Rhizobium tubonense sp. nov., isolated from root nodules of Oxytropis glabra

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Four rhizobial strains, designated CCBAU 85046T, CCBAU 85051, CCBAU 85048 and CCBAU 85049, isolated from root nodules of Oxytropis glabra grown in Tibet, China, were previously defined, using amplified 16S rRNA gene restriction analysis, as a novel group within the genus Rhizobium. To clarify their taxonomic position, these strains were further analysed and compared with reference strains of related bacteria using a polyphasic approach. The 16S rRNA gene analysis showed that the four isolates formed a distinct phylogenetic lineage in the genus Rhizobium. The isolates showed highest sequence similarity (97.8%) to Rhizobium indigoferae CCBAU 71042T. Phenotypic and physiological tests, DNA–DNA hybridization, phylogenetic analyses of housekeeping genes recA, atpD and glnII and fatty acid profiles also indicated that these four strains constitute a novel group distinct from recognized species of the genus Rhizobium. Based on this evidence, strains CCBAU 85046T, CCBAU 85051, CCBAU 85048 and CCBAU 85049 represent a novel species in the genus Rhizobium, for which the name Rhizobium tubonense sp. nov. is proposed. The type strain is CCBAU 85046T (＝LMG 25225T =HAMI 3066T) and its DNA G + C content is 59.52 mol% (Tm). Strain CCBAU 85046T could form effective nodules on plant species Vigna unguiculata and Medicago sativa but not on its host of origin Oxytropis glabra.

The genus Rhizobium was first proposed by Frank (1889) to accommodate all of the root-nodule bacteria. These bacteria have now been divided into different genera, including Rhizobium, Mesorhizobium, Bradyrhizobium and Sinorhizobium, based on their phylogenetic relationships. Recently, some non-symbiotic species, such as Micromonospora lupini, Micromonospora saelicesensis (Trujillo et al., 2007), Micromonospora pisi (Garcia et al., 2010) and Labrys neptuniae (Chou et al., 2007), have also been isolated from root nodules. To date, more than 40 species have been described in the genus Rhizobium (http://www.bacterio.cict.fr/qr/rhizobium.html), all of which are fast-growing, acid-producing bacteria and are mainly symbiotic nitrogen fixers; however, some are endophytic or non-symbiotic species (Hunter et al., 2007; García-Fraile et al., 2007; Peng et al., 2008; Quan et al., 2005).

Previously, Hou et al. (2009) defined the strains CCBAU 85046T, CCBAU 85048, CCBAU 85049 and CCBAU 85051 as a novel group belonging to the genus Rhizobium based on restriction fragment length polymorphism analysis of amplified 16S rRNA genes. To clarify the taxonomic status of this group, in the present study the four strains were further characterized and compared with reference strains by using a polyphasic approach.

The four test strains and reference strains for species of the genus Rhizobium were obtained from the corresponding culture collection centres and maintained on YMA medium (Vincent, 1970) at 4 °C for temporary storage. Genomic DNA was extracted from the strains according to the protocol of Terefework et al. (2001) and was used as a template in the amplification of different genes or specific...
DNA fragments. The 16S rRNA gene of each of the four strains was amplified using primers fD1 and rD1 as described previously (Weisburg et al., 1991) and sequenced directly (van Berkum et al., 1996). The 16S rRNA gene sequences of type strains of species of the genus *Rhizobium* were obtained from the GenBank database. All sequences were aligned using the CLUSTAL W program in MEGA 4.0 software (Tamura et al., 2007). Phylogenetic distances were calculated according to the model of Jukes & Cantor (1969). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1985) methods with bootstrap values based on 1000 replicates. The topology of the maximum-likelihood tree (not shown) was similar to that of the neighbour-joining tree (Fig. 1). In the phylogenetic tree (Fig. 1), strains CCBAU 85046T, CCBAU 85048, CCBAU 85049 and CCBAU 85051 formed a distinct lineage in the genus *Rhizobium* and the closest reference strain was *Rhizobium indigoferae* CCBAU 71042T with 97.8% 16S rRNA gene sequence similarity; this was followed by *Rhizobium fabae* CCBAU 33202T, *Rhizobium pisi* DSM 30132T, *Rhizobium phaseoli* ATCC 14482T, *Rhizobium leguminosarum* USDA 3270T, *Rhizobium huautlense* S02T, *Rhizobium etli* CNF 42T, *Rhizobium tropici* USDA 3270T, *Rhizobium galegae* HAMBI 540T, *Rhizobium sullae* IS123T, *Rhizobium miluonenense* CCBAU 41251T, *Rhizobium tropici* LMG 9517, *Rhizobium yanglingense* CCBAU 71623T and *Rhizobium loessense* CCBAU 7190B, which showed sequence similarities ranging between 97.6 and 96.9%.

