Rhizobium fabae sp. nov., a bacterium that nodulates *Vicia faba*

Chang Fu Tian,¹ En Tao Wang,^{1,2} Li Juan Wu,¹ Tian Xu Han,¹
Wen Feng Chen,¹ Chun Tao Gu,¹ Jin Gang Gu³ and Wen Xin Chen¹

Correspondence

Wen Xin Chen

wxchencau@yahoo.com.cn

¹Key Laboratory of Agro-Microbial Resource and Application, Ministry of Agriculture/College of Biological Sciences, China Agricultural University, 100094 Beijing, PR China

²Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, 11340 México D. F., Mexico

³Institute of Agricultural Resources and Regional Planning of Chinese Academy of Agricultural Sciences, 100081 Beijing, PR China

Six strains were isolated from root nodules of *Vicia faba* grown in Nanchang, Yifeng, Taihu, Huaibei, Bengbu and Lujiang, in the middle and lower reaches of the Yangtze River. According to phylogenetic analyses of 16S rRNA gene, *atpD* and *recA* sequences, these strains belong to the genus *Rhizobium*, with *Rhizobium etli* and *Rhizobium leguminosarum* as the closest related species. CCBAU 33202^T, a representative of these novel isolates, showed sequence similarity to its closest relatives *R. etli* CFN 42^T and *R. leguminosarum* USDA 2370^T of 99.5 and 99.1 % for the 16S rRNA gene, 91.9 and 91.9 % for *atpD* and 90.3 and 93.2 % for *recA*. The strains from this study could also be differentiated from *R. etli* CFN 42^T and *R. leguminosarum* USDA 2370^T by 16S-IGS RFLP and SDS-PAGE of whole-cell proteins, fatty acid profiles and several phenotypic characteristics. DNA–DNA hybridization yielded relatedness of 19 and 14–43 %, respectively, with *R. etli* CFN 42^T and strains representing different biovars of *R. leguminosarum*. All data obtained in this study showed that these *V. faba* isolates belong to a novel species, for which the name *Rhizobium fabae* sp. nov. is proposed. The type strain, CCBAU 33202^T (=LMG 23997^T =JCM 14381^T), was isolated from Nanchang.

It is well known that *Vicia faba* and other species of *Vicia*, *Pisum*, *Lathyrus* and *Lens* form nodules with *Rhizobium leguminosarum* bv. *viciae*, one of the three biovars of *R. leguminosarum* (Jordan 1984). Earlier studies revealed that rhizobia isolated from nodules of *V. faba* in different geographical locations were diverse in their chromosomal background and plasmid content, and rhizobial strains of *V. faba* were distinguishable from rhizobial strains of *Pisum sativum*, especially in the nodulation genotypes (van Berkum *et al.*, 1995; Laguerre *et al.*, 2003; Young *et al.*, 2003; Mutch *et al.*, 2003; Mutch & Young, 2004). However, no species other than *Rhizobium leguminosarum* bv. *viciae* has been reported to nodulate *V. faba*. In our previous

study, wide diversity was revealed amongst rhizobia associated with *V. faba* in Chinese fields (Tian *et al.*, 2007). Six strains isolated from six sites in the middle and lower reaches of the Yangtze River formed a distinct group belonging to *Rhizobium* based upon analysis of BOX-A1R PCR, amplified fragment length polymorphism analysis, amplified 16S rDNA restriction analysis (ARDRA), 16S rRNA gene phylogeny and DNA–DNA hybridization (Tian *et al.*, 2007). In the present study, a polyphasic approach was used to clarify the taxonomic affiliation of this *Rhizobium* group.

Strains CCBAU 33202^T and CCBAU 33201, isolated from Nanchang and Yifeng in Jiangxi province, and CCBAU 23123, CCBAU 23122, CCBAU 23127 and CCBAU 23132, isolated from Taihu, Huaibei, Bengbu and Lujiang in Anhui province, all isolated from *V. faba*, were used in this study. Standard procedures and YMA medium (Vincent, 1970) were used to isolate and cultivate the strains. DNA samples were prepared as described by Terefework *et al.* (2001).

Previously, these six strains have been grouped in the same ARDRA type, and phylogenetic analysis of 16S rRNA gene

The GenBank/EMBL/DDBJ accession numbers for the *recA* and *atpD* sequences of strains CCBAU 23122, CCBAU 23123, CCBAU 23127, CCBAU 23132, CCBAU 33201 and CCBAU 33202^T are respectively EF579936–EF579941 (*recA*) and EF579924–EF579929 (*atpD*). Those for the *nodD* sequences of strains CCBAU 33202^T and CCBAU 23123 are EU430078 and EU430079.

