Rhizobium pseudoryzae sp. nov., isolated from the rhizosphere of rice

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A Gram-stain-negative, aerobic, rod-shaped bacterium, designated strain J3-A127^T, was isolated from the roots of fresh rice plants (*Oryza sativa*). Cells were non-motile and no flagellum was detected. Comparison of 16S rRNA gene sequences indicated that the strain was phylogenetically related to species of the genus *Rhizobium*, with closest similarity to *Rhizobium oryzae* Alt 505^T (96.4 %). The low levels of 16S rRNA gene sequence similarity (<90 %) found between the *gyrB*, *atpD*, *recA* and *glnII* gene sequences of strain J3-A127^T and the type strains of recognized species of the genus *Rhizobium* also indicated that it represented a separate species. The temperature range for growth was 10–40 °C (optimum around 28 °C) and the pH range was 6.0–11.0 (optimum pH 7.0–8.0). Strain J3-A127^T tolerated NaCl concentrations up to 5.0 % (w/v). The strain was catalase- and oxidase-positive. The main cellular fatty acids were summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6; 46.7 %). The DNA G + C content of strain J3-A127^T was 59.5 mol%. Strain J3-A127^T did not form any nodules on four different legumes and the *nodD* and *nifH* genes were not detected by PCR. According to physiological and biochemical characteristics and genotypic data, strain J3-A127^T is considered to represent a novel species of the genus *Rhizobium*, for which the name *Rhizobium pseudoryzae* sp. nov. is proposed. The type strain is J3-A127^T (=ACCC 10380^T=KCTC 23294^T).

The taxonomy of rhizobia is in a state of flux. Since the first description of the genus *Rhizobium* to accommodate root and/or stem-nodule bacteria by Frank (1889), there have been a number of revisions to its description and the addition of many novel species. At the time of writing, the genus *Rhizobium* comprises 43 recognized species (Euzéby, 1997; <http://www.bacterio.cict.fr/>). Species of the genus *Rhizobium* have traditionally been considered as legume endosymbionts and have generally been isolated from nodules on leguminous plants. More recently, novel species of this genus have also been found in different environments, such as *Rhizobium selenitireducens* and *Rhizobium daejeonense* isolated from bioreactors (Quan *et al.*, 2005; Hunter *et al.*, 2007), *Rhizobium cellulosilyticum* from sawdust of *Populus alba* (García-Fraile *et al.*, 2007), *Rhizobium oryzae* from wild rice (Peng *et al.*, 2008) and

strains of *Rhizobium alarii* from the rhizosphere of sunflowers (Berge *et al.*, 2009). A novel bacterial strain, J3-A127^T, isolated during the course of studies of the bacterial diversity in rice (*Oryza sativa*) roots, was studied using a polyphasic taxonomic approach. It is proposed that this strain represents a novel species of the genus *Rhizobium*.

Strain J3-A127^T was isolated from tillering-stage rice roots from the Agricultural Experimental Demonstration Base at the village of Sangyuan, Luannan County, Hebei Province, China. Methods for the isolation and purification of nitrogen-fixing bacteria on YMA medium (Vincent, 1970) were as described previously (Zhang *et al.*, 2008). Cellular morphology was observed by transmission electron microscopy of bacterial cells grown for 18 h on YMA (see Supplementary Fig. S1 in IJSEM Online). Utilization of various carbon sources was investigated on GN2 microplates (Biolog) as described by the manufacturer. Growth at 0, 4, 10, 25, 30, 37, 40, 45 and 50 °C was tested on YMA agar. NaCl and pH tolerance were tested by using YMA broth adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0, and 1, 3, 4, 5, 7 and 10 % (w/v) NaCl (Gao *et al.*,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain J3-A127^T is DQ454123; accession numbers for the *atpD*, *recA*, *gyrB* and *glnII* gene sequences of strain J3-A127^T are EU732562, EU732539, HM132107 and HM132108, respectively.

A supplementary figure and a supplementary table are available with the online version of this paper.

