Rhizobium oryzae sp. nov., isolated from the wild rice *Oryza alta*

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During a study of endophytic nitrogen-fixing bacteria present in the wild rice species *Oryza alta*, eight novel isolates were obtained from surface-sterilized roots and classified in the genus *Rhizobium* on the basis of almost-complete 16S rRNA gene sequence analysis. These strains can nodulate *Phaseolus vulgaris* and *Glycine max*. The highly similar protein patterns, DNA fingerprint patterns of insertion sequence-based PCR (IS-PCR) and DNA–DNA hybridizations showed that these novel isolates were members of the same species. The closest phylogenetic relatives of the representative strain Alt 505^T of the novel group were *Rhizobium etli* CFN 42^T and *Rhizobium indigoferae* CCBAU 71714^T, with 96.2 and 96.0% 16S rRNA gene sequence similarity, respectively. Low DNA–DNA relatedness with the type strains of *R. etli*, *R. indigoferae*, *Rhizobium hainanense*, *Rhizobium mongolense* and *Rhizobium galegae* and differences in IS-PCR fingerprinting patterns, SDS-PAGE of proteins, antibiotic resistance, phenotypic tests and comparison of cellular fatty acids with *Rhizobium* species indicated that the novel group of isolates were distinct from previously described species. Based on these results, we propose to place them in a novel species, as *Rhizobium oryzae* sp. nov. The type strain is Alt 505^T (=LMG 24253^T =CGMCC 1.7048^T).

Rhizobium species are traditionally considered as legume endosymbionts and have generally been isolated from nodules on leguminous plants (Amarger *et al.*, 1997; de Lajudie *et al.*, 1998; Lindström, 1989; Squartini *et al.*, 2002; Valverde *et al.*, 2006; van Berkum *et al.*, 1998; Wang *et al.*, 1998; Wei *et al.*, 2002, 2003), with the exception of *Rhizobium selenitireducens* and *Rhizobium daejeonense*, isolated from bioreactors (Hunter *et al.*, 2007; Quan *et al.*, 2005), and *Rhizobium cellulosilyticum*, from sawdust of *Populus alba* (García-Fraile *et al.*, 2007). Large populations of rhizobia are found both in the bulk soil and in the rhizospheres of legumes and other plants (Schloter *et al.*, 1997; Segovia *et al.*, 1991; Sullivan *et al.*, 1996). Rhizobia are also found as viable cells in water, where they are able to infect and nodulate aquatic legumes, such as species of *Neptunia* (Zurdo-Piñeiro *et al.*, 2004), *Aeschynomene* and

Sesbania (Chaintreuil *et al.*, 2000). More recently, it has been reported that these legume symbionts may also occur as endophytes in the roots of cereals, such as rice (*Oryza breviligulata* and *Oryza sativa*) (Chaintreuil *et al.*, 2000; Elbeltagy *et al.*, 2001; Engelhard *et al.*, 2000; Ueda *et al.*, 1995; Yanni *et al.*, 1997), wheat and maize (Rosenblueth & Martinez-Romero, 2004; Schloter *et al.*, 1997). These findings have shown that rhizobia may colonize non-legume plants, especially cereals, and can promote rice growth (Tan *et al.*, 2001b; Yanni *et al.*, 1997).

Wild rice is closely related to cultivated rice, *O. sativa*, which is the most important food crop in developing countries. During a study of endophytic nitrogen-fixing bacteria present in different wild rice species, a group of novel *Rhizobium* isolates was obtained from surface-sterilized roots of the wild rice *Oryza alta*. These *Rhizobium* isolates from wild rice potentially supply nitrogen to the rice plant and promote growth, and may serve as a valuable bioresource.

In this study, eight strains (Alt 501, Alt 503, Alt 505^T, Alt 509, Alt 521, Alt 525, Alt 530 and Alt 533) were isolated from *O. alta* growing in the Wild Rice Core Collection Nursery of South China Agricultural University. Methods

Abbreviation: IS-PCR, insertion sequence-based PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Alt 505^T and Alt 501 are respectively EU056823 and EU056822.