Neighbour-joining trees based on *recA*, *atpD* and *nodD* sequences and dendrograms based on 16S-IGS RFLP and whole-cell protein SDS-PAGE profiles are available as supplementary material with the online version of this paper.

sequences of CCBAU 33202^T and CCBAU 23132 showed that they belong to the genus *Rhizobium* (Tian *et al.*, 2007). According to the 16S rRNA gene sequence analysis, the closest relative of CCBAU 33202^T is *Rhizobium etli* CFN 42^T (99.5 % similarity), followed by *R. leguminosarum* bv. *trifolii* T24 (99.3 %), *R. leguminosarum* bv. *viciae* USDA 2370^T (99.1 %), *R. leguminosarum* bv. *phaseoli* USDA 2671 (99.1 %), *Rhizobium rhizogenes* IAM 13570^T (98.1 %), *Rhizobium lusitanum* P1-7^T (98.0 %), *Rhizobium tropici* type B CIAT 899^T (97.5 %) and *R. tropici* type A LMG 9517 (96.8 %).

In order to confirm the phylogenetic position of the six new isolates, in addition to the 16S rRNA gene sequence, the core housekeeping genes *atpD* and *recA* were sequenced and their phylogenies were compared with that of the 16S rRNA gene. The two housekeeping genes are unlinked and provide independent genealogies from which to infer a species tree (Nichols, 2001; Rosenberg, 2002; Vinuesa *et al.*, 2005). The methods and primers *atpD*273F (5'-SCTGGG-SCGYATCMTGAACGT-3') and *atpD*771R (5'-GCCGACACTTCCGAACCGCCTG-3') (Gaunt *et al.*, 2001) for *atpD* and *recA*63F (5'-ATCGAGCGGTCGTTCCGGCAAGGG-3') and *recA*555R (5'-CGRATCTGGTTGATGAAGATCACCAT-3') for *recA* described by Gaunt *et al.* (2001) were used to amplify the corresponding genes. The PCR products were purified and sequenced directly. CLUSTAL W (Jeanmougin *et al.*, 1998; Thompson *et al.*, 1994) integrated in MEGA3.1 (Kumar *et al.*, 2004) and BioEdit (Hall, 1999) were used to align and edit the acquired sequences and the related sequences obtained from GenBank. Phylogenetic trees were then reconstructed by the neighbour-joining method with 1000 bootstrap replications using MEGA3.1. As shown in Supplementary Figs S1 and S2, available in IJSEM Online, the results obtained further confirm the distinct phylogenetic position of these test strains within the group of *R. leguminosarum*–*R. etli*. Sequence similarity among the strains isolated from *V. faba* was above 99.8 % for *atpD* and more than 97.9 % for *recA*. The similarity between CCBAU 33202^T and *R. etli* CFN 42^T and *R. leguminosarum* USDA 2370^T was 91.9 % for both strains for *atpD* and respectively 90.3 and 93.2 % for *recA*. These values are similar to those found between other closely related *Rhizobium* species, such as 94.1, 92.3 and 91.8 % for *atpD* and 91.9, 92.0 and 90.6 % for *recA*, respectively, between *R. lusitanum* P1-7^T and *R. rhizogenes* IAM 13570^T, *R. tropici* A CFN 299 and *R. tropici* B CIAT 899^T (Valverde *et al.*, 2006). These sequence similarities with respect to *R. etli* and *R. leguminosarum* suggest that the strains isolated in this study may belong to a novel species.

The G + C content of the novel strains was 61.5–61.9 mol% (Tian *et al.*, 2007), within the range reported for the genus *Rhizobium* (Jordan, 1984). DNA–DNA reassociation analysis was carried out as described by De Ley *et al.* (1970). The DNA–DNA relatedness of strain CCBAU 33202^T with the other five test strains was ≥ 73 %, and strain CCBAU 33202^T showed 19 and 14–43 % relatedness, respectively, with *R. etli* CFN 42^T and strains of the three biovars of *R.*

leguminosarum, including the type strain, USDA 2370^T (Supplementary Table S1). These results indicate that the six strains from this study do not belong to these recognized species when a value of 70 % or higher DNA–DNA relatedness for rhizobial species definition was considered (Graham *et al.*, 1991).