1994). Gram-staining and other phenotypic characteristics were determined according to the methods described by Dong & Cai (2001) and Gao *et al.* (1994). Discriminative features between the novel isolate and its closest relatives are detailed in Table 1 and in the species description below. Strain J3-A127^T could be distinguished on the basis of these characteristics from representatives of its closest phylogenetic relatives, namely *Rhizobium oryzae* Alt 505^T, *Rhizobium sulae* IS123^T, *Rhizobium mesosinicum* CCBAU 25010^T and *Rhizobium yanglingense* CCBAU 71623^T. In contrast to these type strains, strain J3-A127^T did not show a flagellum and was able to grow in the presence of 3 % NaCl. Strain J3-A127^T differed from *R. oryzae* Alt 505^T in metabolism of glycogen, Tween 40, Tween 80, adonitol,

Table 1. Differential characteristics between strain J3A-127^T and the type strains of closely related species of the genus *Rhizobium*

Strains: 1, J3A-127^T; 2, *R. oryzae* Alt 505^T (Peng *et al.*, 2008); 3, *R. sulae* IS123^T (Squartini *et al.*, 2002); 4, *R. mesosinicum* CCBAU 25010^T (Lin *et al.*, 2009); 5, *R. yanglingense* CCBAU 71623^T (Tan *et al.*, 2001). Data for the reference species are from the present study except for the presence of flagellum and DNA G+C content, which are from the original sources. +, Positive; −, negative; w, weakly positive.

Characteristic	1	2	3	4	5
Flagellum	−	+	+	+	+
DNA G+C content (mol%)	59.5	64.1–64.4	61.6	68.1	59–63
Growth at/with:					
pH 5	−	+	−	+	+
pH 10	+	−	+	+	−
pH 11	+	−	+	−	−
40 °C	+	+	−	+	−
3 % NaCl	+	−	−	−	−
Growth in Luria–Bertani medium	+	+	+	+	−
Utilization as carbon source					
Glycogen	−	+	−	w	−
Tween 40	−	+	−	−	−
Tween 80	−	+	−	−	−
Adonitol	−	−	+	+	+
i-Erythritol	−	−	+	−	+
myo-Inositol	+	−	+	+	−
α-Lactose	+	−	+	+	+
Raffinose	−	+	+	w	−
L-Rhamnose	+	−	+	+	+
D-Galacturonic acid	+	−	−	w	−
Propionic acid	+	+	−	−	−
Quinic acid	+	−	w	+	−
D-Saccharic acid	+	−	−	+	−
D-Alanine	+	+	+	−	−
L-Leucine	−	+	−	−	−
L-Phenylalanine	−	+	−	−	−
Glucose 1-phosphate	+	−	−	−	−
Glucose 6-phosphate	+	−	−	−	−

i-erythritol, myo-inositol, α-D-lactose, D-raffinose, L-rhamnose, D-galacturonic acid, quinic acid, D-saccharic acid, L-leucine, L-phenylalanine, glucose 1-phosphate and glucose 6-phosphate and growth at pH 11. It differed from *R. sulae* IS123^T in metabolism of adonitol, i-erythritol, D-raffinose, D-galacturonic acid, propionic acid, D-saccharic acid, glucose 1-phosphate and glucose 6-phosphate and growth at 40 °C. It differed from *R. mesosinicum* CCBAU 25010^T in the metabolism of adonitol, i-erythritol, propionic acid, D-alanine, glucose 1-phosphate and glucose 6-phosphate and growth at pH 11 and with 3 % NaCl. It differed from *R. yanglingense* CCBAU 71623^T in the metabolism of adonitol, i-erythritol, myo-inositol, D-galacturonic acid, propionic acid, quinic acid, D-saccharic acid, D-alanine, glucose 1-phosphate and glucose 6-phosphate and growth at pH 11 and with 3 % NaCl (Table 1). The results of a comparison of carbon source utilization with other related species of the genus *Rhizobium* are shown in Supplementary Table S1.

Fatty acid profiling is a useful tool for the identification of members of the genus *Rhizobium* and related genera (Tighe *et al.*, 2000). In this study, strain J3-A127^T and the four reference strains were grown on YMA medium for 2 days at 30 °C. Fatty acid methyl esters were prepared and separated according to method described by Sasser (1990) and were identified with the MIDI Sherlock Microbial Identification System. Strain J3-A127^T had a cellular fatty acid profile that was similar to those of other reference strains of the genus *Rhizobium* (Table 2). The major fatty acids of strain J3-A127^T were C_{18:1}ω7c and/or C_{18:1}ω6c, which are typical components of root-nodule bacteria (Tighe *et al.*, 2000).

