50)	–	–	+	+	–	+	–	+	+
Streptomycin sulfate (100)	–	–	–	+	–	–	–	+	+
Chloramphenicol (5)	–	+	+	+	+	–	+	+	+
Chloramphenicol (50)	–	–	+	+	–	–	–	+	+
Tolerance to 1% NaCl (w/v)	–	+	+	–	–	+	–	–	+
Acid production in litmus milk	+	+	+	+	–	+	–	+	+
Alkaline production in litmus milk	–	–	–	–	+	–	+	–	–
Litmus acid coagulation	+	–	+	+	–	+	–	–	+

28 °C on YMA, well-grown cells were harvested and fatty acids were extracted (Hou *et al.*, 2009) and identified by the standard method of the Microbial Identification System (MIDI) with an HP 6890 GC using the database TSBA6 for identification of the peaks. The cellular fatty acid profile of the novel group was compared with those of related species of the genus *Rhizobium* (Supplementary Table S2). The novel strains could be assigned to the genus *Rhizobium* because they possessed the major fatty acids 16:0, 16:0

3-OH, 18:0, 19:0 cyclo ω 8c, summed feature 2 and summed feature 8 (Hou *et al.*, 2009), which are common in members of the genus *Rhizobium*. The novel strain could be differentiated from other species of the genus *Rhizobium* particularly by the presence of relatively smaller amounts of 17:0 cyclo, 14:0 and summed feature 5 and larger amounts of 15:0 2-OH, 18:0 3-OH, 18:1 ω 9c, 11-methyl 18:1 ω 7c and 19:0 cyclo ω 8c. The novel strain does not contain 13:0 2-OH, iso-15:0, iso-15:0 3-OH, anteiso-15:0, iso-15:1 G or 20:2 ω 6,9c (Supplementary Table S2).

Nodulation and nitrogen fixation abilities are important characteristics for species of the genus *Rhizobium* and host range is an important feature for the description of novel rhizobial species (Graham *et al.*, 1991). To check the host range and cross-nodulation, seeds of *Phaseolus vulgaris*, *Mimosa pudica*, *Medicago sativa*, *Trifolium repens*, *Leucaena leucocephala* and *Indigofera spicata* were surface-sterilized, germinated and inoculated with strain CCBAU 65647^T by the methods of Trujillo *et al.* (2005). The seeds of *Mimosa pudica* and *Indigofera spicata* were also inoculated with strains CCBAU 65643 and CCBAU 65751. After growing for 7 weeks under natural sunlight in a greenhouse, effective nodules were observed on roots of *Phaseolus vulgaris* inoculated with strain CCBAU 65647^T, while no nodules were found on *Mimosa pudica*, *Medicago sativa*, *Trifolium repens* or *Leucaena leucocephala* inoculated with strain CCBAU 65647^T or on *Mimosa pudica* or *Indigofera spicata* inoculated with strains CCBAU 65643 or CCBAU 65751. This host range differed from those of *R. multihospitium* and *R. miluonense*, which failed to nodulate *Phaseolus vulgaris* effectively (Han *et al.*, 2008; Gu *et al.*, 2008). The cross-nodulation tests and *nodC* analysis demonstrated that the test strains were not symbionts of *Mimosa pudica* or *Indigofera spicata*, but they might be nodule endophytes of these plants. In addition, strains CCBAU 65643 and CCBAU 65751 might be non-symbiotic bacteria.

Taking into consideration the data from this study and the current criteria of rhizobial species description (Graham *et al.*, 1991; Wayne *et al.*, 1987), we propose the novel group as a novel species of the genus *Rhizobium* with the name *Rhizobium vallis* sp. nov. This species can be differentiated genotypically and phenotypically from previously defined species.

Description of *Rhizobium vallis* sp. nov.

Rhizobium vallis (val'lis. L. gen. n. *vallis* of a valley, referring to the fact that the strains were isolated from the Valley of Red River in Yunnan province, China).

Cells are Gram-negative, motile, aerobic, non-spore-forming rods, 1.0–1.5 $\mu\text{m} \times 2.0$ –3.5 μm . Colonies on YMA are circular, convex, pearl-white, translucent and usually 2–3 mm in diameter after 3 days of incubation at 28 °C. Strains can grow at pH 6.0–9.0 and cannot grow with up to 1% (w/v) NaCl. Optimum growth temperature is 28 °C; cells can grow after exposure to 60 °C for 10 min and cannot grow at 10 °C

or 37 °C. Tests for catalase, urease, oxidase and litmus milk acid production, acid coagulation and peptonization are positive. Tests for nitrate reductase, cellulose hydrolase, L-phenylalanine deaminase, H₂S production and hydrolysis of starch, gelatin, DNA and Tween 80 are negative. Reduces litmus milk, but methyl red and Voges–Proskauer reactions are negative. D-Arabinose, galactitol, D-fructose, D-galactose, glucose, inositol, lactose, sodium malate, maltose, D-mannose, melibiose, raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, sucrose, trehalose, D-xylose, and DL-proline can be used as sole carbon sources. Strains grow weakly on media with adipate, melezitose, D-amygdalin, calcium gluconate, calcium malonate, dextrin, meso-erythrose, inulin, sodium pyruvate, sodium acetate, sodium citrate, sodium formate, sodium gluconate, sodium succinate, sodium hippurate, sorbose, starch, syringic acid, potassium sodium tartrate or vanillic acid as sole carbon source. DL-Alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, hypoxanthine, L-isoleucine, L-lysine, L-phenylalanine, L-threonine, L-methionine, D-valine and DL-proline are used as sole nitrogen sources. Resistant to (μg ml⁻¹) kanamycin (5) and neomycin (5); and sensitive to ampicillin (50), kanamycin (50), neomycin (50), erythromycin (5), gentamicin sulfate (5) and streptomycin sulfate (5). The pattern of cellular fatty acids of the type strain is given in Supplementary Table S2.

The type strain, CCBAU 65647^T (=LMG 25295^T =HAMBI 3073^T), was isolated from root nodules of *Phaseolus vulgaris* and its DNA G+C content is 60.9 mol% (T_m).

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