In addition to 16S rRNA gene and housekeeping gene analyses and DNA–DNA reassociation, DNA profiling methods that discriminate at the subspecific level also show great promise in the definition of bacterial species (Stackebrandt *et al.*, 2002). In this study, the 16S–IGS region was amplified as described by Rasolomampianina *et al.* (2005) and the PCR products were digested with *DdeI*, *HaeIII* and *MspI*. A UPGMA dendrogram of 16S–IGS RFLP profiles was obtained with the same method described previously for ARDRA (Tian *et al.*, 2007). Similarities of more than 93.2 % among the *V. faba* isolates and less than 72.8 % between the test strains and reference strains for defined *Rhizobium* species were observed in the 16S–IGS RFLP UPGMA dendrogram (based on the Dice correlation similarity coefficient) (Supplementary Fig. S3). Moreover, whole-cell protein SDS-PAGE, a subspecific discriminating method at the protein level, was performed to analyse these novel strains and related *Rhizobium* species with methods described previously (Tan *et al.*, 1997). Normalized densitometric traces of the electrophoretic protein patterns were grouped with Pearson's correlation coefficient and the UPGMA method integrated in the GelCompar software package. The test strains grouped at a similarity of 83 % and clustered with other *Rhizobium* strains at a similarity of 29 % (Supplementary Fig. S4).

Strains CCBAU 33202^T and CCBAU 23123, representing the *nodC*–*nodD* RFLP symbiotic genotypes *B* and *M* identified previously in the novel strains (Tian *et al.*, 2007), were chosen for *nodD* sequence analysis with the same procedure described above for housekeeping gene sequences. The *nodD* primers used herein were the same as those in our earlier study (Tian *et al.*, 2007). In the phylogenetic tree (Supplementary Fig. S5), the novel strains were grouped with *R. leguminosarum* bv. *viciae* strains nodulating various legume hosts, such as *Vicia sativa*, *V. cracca*, *V. hirsuta*, *V. faba*, *Lathyrus aphaca*, *L. nissolia*, *L. pratensis* and *Pisum sativum*. The *nodD* phylogeny was correlated with the host range of the test strains, since all these novel strains formed effective nodules on *V. faba* (Tian *et al.*, 2007) and CCBAU 33202^T and CCBAU 23123 could form effective pink nodules on *Pisum sativum* and *Lathyrus* species and did not nodulate *Phaseolus vulgaris*, *Trifolium*, *Glycine max* or *Medicago sativa*. In short, nodulation gene analysis and nodulation ability tests demonstrated that these novel strains have similar symbiotic characteristics to *V. faba* isolates identified as *R. leguminosarum* bv. *viciae* (Jordan, 1984).

It has been found that similarities derived from fatty acid analysis are in broad agreement with 16S rRNA gene sequence similarities and appear to distinguish accurately between most rhizobial species (Tighe *et al.*, 2000). In the

genus *Rhizobium*, *R. etli* and *R. leguminosarum* are discriminated less clearly from each other and form a subcluster with similar fatty acid profiles (Tighe *et al.*, 2000). Using a previously described method (Graham *et al.*, 1995; Jarvis & Tighe, 1994; Tighe *et al.*, 2000), fatty acid profiles of strains CCBAU 33202^T and CCBAU 23123 from *V. faba* were determined in this study and were compared with those of the related *Rhizobium* species (Table 1; Tighe *et al.*, 2000). Major fatty acids found in *Rhizobium*, such as 16:0, 18:0, 19:0 cyclo ω 8c, summed feature 3 and summed feature 7, were detected in the test strains, and the novel strains could be differentiated from members of other rhizobial genera. Within the genus *Rhizobium*, the *V. faba* strains could also be differentiated from related species (Table 1). For example, they have 15:0 2-OH, 18:0 3-OH

and 18:1 ω 9c, but not iso-15:0 3-OH, 18:1 2-OH or 20:3 ω 6,9,12c, and they have relatively larger amounts of summed feature 7 and smaller amounts of 19:0 cyclo ω 8c.

