Rhizobium yantingense sp. nov., a mineral-weathering bacterium

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A Gram-stain-negative, rod-shaped bacterial strain, H66<sup>T</sup>, was isolated from the surfaces of weathered rock (purple siltstone) found in Yanting, Sichuan Province, PR China. Cells of strain H66<sup>T</sup> were motile with peritrichous flagella. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain H66<sup>T</sup> belongs to the genus *Rhizobium*. It is closely related to *Rhizobium huautlense* SO2<sup>T</sup> (98.1 %), *Rhizobium alkalisoli* CCBAU 01393<sup>T</sup> (98.0 %) and *Rhizobium cellulosilyticum* ALA10B2<sup>T</sup> (98.0 %). Analysis of the housekeeping genes, *recA*, *glnII* and *atpD*, showed low levels of sequence similarity (<92.0 %) between strain H66<sup>T</sup> and other recognized species of the genus *Rhizobium*. The predominant components of the cellular fatty acids were summed feature 8 (C<sub>18:1</sub>ω7c and/or C<sub>18:1</sub>ω6c) and C<sub>16:0</sub>. The G+C content of strain H66<sup>T</sup> was 60.3 mol%. Strain H66<sup>T</sup> is suggested to be a novel species of the genus *Rhizobium* based on the low levels of DNA–DNA relatedness (ranging from 14.3 % to 40.0 %) with type strains of species of the genus *Rhizobium* and on its unique phenotypic characteristics. The name *Rhizobium yantingense* sp. nov. is proposed for this novel species. The type strain is H66<sup>T</sup> (=CCTCC AB 2014007<sup>T</sup> =LMG 28229<sup>T</sup>).
Cellular fatty acids of strain H66<sup>T</sup> and the type strains of species of the genus *Rhizobium*

**Strains:** 1, H66<sup>T</sup>; 2, *R. huautense* SO2<sup>T</sup>; 3, *R. alkalisoli* CCBAU 01393<sup>T</sup>; 4, *R. cellosolityicum* ALA1082<sup>T</sup>; 5, *R. vignae* CCBAU 05716<sup>T</sup>; 6, *R. galegae* ATCC 43677<sup>T</sup>. Characteristics of the reference strains were analysed in this study and the results were the same as those from Lindstrom (1989), Lu et al. (2009), Wang et al. (1998), Ren et al. (2011) and García-Fraile et al. (2007). +, Positive; −, negative, s, sensitive; r, resistant.

**Table 1. Differential phenotypic characteristics between strain H66<sup>T</sup> and the type strains of species of the genus *Rhizobium***

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>3</th>
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<tbody>
<tr>
<td><strong>Used as sole nitrogen source:</strong></td>
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<tr>
<td>l-Arginine</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>N-Acetyl-glucosamine</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Urea</td>
<td>+</td>
<td>–</td>
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<td><strong>Resistance to (mg ml&lt;sup&gt;−1&lt;/sup&gt;):</strong></td>
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<td>Ampicillin (100)</td>
<td>R</td>
<td>S</td>
<td>R</td>
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<td>Bacitracin (300)</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
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<td>R</td>
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<td>Erythromycin (50)</td>
<td>S</td>
<td>R</td>
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<td>Neomycin sulfate (50)</td>
<td>R</td>
<td>S</td>
<td>S</td>
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<td><strong>Growth in NaCl (3 %, w/v):</strong></td>
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<td>+</td>
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<td>+</td>
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<td>Growth at pH 10.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td><strong>DNA G+C content (mol%):</strong></td>
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<tr>
<td>60.3</td>
<td>57.0</td>
<td>65.5</td>
<td>57.0</td>
<td>60.8</td>
<td>63.0</td>
<td>63.0</td>
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</table>

Strain H66<sup>T</sup> was identified as a member of the genus *Rhizobium* based on the pairwise analysis of the 1430 bp 16S rRNA gene sequence using the EzTaxon server (Kim et al., 2012). The sequences of the 16S rRNA gene of other species of the genus *Rhizobium* were obtained from the GenBank database, and multiple alignment analysis was performed using the CLUSTAL W program, version 1.81 (Thompson et al., 1994). Phylogenetic trees were reconstructed using the neighbour-joining, maximum-likelihood and minimum-evolution methods according to the procedures in the program MEGA version 4 (Tamura et al., 2007). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980). The confidence levels of the clusters were determined by using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. As no significant topological differences were found between the phylogenetic trees reconstructed using the two methods, only the tree reconstructed by using the NJ method after distance analysis of aligned sequences is shown (Fig. 1).