Since the 16S–23S intergenic spacer (IGS) has been shown to be a valuable marker for species definition (Jensen et al., 1993; Laguerre et al., 1996; Tan et al., 2001; Hurek et al., 1997), it was amplified using the forward and reverse primers 926f and 115r/23s (Tan et al., 2001) and sequenced as described previously (van Berkum et al., 1996). A phylogenetic tree based on 16S–23S IGS sequences was reconstructed using the same method as in the 16S rRNA gene analysis and also showed that strain CCBAU 85046T formed a distinct lineage but with 90.5% similarity to *R. fabae* CCBAU 33202T as the closest relative (Supplementary Fig. S1, available in IJSEM Online).

Recently, some housekeeping genes have been used as an important tool in bacterial taxonomy (Gaunt et al., 2001; Turner & Young, 2000; Vinuesa et al., 2005a). In the present study, atpD, recA and glnII genes were amplified using primer pairs atpD255F and atpD782R, recA41F and recA640R and glnII12F and glnII689R (Gaunt et al., 2001; Turner & Young, 2000; Vinuesa et al., 2005a, b), respectively. The PCR products were sequenced directly and phylogenetic trees were reconstructed as before using MEGA 4.0 software, (Supplementary Figs S2, S3 and S4).

![Table 1. DNA–DNA relatedness values between strain CCBAU 85046T and phylogenetically related representatives of species of the genus *Rhizobium*](http://ijs.sgmjournals.org)
represented a distinct lineage, the highest recA, atpD and glnII gene sequence similarities being 84.4% to *R. fabae* CCBAU 33202T, 91.3% to *R. rhizogenes* NCPPB 2991T and 89.1% to *R. fabae* CCBAU 33202T, respectively.

DNA–DNA hybridization is often used as a method of defining rhizobial species (Wayne et al., 1987; Graham et al., 1991) and it was also used in the present study. Total DNA from the four strains and the reference strains was prepared according to the method of Marmur (1961). The genomic DNA G+C content was measured according to the thermal melting protocol of De Ley (1970) using *Escherichia coli* DH5α as a reference. The DNA G+C content of CCBAU 85046T was 59.52 mol%, which is within the range typical of species of the genus *Rhizobium* (56.9–60.9 mol%). The novel group always formed a distinct lineage but had different positions in different phylogenetic trees. Considering that organisms with less than 97% 16S rRNA sequence similarity do not give a DNA–DNA reassociation of more than 60% (Vandamme et al., 1996), closely related strains from the phylogenetic tree based on 16S rRNA gene sequences were selected for use in the DNA–DNA hybridization studies. DNA–DNA hybridization was performed using the initial renaturation rate method (De Ley et al., 1970) and DNA relatedness values are presented in Table 1. The DNA–DNA hybridization values presented in Table 1 and the current phylogenetic analyses suggest that the novel group forms a distinct lineage within the genus *Rhizobium*.