Phenotypic characteristics were tested for the novel strains and reference strains of the closest related species of genus *Rhizobium* using the methods of Gao *et al.* (1994). Forty-two different carbon sources and 15 amino acids as sole nitrogen sources were tested. Antibiotic resistance was investigated with ampicillin, bacitracin, chloramphenicol, erythromycin, kanamycin, neomycin and streptomycin at concentrations of 300, 100, 50 and 5 μ g ml⁻¹ using YMA medium. The temperature range for growth was determined by incubating cultures in YMA plates between 4 and 37 °C. The pH range was measured in YMA or TY

Table 1. Major fatty acids of the novel strains (*Rhizobium fabae* sp. nov.) and related *Rhizobium* species

Strains: 1, *R. galegae* (n=19); 2, *R. giardinii* (n=20); 3, *R. gallicum* (n=17); 4, *R. hainanense* (n=13); 5, *R. tropici* (n=18); 6, *R. etli* (n=30); 7, *R. leguminosarum* (n=28); 8, *R. fabae* CCBAU 33202^T; 9, *R. fabae* CCBAU 23123. Values are percentages of total fatty acids; values below 0.1 % are not shown. Data for reference species were taken from Tighe *et al.* (2000) and are means for the number of strains shown. ECL, Equivalent chain length.

Fatty acid	1	2	3	4	5	6	7	8	9
8:0 3-OH								0.13	
10:0 iso								0.11	
Unknown ECL 11.541				0.54					
Unknown ECL 14.952		0.21	0.25	1.09		0.20			
13:1 at 12–13			0.48	0.85					
14:0								0.13	
Unknown ECL 14.780		0.10							
15:0 2-OH			0.48			0.70	0.87	0.80	2.38
15:0 iso 3-OH				2.94	3.40				
Unknown ECL 15.953					0.11				
16:0	9.72	11.39	3.22	6.33	7.70	2.53	4.08	2.37	2.04
16:0 3-OH	3.30		2.29	3.53	3.87	1.20	1.32	0.96	0.88
17:0	0.43	0.58		0.33		0.32	0.32		
17:0 iso				1.32	0.93				
17:0 iso 3-OH				0.15	0.31				
17:0 cyclo	0.11	1.77		0.20	0.68		0.15		
17:1 ω 8c		0.12							
18:0	1.20	2.35	9.26	3.98	2.87	8.71	7.83	6.75	5.52
18:0 3-OH								1.41	1.57
11-Methyl 18:1 ω 7c	0.10	2.87		0.89	0.55	1.48	4.70	0.89	1.28
18:1 2-OH				1.95	0.82				
18:1 ω 9c						0.12		0.92	1.67
19:0 cyclo ω 8c	31.12	2.78	7.30	38.84	49.29	10.19	13.91	4.89	1.58
10-Methyl 19:0	2.32	1.53	0.10					0.18	
20:1 ω 7c								0.28	
20:1 ω 9t			0.23						
20:2 ω 6,9c	0.11			0.57	0.39			0.34	
20:3 ω 6,9,12c	1.25	2.66	0.77	2.80	1.83	1.95	2.31		
Summed features*									
Summed feature 3	4.82	10.45	6.25	3.30	2.33	5.95	6.46	6.75	8.67
Summed feature 4	0.57	4.37	0.58	0.09	0.16	0.22	0.52	0.53	
Summed feature 7	44.78	58.80	68.65	30.16	24.61	66.17	57.22	72.55	74.42

*Summed features represent groups of two or more fatty acids that could not be separated by the MIDI System. Summed feature 3 contains one or more of 12:0 (aldehyde?), unknown ECL 10.928, 16:1 iso I and/or 14:0 3-OH; summed feature 4 contains 15:0 iso 2-OH and/or 16:1 ω 7c; summed feature 7 contains one or more of 18:1 ω 7c/ ω 9t/ ω 12t and/or 18:1 ω 7c/ ω 9c/ ω 12t.

medium with a final pH between 4.0 and 11.2. Salt tolerance was studied in YMA medium containing 0–4% (w/v) NaCl. Aerobic and anaerobic growth in semisolid YMA medium, indole production, reactions in the methyl red and Voges–Proskauer tests and hydrolysis of starch, gelatin, DNA and Tween 80 were also determined. The six novel strains from this study had the same characteristics in most cases except that CCBAU 23123 could grow at pH 9.0–9.4 and resist 300 µg bacitracin ml⁻¹. Strains CCBAU 33202^T, CCBAU 23123 and CCBAU 23127 were further compared with *R. etli* CFN 42^T and *R. leguminosarum* USDA 2370^T in the following properties: activities of catalase, urease, oxidase and nitrate reductase, reduction of litmus milk, Nile blue and methylene blue and litmus milk acid production, acid coagulation, alkali production and peptonization. Strains CCBAU 33202^T, CCBAU 23123 and CCBAU 23127 had the same characteristics in these tests. The novel strains could be differentiated from their closest relatives, *R. etli* CFN 42^T and *R. leguminosarum* USDA 2370^T, by several phenotypic characteristics (Table 2).

