Rhizobium wenxiniae sp. nov., an endophytic bacterium isolated from maize root

Jun-lian Gao,† Pengbo Sun,‡ Xu-ming Wang,† Fan-yang Lv,§ Xiao-jie Mao§ and Jian-guang Sun§,*

Abstract

A novel Gram-stain-negative, aerobic, rod-shaped strain designated 166T was isolated from surface-sterilized root tissue of maize planted in the Fangshan District of Beijing, PR China. The 16S rRNA gene sequence analysis indicated that strain 166T belongs to the genus Rhizobium and is closely related to Rhizobium celulasltyicum ALA10B2T and Rhizobium yantingense H66T with sequence similarities of 98.8 and 98.3 %, respectively. According to atpD and recA sequence analysis, the highest sequence similarity between strain 166T and R. celulasltyicum ALA10B2T is 93.8 and 84.7 %, respectively. However, the new isolate exhibited relatively low levels of DNA–DNA relatedness with respect to R. celulasltyicum DSM 18291T (20.8±2.3 %) and Rhizobium yantingense CCTCC AB 2014007T (47.2±1.4 %). The DNA G+C content of strain 166T was 59.8 mol%. The main polar lipids consisted of phosphatidyl ethanolamine, phosphatidylglycerol, phosphatidylcholine, diphasphatidylglycerol, an unidentified aminophospholipid and an unidentified aminolipid. The major fatty acids of strain 166T were summed feature 8 (C18:1ω7c and/or C18:1ω6c). The results of the physiological and biochemical tests and minor differences in the fatty acid profiles allowed a clear phenotypic differentiation of strain 166T from the type strains of closely related species, R. celulasltyicum DSM 18291T and R. yantingense CCTCC AB 2014007T. Strain 166T represents a novel species within the genus Rhizobium, for which the name Rhizobium wenxiniae sp. nov. is proposed, with the type strain 166T (=CGMCC 1.15279T=DSM 100734T).

The genus Rhizobium, first described by Frank [1] and updated by Kuykendall et al. [2], is a member of the family Rhizobiaceae within the order Rhizobiales of the class Alphaproteobacteria [2, 3]. Members of this genus include species that are capable of forming symbiotic nitrogen-fixing nodules together with leguminous plant [3], species that are capable of forming pathogenic tumours [4], species that are free-living bacteria in soil [5–7] and species that are endophytic bacteria found in different crops, including cotton [8], maize [9], wheat [10], sugar cane [11] and rice [12–14]. At the time of writing, this genus comprises 89 recognized species (http://www.bacterio.net/ rhizobium.html). Members of the genus Rhizobium have certain characteristics in common; they are all Gram-stain-negative, non-spore-forming, rod-shaped, aerobic and chemo-organotrophic, with C18:1ω7c as the predominant fatty acid and a DNA G+C content of between 57 and 66 mol% [4, 15]. During an investigation on the diversity of endophytic bacteria in maize, a novel strain, designated 166T, was obtained. Data from a polyphasic taxonomic study indicated that this strain represents a novel species of the genus Rhizobium.

Strain 166T was isolated from surface-sterilized root tissue of maize planted in the Fangshan District of Beijing, PR China. The entire maize plant was dug out at tasselling time and washed with tap water; the roots were then cut and their surface was sterilized by immersion in 70 % ethanol for 1 min, immersion in 2 % sodium hypochlorite for 10 min and three 10-minute rinses in sterile distilled water. To check for surface contaminations, samples were imprinted on triplicate plates of tryptic soy agar (TSA) (BD Diagnostics). Tissues found to be free of surface contamination were ground in a sterile mortar with a sterile pestle to be further diluted with sterile water, and then plated onto LB medium [16]. After aerobic incubation at room temperature for 2–3 days, colonies that appeared on the LB agar plates were then picked and re-streaked on fresh media until pure cultures were obtained. Strain 166T was selected, and pure culture was maintained at −80 °C in 30 % (v/v) glycerol. The type strains of closely related species, namely
Rhizobium cellulosilyticum DSM 18291T [17] and Rhizobium yantingense CCTCC AB 2014007T [18], were used as references. Routine cultivation was performed on YMA medium [19] at 30 °C for 3 days.