A transmission electron micrograph of cells of strain Alt 505^T, detailed DNA–DNA hybridization results and a list of characters of two representative novel isolates are available as supplementary material with the online version of this paper.

of isolation and purification of nitrogen-fixing bacteria on YMA medium (Vincent, 1970) were described previously (Peng *et al.*, 2006). To test the symbiotic characteristics of the novel isolates, *Phaseolus vulgaris* and *Glycine max* plants were inoculated as described by Chen *et al.* (1991), using *Rhizobium etli* CFN 42^T and *Sinorhizobium fredii* USDA 205^T inoculant, respectively, as positive controls. Non-inoculated plants were used as negative controls. All eight novel strains were able to elicit effective nodules in *P. vulgaris* and *G. max*.

Whole-cell protein SDS-PAGE pattern analysis (Tan *et al.*, 2001a) was used to group the novel *Rhizobium* isolates. The eight diazotrophic isolates had almost identical protein patterns, different from reference *Rhizobium* species (Fig. 1).

To evaluate the genotypic diversity of the novel isolates further, IS-PCR (insertion sequence-based PCR) fingerprinting was performed. The conditions of PCR amplification and electrophoresis were described previously (Peng *et al.*, 2006). The eight novel isolates showed very similar patterns, but the patterns differed from those of strains of related *Rhizobium* species (Fig. 2). The amplified fragments of the novel isolates were 500–1000 bp long, whereas most of the reference *Rhizobium* strains yielded fragments of 600–2500 bp.

Nearly complete 16S rRNA gene sequences of representative strains Alt 505^T and Alt 501 were obtained in this study according to the method described previously by Peng *et al.* (2006). The 16S rRNA gene sequences obtained and related reference sequences from GenBank were aligned using the CLUSTAL W software (Thompson *et al.*, 1994). The tree topology was calculated by the maximum-parsimony, maximum-likelihood (using PAUP 4.0; Swofford, 1998) and neighbour-joining (Saitou & Nei, 1987) tree-building methods. The neighbour-joining tree was drawn and bootstrapped by using the TREECON

software package (Van de Peer & de Wachter, 1994) using 1000 replicates.

16S rRNA gene sequences of strains Alt 505^T (1425 bp) and Alt 501 (1422 bp) were almost identical. The results of the phylogenetic analysis indicate that the strains from this study are related to members of the genus *Rhizobium* within the family *Rhizobiaceae* (Fig. 3). Their closest phylogenetic relatives are *R. etli* CFN 42^T and *Rhizobium indigoferae* CCBAU 71714^T, with 96.2 and 96.0 % 16S rRNA gene sequence similarity, respectively.

Extraction and purification of total genomic DNA were carried out by using the method of Marmur (1961) and DNA base composition was determined by the thermal denaturation method (T_m) (De Ley, 1970; Marmur & Doty, 1962). DNA from *Escherichia coli* K-12 was used as the standard for estimation of G + C content. DNA–DNA relatedness was determined by the initial renaturation rate method (De Ley, 1970) in 2 × SSC. The G + C content of the genomic DNA of strain Alt 505^T was 64.1 ± 0.4 mol%. This value is in the range reported for other *Rhizobium* species (Jordan, 1984).

DNA–DNA relatedness among the novel isolates varied from 90 to 100 %, indicating that they were members of the same genomic species. DNA–DNA hybridization between Alt 505^T and reference *Rhizobium* strains yielded low values (≤ 33 %; see Supplementary Table S1, available in IJSEM Online), well below the recommended value of 70 % for species definition (Wayne *et al.*, 1987).