The maximum-likelihood and minimum-evolution trees are
shown in Figs S2 and S3. The 16S rRNA gene sequence of strain H66\textsuperscript{T} was closely related to *Rhizobium huautlense* S02\textsuperscript{T}, *Rhizobium* *alkalisolii* CCBAU 01393\textsuperscript{T}, *Rhizobium* *cellulosilyticum* ALA10B2\textsuperscript{T}, *Rhizobium* *vignae* CCBAU 05716\textsuperscript{T} and *Rhizobium* *galegae* ATCC 43677\textsuperscript{T} with gene sequence similarities of 98.1\%, 98.0\%, 98.0\%, 97.8\% and 97.5\%, respectively.

Housekeeping genes (*atpD*, *glnI*, *glnII*, *recA*, *dnaK* etc.) throughout the genome are being used increasingly to investigate the phylogeny of bacteria. We determined partial *atpD*, *recA* and *glnII* sequences for strain H66\textsuperscript{T} using the methods reported previously (Martens et al., 2007, 2008). Distances were calculated and clustering was performed with the neighbour-joining algorithm as described above. Bootstrap analysis was performed using 1000 replications. In this study PCR amplifications and sequencing of partial *atpD* (510 bp) and *recA* (530 bp) genes were performed according to Gaunt et al. (2001) and a 660 bp intragenic fragment of *glnII* was amplified according to Turner & Young (2000). Sequence similarities of *atpD*, *recA* and *glnII* genes between strain H66\textsuperscript{T} and reference strains are given in Table S2.

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain H66\textsuperscript{T} and recognized species of the genus *Rhizobium*. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at the nodes. *Novosphingobium panipatense* SM16\textsuperscript{T} was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.](image-url)

The DNA G + C content of strain H66\textsuperscript{T} was determined by the thermal denaturation method (Marmur & Doty, 1962) using *Escherichia coli* K-12 as a reference. DNA–DNA hybridizations among strain H66\textsuperscript{T} and type strains of the genus *Rhizobium* were determined using a UV/VIS spectrophotometer (UV1201; Rayleigh) as described by De Ley et al. (1970). The genomic DNA G + C content of strain H66\textsuperscript{T} was 60.3 mol\%, which was in the range previously determined for members of the genus *Rhizobium* (57–66\%; Jordan, 1984). DNA–DNA hybridization studies showed relatively low relatedness values of strain H66\textsuperscript{T} with *R. huautlense* S02\textsuperscript{T} (14.3\%), *R. alkalisolii* CCBAU 01393\textsuperscript{T} (26.4\%), *R. cellulosilyticum* ALA10B2\textsuperscript{T} (27.3\%), *R. vignae* CCBAU 05716\textsuperscript{T} (40.0\%) and *R. galegae* ATCC 43677\textsuperscript{T} (40.0\%). All of the values were significantly lower than 70\%, the threshold value recommended for the assignment of genomic species (Wayne et al., 1987). These results indicate that strain H66\textsuperscript{T} represents a novel species of the genus *Rhizobium*.

Nodulation and nitrogen fixation abilities are important characteristics for *Rhizobium* species and the host range is an important feature for the description of novel rhizobial species (Graham et al., 1991). In this study, the *nifH* and *nodC* genes of strain H66\textsuperscript{T} were analysed according to the methods of Ueda et al. (1995) and Laguerre et al. (2001). However, the *nifH* and *nodC* genes could not be amplified from strain H66\textsuperscript{T}.
A nodulation test was carried out as described by Zhang et al. (2012). Six legume plant species were selected: *Leucaena leucocephala*, *Phaseolus vulgaris*, *Pisum sativum*, *Medicago sativa*, *Astragalus sinicus* and *Vicia faba*. Strain H66T could not nodulate any of the plants tested.