Sequencing of the 16S rRNA gene was performed as described previously (Zhang *et al.*, 2008). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity values were achieved by using the EzTaxon server (Chun *et al.*, 2007; <http://147.47.212.35:8080/>). Sequence data were analysed by using the software package MEGA version 4.0 (Tamura *et al.*, 2007), after multiple alignments of the data with CLUSTAL_X 1.83 (Thompson *et al.*, 1997). Distance matrix methods (distance options according to the Kimura two-parameter model), including clustering by the neighbour-joining (Fig. 1) and minimum-evolution methods (data not shown), were used. In each case, bootstrap values were calculated based on 1000 replications. The 16S rRNA gene sequence of strain J3-A127^T was a continuous stretch of 1443 bp. Similarity values were calculated by using the same software. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain J3-A127^T showed the greatest degree of similarity to *R. oryzae* Alt 505^T (96.4 %), with bootstrap confidence values of 93 % (neighbour-joining method; Fig. 1) and of 95 and 97 % for the minimum-evolution and maximum-parsimony methods, respectively (data not shown). Strain J3-A127^T shared lower levels of 16S rRNA gene sequence similarity (<95.6 %) with the type strains of all other recognized species of the genus *Rhizobium*.

Table 2. Cellular fatty acids of strain J3A-127^T and the type strains of related *Rhizobium* species

Strains: 1, J3A-127^T; 2, *R. oryzae* Alt 505^T; 3, *R. sullae* IS123^T; 4, *R. mesosinicum* CCBAU 25010^T; 5, *R. yanglingense* CCBAU 71623^T. All data were obtained in this study. A dash indicates <2% or not detected.

Fatty acid	1	2	3	4	5
Summed feature 2*	10.40	6.30	4.83	4.51	6.49
Summed feature 3*	3.08	—	—	—	—
C _{16:0}	12.73	28.71	9.98	9.94	4.82
C _{17:0} cyclo	—	3.78	—	—	—
C _{16:0} 3-OH	3.64	—	—	—	2.72
Summed feature 8*	46.71	32.89	63.81	44.62	74.47
C _{18:0}	—	2.35	9.41	9.09	7.66
C _{18:1} ω7c 11-methyl	—	9.48	—	—	—
C _{18:1} ω9c	—	—	3.13	—	—
C _{19:0} cyclo ω8c	12.96	—	—	18.2	2.27
C _{19:0} 10-methyl	2.36	—	—	—	—
C _{18:0} 3-OH	2.72	—	—	1.77	—

*Summed features consist of two or more fatty acids that could not be separated by the Microbial Identification System. Summed feature 2 comprised C_{12:0} aldehyde, C_{14:0} 3-OH/iso-C_{16:1} I and/or unknown ECL 10.928; summed feature 3 comprised C_{16:1}ω7c and/or C_{16:1}ω6c; summed feature 8 comprised C_{18:1}ω7c and/or C_{18:1}ω6c.

Partial *recA* (530 bp), *atpD* (533 bp), *gyrB* (756 bp) and *glnII* (552 bp) gene sequences were also determined for the new isolate by using the methods and primers reported by Martens *et al.* (2007). Analysis of these housekeeping gene sequences supported the separate phylogenetic position of the novel strain within the genus *Rhizobium*. Strain J3-A127^T shared highest *recA* gene sequence similarity with *Rhizobium etli* CFN 42^T (89%) and less than 88% with the type strains of other species of the genus *Rhizobium*. The *atpD* gene sequence of strain J3-A127^T showed the greatest degree of similarity to that of *R. oryzae* Alt 505^T (89%). In the analysis of partial *gyrB* gene sequences, strain J3-A127^T showed similarity levels of less than 85% with the type strains of recognized species of the genus *Rhizobium*. The *glnII* gene sequence of strain J3-A127^T showed the greatest degree of similarity to that of *R. sullae* IS123^T (89%). The low levels of similarity between the housekeeping gene sequences of the novel strain and those of recognized species of the genus indicate that this strain may represent a separate species of the genus *Rhizobium*.