### Table 2. Distinctive features of strains of *Rhizobium tubonense* sp. nov. and species of the genus *Rhizobium* that represent different taxonomic sub-branches based on 16S rRNA gene sequence analysis

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<td>Erythrosine (0.1%)</td>
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<td>Neutral red (0.1%)</td>
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<td>NaCl (1%)</td>
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relatedness values between strain CCBAU 85046T and the other three strains ranged from 94.2 to 100%. The DNA relatedness values between strain CCBAU 85046T and the closely related species *R. huautlense* S02T and *R. indigoferae* CCBAU 71042T were 30.9% and 30.4%, respectively, which are much lower than 70%, the recommended threshold for the delineation of bacterial species (Wayne et al., 1987; Graham et al., 1991), and are believed to be taxonomically insignificant (Vandamme et al., 1996).

Phenotypic, physiological and biochemical features of the four strains and related type strains were determined according to the method described by Gao et al. (1994), including tests for the utilization of sole carbon and nitrogen sources, resistance to antibiotics (at 5, 50, 100 and 300 µg ml⁻¹), tolerance to 1–5% (w/v) NaCl, growth at pH 4–12 and a range of temperatures. Some distinctive features of these strains are listed in Table 2. The results showed that the four test strains had the same features except that strains CCBAU 85051 and CCBAU 85049 were

### Table 3. Cellular fatty acid compositions (%) of strains CCBAU 85046T, CCBAU 85049 and CCBAU 85051 and related species of the genus *Rhizobium*

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**Summed features***

| 1  | 0.1| 0.6| 0.1|
| 2  | 5.8| 6.5| 7.5| 8.8| 5.7| 7.1| 4.7| 12.8| 6.1| 0.5| 4.4| 7.5| 5.5| 2.4|
| 3  | 1.4| 1.3| 0.7| 0.7| 1.8| 1.3| 1.3| 1.3| 1.3| 1.3| 1.3| 2.2|    |
| 4  | 1.6| 0.5| 0.3| 1.6| 0.2|    |    |    |    |    |    |    |    |    |
| 8  | 42.9| 46.1| 52.8| 66.0| 63.9| 30.8| 51.7| 57.9| 43.4| 51.3| 44.2| 66.7| 54.5| 45.2|

*Summed features are combinations of fatty acids that cannot be separated by the MIDI system. Summed feature 1, iso-C15:1 H and/or C13:0 3-OH; summed feature 2, C12:0 aldehyde/unknown ECL 10.928 and/or iso-C16:1 I/C14:0 3-OH; summed feature 3, C16:1 10:7c and/or iso-C16:1 06c; summed feature 4, iso-C17:1 1/e anteiso B and/or anteiso-C17:1 B/iso I; summed feature 8, C18:1 10:7c and/or C18:1 06c.
resistant to 5 µg streptomycin sulfate ml\(^{-1}\), CCBAU 85051 and CCBAU 85048 were resistant to 100 µg erythromycin ml\(^{-1}\) and CCBAU 85049 and CCBAU 85048 were resistant to 50 µg neomycin sulfate ml\(^{-1}\) and 50 µg kanamycin sulfate ml\(^{-1}\). In addition, there were features that distinguished the novel group from other type strains of the genus *Rhizobium*.

Fatty acid profiling is also a useful method for characterising bacteria (Quan et al., 2005; Tighe et al., 2000; Schutter & Dick, 2000). Three of the novel strains were selected at random; these and the closely related reference strains were grown on YMA medium for 72 h at 28 °C. The cells were harvested and the fatty acids were prepared and identified using the standard Microbial Identification System (MIDI) method. Some features are presented in Table 3 and the complete dataset is listed in Supplementary Table S1. Among the three test strains, different proportions of anteiso-C\(_{15}:0\), C\(_{18}:1\)-OH, summed feature 3 and summed feature 4 provided further evidence that they were distinct strains. For most of the reference species of the genus *Rhizobium*, fatty acids C\(_{14}:0\), C\(_{16}:0\)-3-OH, C\(_{18}:0\), summed feature 2 and summed feature 8 were common components.