Harvesting of cells and extraction of fatty acids were carried out as described by Sasser (1990) and fatty acid analysis was carried out as described by Peng *et al.* (2006). The cellular fatty acids of strain Alt 505^T representing the novel group and the related strains *R. etli* CFN 42^T and *R. indigoferae* CCBAU 71714^T have three common components at retention times of 7.5, 10.0 and 10.4 min. These common components together made up 84.4, 73.2 and 30.6 % of the detected components of Alt 505^T, *R. etli* CFN

Fig. 1. SDS-PAGE whole-cell protein patterns of the nitrogen-fixing isolates from wild rice *Oryza alta* and strains of related species. Lanes: M, molecular-mass standards; 1, strain Alt 530; 2, Alt 533; 3, Alt 509; 4, Alt 505^T; 5, Alt 521; 6, Alt 503; 7, Alt 501; 8, Alt 525; 9, *R. sultae* IS123^T; 10, *R. tropici* type B CIAT 899^T; 11, *R. loessense* CCBAU 7190B^T; 12, *R. leguminosarum* USDA 2370^T; 13, *R. giardinii* USDA 2914^T; 14, *R. galegae* HAMBI 540^T; 15, *R. huautlense* SO2^T; 16, *R. yanglingense* CCBAU 71623^T; 17, *R. tropici* type A CFN 299; 18, *R. mongolense* USDA 1844^T; 19, *R. hainanense* CCBAU 57015^T; 20, *R. etli* CFN 42^T; 21, *R. indigoferae* CCBAU 71714^T; 22, *R. undicola* LMG 11875^T.

Fig. 2. IS-PCR fingerprinting patterns of the diazotrophic isolates from wild rice and strains of related species. Lanes: M, standard DNA marker; 1, strain Alt 525; 2, Alt 533; 3, Alt 503; 4, Alt 505^T; 5, Alt 501; 6, Alt 509; 7, Alt 521; 8, Alt 530; 9, *R. mongolense* USDA 1844^T; 10, *R. giardinii* USDA 2914^T; 11, *R. indigoferae* CCBAU 71714^T; 12, *R. loessense* CCBAU 7190B^T; 13, *R. leguminosarum* USDA 2370^T; 14, *R. sullae* IS123^T; 15, *R. tropici* type A CFN 299; 16, *R. yanglingense* CCBAU 71623^T; 17, *R. undicola* LMG 11875^T; 18, *R. etli* CFN 42^T; 19, *R. huautlense* SO2^T; 20, *R. galegae* HAMBI 540^T; 21, *R. hainanense* CCBAU 57015^T; 22, *R. tropici* type B CIAT 899^T.

42^T and *R. indigoferae* CCBAU 71714^T (Table 1). Other components were present in some or all of strain Alt 505^T, *R. etli* CFN 42^T and *R. indigoferae* CCBAU 71714^T. The relative abundance of each compound was also different in the tested strains.

Negatively stained cells (Cole & Popkin, 1981) harvested from cultures grown in YMA liquid medium were used for the examination of flagella under a transmission electron microscope. Cell morphology was observed by using light microscopy and transmission electron microscopy

Fig. 3. Phylogenetic tree of 16S rRNA gene sequences constructed with the neighbour-joining method showing relationships among strain Alt 505^T and members of related genera. The sequence of *Bradyrhizobium japonicum* USDA 6^T was used as an outgroup. Bootstrap percentages calculated by the neighbour joining/maximum-parsimony methods $\geq 55\%$ are indicated at nodes. Bar, 1% nucleotide substitution. GenBank/EMBL/DBJ accession numbers are shown in parentheses.

Table 1. Cellular fatty acid compositions of strain Alt 505^T and related strains

Fatty acids were determined as methyl esters by gas chromatography; values are percentages of total peak area.

Retention time (min)	Alt 505 ^T	<i>R. etli</i> CFN 42 ^T	<i>R. indigoferae</i> CCBAU 71714 ^T
3.9			2.4
5.1			1.6
6.0	1.1		28.9
6.3	1.0		0.8
7.0	1.8	9.2	
7.1			7.3
7.3	7.7		5.4
7.5	20.4	6.8	8.1
8.5			21.6
8.9			1.4
10.0	64.0	61.5	21.2
10.4	4.0	4.9	1.3
11.7		12.5	
13.0		5.1	

(Hitachi-600). Cells are straight rods with polar flagella (Supplementary Fig. S1).