Many well-defined nitrogen fixation genes are clustered on large plasmids or MEGA plasmids, which are not very stable during long-term evolution; they may be lost or horizontally transferred across species boundaries (Kuykendall et al., 2005). This may explain why strain H66T could not form nodules with legume plants and why the *nifH* and *nodC* genes could not be amplified from strain H66T.

Phylogenetic analysis, enzyme activities and differences in other physiological and biochemical characteristics during long-term evolution; they may be lost or horizontally transferred across species boundaries (Kuykendall et al., 2005). This may explain why strain H66T could not form nodules with legume plants and why the *nifH* and *nodC* genes could not be amplified from strain H66T.

Phylogenetic analysis, enzyme activities and differences in other physiological and biochemical characteristics during long-term evolution; they may be lost or horizontally transferred across species boundaries (Kuykendall et al., 2005). This may explain why strain H66T could not form nodules with legume plants and why the *nifH* and *nodC* genes could not be amplified from strain H66T.

Fig. 2. Neighbour-joining phylogenetic trees based on partial *recA* (a), *atpD* (b), and *glnII* (c) gene sequences showing the position of strain H66T and recognized species of the genus *Rhizobium*. Bootstrap values (expressed as percentages of 1000 replications) are shown at each node. Bars, 0.2, 0.1 or 0.01 substitutions per nucleotide position.
(Table 1) together with the fatty acid profile (Table S1) clearly distinguish strain H66T from closely related species of the genus Rhizobium. Thus, on the basis of the data presented, we suggest that strain H66T represents a novel species of the genus Rhizobium, for which the name Rhizobium yantingense H66T sp. nov. is proposed.

**Description of Rhizobium yantingense sp. nov.**

*Rhizobium yantingense* (yan. ting. en’se. N.L. neut. adj. yantingense referring to Yanting district, Sichuan Province, PR China, where the organism was isolated).

Cells are Gram-stain-negative, aerobic, non-motile, non-spore-forming and rod-shaped (0.6–0.8 μm x 1.2–2.8 μm). Colonies are circular, convex, white and opaque with a colony diameter of 3 mm after growth on YMA agar at 28 °C for 3 days. Growth occurs at 10–40 °C with an optimum temperature of 28 °C. Growth occurs at pH 7.0–10.0 (optimum, pH 7.0) and with 0–3% (w/v) NaCl. Positive for alkaline phosphatase, esterase, leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and 3-glucosidase activities. Negative for β-galactosidase and β-glucosidase activities. Oxidase- and urease-positive, but catalase-negative. Nitrate is not reduced to either nitrite or nitrogen gas. Utilizes D-arabinose, D-mannitol, D-xylose, D-galactose, D-fructose, D-glucose, D-mannose, ascellin ferric citrate, maltose and malic acid as single carbon sources. Utilizes B-carabinose, L-arabinose, D-mannitol, D-xylose, D-galactose, D-fructose, D-glucose, D-mannose, ascellin ferric citrate, maltose and malic acid as single carbon sources. Utilizes urea, N-acetylglucosamine, L-aspartic acid, L-glutamic acid, D-glutamic acid, L-alanine, DL-threonine, hypoxanthine, L-cystine, L-isoleucine, L-lysine, L-phenylalanine, L-threonine, L-methionine and L-valine as sole nitrogen sources. The major fatty acids (>10% of total) are summed feature 8 (comprising C16:1ω7c and/or C18:1ω6c) and C16:0.

The type strain is H66T (=CCTCC AB 2014007T =LMG 28229T), which was isolated from the surfaces of weathered rock (purple siltstone) in Yanting (Sichuan, PR China). It can weather silicate minerals and release Si, Al, and Fe from silicate minerals. The genomic DNA G+C content of the type strain is 60.3 mol%.

**Acknowledgements**

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**References**


Rhizobium yantingense sp. nov.