For construction of the template for 16S rRNA gene PCR, a loop-full of biomass was scraped off the YMA medium plate, suspended in 100 µl double-distilled H2O and lysed by 10 min of boiling and afterwards 5 min of freezing. After centrifugation, the supernatant was used as the template for PCR. The 16S rRNA gene was amplified using the universal primers 27F and 1492R [20], whereas partial atpD and recA gene sequences were determined as described by Vinuesa et al. [21]. All the purified PCR products were sequenced with an ABI 3730 sequencer following the manufacturer's protocols. The almost complete 16S rRNA gene sequence (1348 bp) of strain 166 was determined by direct sequencing and compared against related available 16S rRNA gene sequences in the EzBioCloud database (http://www.ezbiocloud.net/) [22]. The partial atpD gene (486 bp) and recA gene (504 bp) sequences of strain 166 were determined by direct sequencing and compared with related sequences of the reference strains found by BLAST searching the NCBI sequence database [23]. Multiple alignments were performed using the CLUSTAL X program [24], and the model selection test was used to determine the best model by using the software package MEGA version 6.0 [25]. The Tamura 3-parameter model was used to calculate evolutionary distances. The phylogenetic trees were reconstructed with the neighbour-joining method [26], maximum-likelihood method [27] and minimum-evolution method [28, 29] with the MEGA6 program [25]. In each case, bootstrap values were calculated based on 1000 replications. Nearest-neighbour-interchange was applied to the maximum-likelihood analysis. The neighbour-joining phylogenetic tree (Fig. 1) reconstructed from 16S rRNA gene sequences of strain 166T and related species in the genus Rhizobium showed that strain 166T was closely related to R. cellulosilyticum ALA10B2T and R. yantingense H66T with sequence similarities of 98.8 and 98.3 %, respectively. The topologies of the 16S rRNA gene phylogenetic trees built by using the maximum-likelihood and minimum-evolution algorithms also supported the result that strain 166T formed a stable clade together with the two reference strains. The neighbour-joining phylogenetic trees of both the atpD gene sequences (Fig. S1, available in the online Supplementary Material) and recA gene sequences (Fig. S2) displayed that strain 166T was closely related to R. cellulosilyticum ALA10B2T with sequence similarities of 93.8 % (atpD) and 84.7 % (recA), respectively. Phylogenetic trees of the partial atpD and recA gene sequences built by the maximum-likelihood method shared similar topologies with the phylogenetic trees reconstructed by the neighbour-joining method. The low similarities of the atpD and recA gene sequences between strain 166T and the type strain of its most closely related species, R. cellulosilyticum ALA10B2T, indicated that strain 166T represents a novel species of the genus Rhizobium.

Attempts were made to amplify intragenic fragments of nifH and nodC genes of strain 166T, to determine its symbiotic properties. PCR amplification of the nifH gene was performed by using the method described by Laguerre et al. [30] and Poly et al. [31]. PCR amplification of the nodC gene was performed by using the method described by Laguerre et al. [30].

**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain 166T among species of the genus Rhizobium. Asterisks indicate branches that were also recovered using maximum-likelihood and minimum evolution algorithms. Bootstrap analyses were determined based on 1000 resamplings; values >70 % are shown. Bar, 0.01 substitutions per nucleotide position.
and Sarita et al. [32]. Both the \textit{nifH} gene encoding denitrogense reductase and the \textit{nodC} gene encoding \textit{N}-acyethylglucosaminyltransferase were not detected by PCR.

Colonial properties of the isolate were observed on YMA agar medium. Cell morphology was examined using scanning electron microscopy (S-3400N, Hitachi) after 3 days of incubation of strain 166$^T$ on YMA agar medium at 30°C. Cells were approximately 0.3–0.4 µm in width and 1.2–2.2 µm in length (Fig. S3). Gram staining was determined by using the bioMérieux Gram Stain kit according to the manufacturer’s instructions. Growth was tested at 5, 10, 15, 20, 25, 30, 35, 37 and 40°C on YMA agar medium. The pH range for growth was determined by measuring the optical densities (wavelength 600 nm) of YMA broth cultures after 3 days. pH was adjusted prior to sterilization to pH 4–10 (at intervals of 1.0 pH unit) using appropriate biological buffers [33, 34]: citrate/Na$_2$HPO$_4$ buffer, pH 4.0–5.0; phosphate buffer, pH 6.0–7.0; Tris buffer, pH 8.0–9.0; and NaHCO$_3$/Na$_2$CO$_3$ buffer, pH 10.0. Verification of the pH after autoclaving revealed only minor changes. Tolerance of NaCl was determined using YMA agar medium adjusted to various NaCl concentrations in the range of 1.0–7.0 % (w/v; intervals of 1 %). Oxidase was tested using oxidase reagent (bioMérieux) according to the instructions of the manufacturer. Catalase activity was determined by assessing bubble production in 3.0 % (w/v) H$_2$O$_2$. Hydrolysis of starch and casein was tested using standard methods [35]. Hydrolysis of carboxymethylcellulose was tested as described by García-Fraile et al. [17]. Anaerobic growth was tested as described by Gao et al. [36].