To confirm the symbiotic properties of the four test strains, a cross-nodulation test was performed. Seeds of *Leucaena leucocephala*, *Phaseolus vulgaris*, *Pisum sativum*, *Medicago sativa*, *Glycine max*, *Trifolium pretense*, *Vigna unguiculata* and *Oxytropis glabra* were surface-sterilized, pre-germinated and inoculated according to the protocol of Vincent (1970). After 2 months of growth in a greenhouse with conditions of natural temperature and sunlight, it was found that strain CCBAU 85046\(^{T}\) could form nodules on *V. unguiculata* and *M. sativa* but, for unknown reasons, could not form nodules on its original host plant species *O. glabra*. This may be because the inoculation conditions were different from the natural growth conditions of *O. glabra*. Another possibility is that the test strain was an endophyte of *O. glabra* and not a true symbiont, as was the case in studies of *R. oryzae* (Peng et al., 2008).

Taken together, these results suggest that the four test strains represent a novel group that is closely related to, but distinct from, recognized species of the genus *Rhizobium*. Therefore, it is suggested that the group represents a novel species in the genus *Rhizobium*, for which the name *Rhizobium tubonense* sp. nov. is proposed.

**Description of Rhizobium tubonense** sp. nov.

*Rhizobium tubonense* (tu.bo.nen’se. N.L. fem. adj. tubonense pertaining to the ancient name ‘Tubo’ of Tibet, where the bacterium was isolated).

Cells are Gram-reaction-negative, aerobic, motile, non-spore-forming rods, 0.5–0.6 × 1.5–2.3 µm. Colonies on YMA medium are circular, convex, semi-opaque and 2–3 mm in diameter after 2–4 days of growth at 28 °C. Growth occurs at pH 5–11 and 10–37 °C (optimum 25–30 °C). D-Arabinol, dextrin, meso-erythritol, D-fructose, D-galactose, D-glucose, myo-inositol, lactose, sodium-DL-malate, maltose, D-mannose, melibiose, raffinose, L-thamnose, D-ribose, D-sorbitol, sucrose, trehalose and D-xylose are utilized as sole carbon sources but adipic acid, amygdalin, D-arabinose, calcium gluconate, calcium malonate, galactitol, inulin, melizetinose, sodium pyruvate, sodium acetate, sodium citrate, sodium formate, salicin, sodium D-gluconate, sodium hippurate, sodium succinate, sorbose, soluble starch, syringic acid, potassium sodium tartrate, vanillic acid, DL-asparagine, glycine, L-arginine, L-methionine and L-proline are not. DL-α-Aminopropionic acid, (+)-L-glutamic acid and L-methionine are utilized as sole nitrogen sources but L-aspartic acid, cysteine, D-glutamic acid, hypoxanthine, L-isoleucine, L-lysine, L-phenylalanine, D-threonine and L-valine are not. Resistant to chloramphenicol (5 µg ml\(^{-1}\)), neomycin sulfate (5 µg ml\(^{-1}\)), polymyxin B sulfate (5 µg ml\(^{-1}\)), erythromycin (50 µg ml\(^{-1}\)), ampicillin (100 µg ml\(^{-1}\)) and bacitracin (300 µg ml\(^{-1}\)). Catalase- and urease-positive. Negative for L-phenylalanine deaminase and 3-ketolactose production and reduction of methyl blue. Acid production, reduction reaction and peptonization are produced in litmus milk. No growth occurs in nutrient broth. Negative for hydrolysis of starch, DNA and Tween 80. Negative for methyl red reaction, Voges–Proskauer reaction, gelatin liquefaction and production of hydrogen sulfide.

The type strain, CCBAU 85046\(^{T}\) (=LMG 25225\(^{T}\) =HMBI 3066\(^{T}\)), was isolated from root nodules of *Oxytropis glabra* grown in Tibet, China. The DNA G+C mol% content of the type strain is 59.52 mol% (\(T_{\text{m}}\)).

**Acknowledgements**

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**References**