The novel strains that nodulate *V. faba* in the middle and lower reaches of the Yangtze River in China can therefore be differentiated genotypically and phenotypically from previously described species, and we propose the name *Rhizobium fabae* sp. nov. to accommodate them.

Table 2. Distinctive features of *R. fabae* sp. nov. and its closest relatives

Strains: 1, *R. fabae* sp. nov. (six strains were investigated for utilization of carbon sources and antibiotic resistance; three strains were investigated for reduction of dyes and litmus acid coagulation); 2, *R. etli* CFN 42^T; 3, *R. leguminosarum* USDA 2370^T. Data were obtained in this study unless indicated.

Feature	1	2	3
Utilization as sole carbon source of:			
Calcium gluconate	+	–	–
Calcium malonate	–	– ^{a*}	+ ^a
Erythritol	–	– ^b	+ ^b
Inulin	–	–	+
Sodium pyruvate	+	–	+
Sodium D-gluconate	–	–	+
Sodium hippurate	–	–	+
Sodium tartrate	–	–	+
L-Arginine	+	+	–
L-Aspartic acid	+	–	+
Reduction of:			
Nile blue	–	+	– ^a
Methylene blue	+	–	– ^a
Litmus acid coagulation	+	+	– ^a
Antibiotic resistance (µg ml ⁻¹)			
Streptomycin (300)	–	+ ^a	– ^a
Ampicillin (50)	–	+ ^a	+ ^a

*Data from: a, Wei *et al.* (2003); b, Amarger *et al.* (1997) and Chen *et al.* (1997) (confirmed in this study).

Description of *Rhizobium fabae* sp. nov.

Rhizobium fabae [fa'bae. L. gen. n. *fabae* of a bean, referring to the isolation of the first strains from broad bean or fava bean (*Vicia faba*)].

Cells are Gram-negative, motile, aerobic, non-spore-forming rods, 0.5–0.7 µm wide by 2.0–2.5 µm long. Colonies are circular, convex and pearl white on YMA at 28 °C and pH 7, the optimal growth temperature and pH. Strains grow at 10–37 °C and pH 5–8 (pH 5–9.4 for CCBAU 23123) and grow weakly up to 2% (w/v) NaCl. Tests for catalase, urease, oxidase and litmus milk acid production, acid coagulation and peptonization are positive. Tests negative for nitrate reductase, indole and H₂S production and hydrolysis of starch, gelatin, DNA and Tween 80. Reduces litmus milk and methylene blue, but methyl red and Voges–Proskauer reactions are negative. Utilizes D-amylgdalin, D-arabinose, calcium gluconate, D-fructose, D-galactose, D-glucose, inositol, lactose, sodium malate, maltose, D-mannose, turanose, sodium pyruvate, raffinose, L-rhamnose, salicin, sodium acetate, sodium citrate, sodium succinate, sucrose, trehalose, D-xylose, L-arginine, L-aspartic acid and L-proline as carbon sources. Grows on DL-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, hypoxanthine, L-lysine, L-phenylalanine, glycine and L-hydroxyproline and grows weakly on D-glutamic acid, L-isoleucine, L-valine and L-threonine as nitrogen sources. Test strains are resistant to (µg ml⁻¹) ampicillin (5), chloramphenicol (5), kanamycin (5), neomycin (5), streptomycin (100), bacitracin (100) and erythromycin (5). The major fatty acids of *Rhizobium* species, 16:0, 18:0, 19:0 cyclo ω8c and summed features 3 and 7, are detected; 15:0 2-OH, 18:1 ω9c, 11-methyl 18:1 ω7c and 18:0 3-OH are also found. The G+C content of the type strain is 61.9 mol%.

The type strain, CCBAU 33202^T (=LMG 23997^T =JCM 14381^T), was isolated from effective nodules of *Vicia faba* in Nanchang, Jiangxi province, China. Strain CCBAU 33201, isolated from Yifeng in Jiangxi province, and CCBAU 23123 (=LMG 23998 =JCM 14382), CCBAU 23122, CCBAU 23127 and CCBAU 23132, isolated from Taihu, Huaipei, Bengbu and Lujiang in Anhui province, all isolated from *V. faba*, are reference strains.

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