Motility was determined by observing growth of cells in test tubes containing semisolid YMA medium with 0.5 % agar concentration after 3 days of incubation at 28°C [37].
Carbon source utilization, acid production and additional physiological and biochemical characterizations were performed using the Biolog GEN III microtiter system and the API 20E, API 20NE and API ZYM systems (bioMérieux) according to the manufacturers’ instructions. Resistance of strain 166T, *R. cellulolyticum* DSM 18291T and *R. yantingense* CCTCC AB 2014007T to ampicillin, chloromycin, erythromycin, gentamicin, kanamycin, lincomycin, streptomycin and tetracycline (each at concentrations of 300, 100, 50 and 5 µg ml−1 in YMA) was determined by using the method described by Wang *et al.* [38]. Phenotypic characteristics of strain 166T and the reference strains are presented in Table 1 and in the species description.

For determination of the cellular fatty acid composition, strain 166T and the reference strains *R. cellulolyticum* DSM 18291T and *R. yantingense* CCTCC AB 2014007T were incubated for 2–3 days on tryptic soy agar (TSA) (BD Diagnostics) medium at 30 °C. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI) [39]. These were analysed via gas chromatography (model 6890, Hewlett Packard). The fatty acid methyl esters were identified and quantified by using the TSBA 6 database of the Sherlock Microbial Identification System. The major fatty acids of strain 166T were summed feature 8 (C18:1ω7c and/or C18:1ω6c), which was similar to *R. cellulolyticum* DSM 18291T and *R. yantingense* CCTCC AB 2014007T under the same laboratory conditions, indicating that strain 166T should be assigned to the genus *Rhizobium* [15]. The minor differences in the fatty acid profiles among the four strains are shown in Table 2. Polar lipids of strain 166T were extracted according to the method of Bligh and Dyer [40]. The biomass used for lipid extraction was obtained from cultures growing in TY liquid medium that contained 4.5 mM CaCl2 [41] at 30 °C for 3 days. The polar lipids extracted were separated by two-dimensional silica-gel TLC. Chloroform/methanol/water (65:25:4, by vol.) was used as the first solvent system and chloroform/methanol/glacial acetic acid/water (80:12:15:4, by vol.) was used as the second solvent system. Individual lipids were detected by using the following spray reagents: ninhydrin, to detect lipids containing free amino groups [42]; molybdenum blue reagent, to detect molybdophosphoric acid was used to obtain a fingerprint of the total lipid content [43, 44]. The dominant polar lipids of strain 166T were phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, diphosphatidylglycerol, an unidentified amino phospholipid and an unidentified aminolipid, which was similar to those of *R. cellulolyticum* DSM 18291T under the same laboratory conditions (Fig. S4).

Preparation of genomic DNA was carried out according to the method of Marmur [46]. The G+C content of the DNA was determined by using the thermal denaturation method [47] with *Escherichia coli* K-12 as a control. The DNA G+C content of strain 166T was 59.8 mol%. DNA–DNA relatedness was determined by the initial renaturation rate method in 2× SSC [48]. Genomic DNA relatedness between strain 166T and its closest phylogenetic relatives, *R. cellulolyticum* DSM 18291T and *R. yantingense* CCTCC AB 2014007T, was 20.8±2.3 and 47.2±1.4 %, respectively, which is below the recommended value of 70 % for species definition [49].

Based on the results from the present study, it can be concluded that strain 166T represents a novel species of the genus *Rhizobium*, for which the name *Rhizobium wenxiniae* sp. nov., is proposed.