PCR amplifications of the *nodD* and *nifH* genes were performed by using primers and conditions as described by Ueda *et al.* (1995), Laguerre *et al.* (2001) and Raja *et al.* (2006). A nodulation test was carried out in a test tube (18 × 180 mm) containing quarter-strength nitrogen-free plant nutrient solution (Vincent, 1970). Four legume plant species were selected: soybean (*Glycine max*), kidney bean (*Phaseolus vulgaris*), clover (*Trifolium repens*) and alfalfa

(*Medicago sativa*). These plants are grown widely in the north of China. After surface-sterilized seeds had been germinated, seedlings were planted aseptically in each tube after being immersed in the culture broth of strain J3-A127^T with approximately 10⁶ cells ml⁻¹ for 1 h. The plants were grown at 25 °C for 6 weeks in an artificial climate chamber (humidity 55%, light/dark photoperiod of 14/10 h). Non-inoculated seedlings were used as controls and were cultured under the same conditions. Strain J3-A127^T did not form nodules on these four legume plants. The *nodD* and *nifH* genes were also not detected by PCR in this strain.

For determination of the DNA base composition, total DNA was extracted from each strain according to the method of Marmur (1961). By using the thermal denaturation method (Marmur & Doty, 1962) and *Escherichia coli* K-12 as standard, the DNA G + C content of strain J3-A127^T was 59.5 mol% (T_m). This value was within the range reported for members of the genus *Rhizobium* (57–66 mol%; Jordan, 1984).

The results of the present study show that strain J3-A127^T is genotypically and phenotypically distinct, and represents a novel species of the genus *Rhizobium*, for which we propose the name *Rhizobium pseudoryzae* sp. nov.

Description of *Rhizobium pseudoryzae* sp. nov.

Rhizobium pseudoryzae [pseud.o.ry'zae. Gr. adj. *pseudes* false; L. gen. n. *oryzae* of rice and also the name of a bacterial specific epithet; N.L. gen. n. *pseudoryzae* not the true (*Rhizobium*) *oryzae*].

Gram-stain-negative, aerobic, non-spore-forming rods (1.0–1.23 × 2.3–4.3 μm). Non-motile. No flagellum is detected. Colonies are circular and pearl white when grown on YMA at 28 °C, which is the optimal growth temperature. The pH range for growth is 6–11 (optimum pH 7–8). The maximum temperature for growth is 40 °C. Positive for catalase, oxidase and urease. Tolerates NaCl concentrations up to 5% (w/v) (optimal 1%, w/v). Does not reduce nitrate to nitrite. L-Phenylalanine ammonia-lyase is not detected. Does not produce 3-ketolactose from lactose. On GN2 microplates (Biolog), utilizes the following 64 compounds among 95 different carbon sources: dextrin, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, maltose, D-mannitol, D-mannose, D-psicose, D-sorbitol, sucrose, trehalose, turanose, xylitol, formic acid, DL-lactic acid, succinic acid, bromosuccinic acid, L-alanine, L-alanyl glycine, L-glutamic acid, glycerol, uridine, L-proline, L-asparagine, N-acetyl-D-glucosamine, L-arabinose, myo-inositol, α-D-lactose, lactulose, methyl β-D-glucoside, L-rhamnose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, cis-aconitic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinamic acid, D-alanine, glycyl L-aspartic acid, glycyl

Fig. 1. Phylogenetic tree based on analysis of the nearly complete 16S rRNA gene sequences of strain J3-A127^T and representatives of the most closely related cultured species of the genus *Rhizobium*. The branching pattern was generated by the neighbour-joining method. The topology shown was obtained by using 1000 bootstrap replications. GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide position.

L-glutamic acid, L-histidine, L-ornithine, L-pyroglutamic acid, L-serine, L-threonine, urocanic acid, inosine, glucose 1-phosphate and glucose 6-phosphate. The main cellular fatty acids are summed feature 8 (comprising *C*_{18:1ω7c} and/or *C*_{18:1ω6c}, 46.7 %).

The type strain, J3-A127^T (=ACCC 10380^T=KCTC 23294^T), was isolated from the roots of fresh rice plants (*Oryza sativa*). The DNA G + C content of the type strain is 59.5 mol%.

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