**DESCRIPTION OF RHIZOBIUM WENXINIAE**

**SP. NOV.**

*Rhizobium wenxiniae* (wen.xin’i.ae. N.L. fem. gen. n. wenxiniae of Wen-xin, to honour Wen-xin Chen, a respected rhizobial taxonomist, for her great contributions to the

**Table 2.** Cellular fatty acid contents (percentages) of strain 166T and the closely related strains *R. cellulolyticum* DSM 18291T and *R. yantingense* CCTCC AB 2014007T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>C11.1 at 12–13</td>
<td>0.4</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.4</td>
<td>0.3</td>
<td>0.1</td>
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<td>C15:0 3-OH</td>
<td>0.2</td>
<td>0.3</td>
<td>ND</td>
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<tr>
<td>C16:0</td>
<td>7.8</td>
<td>7.3</td>
<td>8.3</td>
</tr>
<tr>
<td>C16:0 3-OH</td>
<td>3.1</td>
<td>2.3</td>
<td>3.0</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.4</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>C17:0 3-OH</td>
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<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Anteiso-C17:1ω9c</td>
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</tr>
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<td>C17:1ω6c</td>
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<td>C17:1ω9c</td>
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<td>ND</td>
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</tr>
<tr>
<td>C18:0</td>
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<td>0.2</td>
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<tr>
<td>C18:0 3-OH</td>
<td>0.2</td>
<td>0.2</td>
<td>0.9</td>
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<tr>
<td>C18:1 2-OH</td>
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<td>ND</td>
<td>0.5</td>
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<tr>
<td>C18:1ω5c</td>
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<td>C18:1ω7c 11-methyl</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>C19:0 10-methyl</td>
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</tr>
<tr>
<td>C19:0 cycloω8c</td>
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<td>0.7</td>
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<td>Summed feature 1*</td>
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<td>0.3</td>
<td>0.1</td>
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<td>Summed feature 2*</td>
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<tr>
<td>Summed feature 3*</td>
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<td>2.5</td>
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<tr>
<td>Summed feature 7*</td>
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<td>Summed feature 8*</td>
<td>71.1</td>
<td>73.7</td>
<td>76.0</td>
</tr>
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</table>

*Summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete equivalent chain lengths (ECLs) as well as those where the ECLs are not reported separately. Summed feature 1 was listed as iso-C15:1 H and/or C13:0 3-OH; summed feature 2 was listed as C12:0 aldehyde and/or unknown 10.928; summed feature 3 was listed as C16:0ω6c and/or C16:1ω7c; summed feature 7 was listed as unknown 18.8ω6 and/or C17:1ω6c; summed feature 8 was listed as C18:1ω7c and/or C18:1ω6c.
investigation and taxonomy of rhizobial resources in China).

Cells are Gram-staining-negative, aerobic, non-motile, rod-shaped and approximately 0.3–0.4 µm in width and 1.2–2.2 µm in length, after 3 days of incubation at 30 °C on YMA agar. Colonies are circular with entire margins, convex, milky, glossy and 3–4 mm in diameter on YMA agar plates after 2 days of incubation at 30 °C. The temperature range for growth is 15–37 °C; optimum growth occurs at 30 °C. The pH range for growth is pH 6.0–9.0; optimum growth occurs at pH 7.0. Growth occurs at a NaCl concentration of 0–2.0% (w/v). Resistant to 300 µg lincomycin ml⁻¹, 5 µg ampicillin ml⁻¹ and 5 µg erythromycin ml⁻¹. Sensitive to (50 µg ml⁻¹ each) ampicillin and erythromycin, and (5 µg ml⁻¹ each) chloramycin, gentamicin, kanamycin, streptomycin and tetracycline. Positive for oxidase and catalase, production of acetoin and hydrolysis of cellulose, but negative for hydrolysis of starch and casein. In the API ZYM system, positive for alkaline phosphatase, C₄ esterase, C₈ esterase lipase, leucine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phospha-

The type strain is 166 T (DSM 100734), isolated from surface-sterilized root tissue of maize planted in the Fangshan District of Beijing, PR China. The DNA G+C content of the type strain is 59.8 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References
8. McNeney JA, Kloepfer JW. Population dynamics of endophytic bacte-

17. Garcia-Fraile P, Rivas R, Willems A, Peix A, Martens M et al. Rhiz-
tions; 1970.
22. Yoon SH, Ha SM, Kwon S, Lim J, Kim Y et al. Introducing EzBio-
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