Utilization of sugars, amino acids, alcohols and organic acids as sole carbon sources was examined as described previously (Chen *et al.*, 1988; Gao *et al.*, 1994). Representative strains Alt 505^T and Alt 501 were also characterized by using Biolog GN2 MicroPlates in comparison with reference *Rhizobium* species. Overnight cultures were used to inoculate the GN2 MicroPlates, according to the manufacturer's instructions. Antibiotic resistance tests and determination of NaCl tolerance and pH range for growth were performed in YMA, as described by Tan *et al.* (1999); phenotypic and biochemical features are summarized in Supplementary Table S2. Discriminative features among the novel isolates and their closest relatives, including *R. etli* and *R. indigoferae*, are shown in Table 2.

Based on the results described above, we propose to place the new isolates from wild rice in a novel species, *Rhizobium oryzae* sp. nov.

Description of *Rhizobium oryzae* sp. nov.

Rhizobium oryzae (o.ry'zae. L. gen. n. *oryzae* of rice, the origin of the first strains).

Cells are Gram-negative, motile, straight, non-spore-forming rods (0.8–1.0 × 1.4–1.8 µm) with polar flagella. Colonies on YMA medium are circular, cream coloured and semi-translucent, with a diameter of 3 mm within 3 days at 28 °C. Growth occurs at 10–40 °C (optimum 28–37 °C). The pH range for growth is pH 5.0–8.0 (optimum pH 6.0–7.0). NaCl inhibits growth above 1.5 %. Resistant to ampicillin (100 µg ml⁻¹) and neomycin (5 µg ml⁻¹). Tests for *o*-nitrophenyl-β-D-galactopyranosidase, urease,

Table 2. Discriminative features of two representative novel isolates and related species of the genus *Rhizobium*

Taxa: 1, strains Alt 505^T and Alt 501; 2, *Rhizobium leguminosarum* (compiled from data for two strains; origin *Phaseolus* sp. and *Vicia* sp., USA); 3, *R. tropici* (n=2; *Leucaena leucocephala* and *Phaseolus vulgaris*, Brazil); 4, *R. galegae* (n=2; *Galega orientalis*, Finland); 5, *R. hainanense* (n=2; *Desmodium sinuatum*, China); 6, *R. etli* (n=2; *Phaseolus vulgaris*, Mexico); 7, *R. indigoferae* (n=8; *Indigofera amblyantha*, China). Numbers are percentages of strains testing positive. +, All strains positive; –, all strains negative. Data for reference species were compiled from Chen *et al.* (1997) and Wei *et al.* (2002).

Feature	1	2	3	4	5	6	7
Growth in/at:							
NaCl (3.0 %)	–	+	+	+	50	–	13
pH 5.0	+	–	+	+	+	–	–
40 °C	+	–	50	–	+	–	–
Utilization as sole carbon source of:							
D-Amygdalin	–	+	–	50	–	–	13
D-Arabinose	+	+	+	+	+	+	–
D-Arabitol	–	–	50	–	+	+	75
Calcium malonate	–	+	–	+	–	–	–
Dulcitol	+	+	–	+	+	–	+
i-Erythritol	+	–	+	50	+	–	25
Lactose	+	+	50	+	–	–	88
Salicin	–	+	–	+	–	+	+
D-Ribose	–	+	+	–	+	+	25
D-Gluconate	+	–	–	50	+	+	75
Sucrose	+	–	–	+	+	+	+
Tartrate	–	50	50	50	–	+	–
Xylose	+	+	+	–	+	+	+
Glycine	–	–	+	50	+	+	+
Antibiotic resistance (µg ml ⁻¹)							
Ampicillin (100)	+	+	+	+	+	+	13
Chloramphenicol (100)	–	–	+	–	+	–	–
Erythromycin (100)	–	–	–	+	+	–	13
Kanamycin (5)	–	+	+	+	+	+	–
Streptomycin (300)	–	–	+	–	–	+	–

cytochrome oxidase and the Voges–Proskauer reaction are positive. The following compounds are used as sole carbon sources: L- and D-arabinose, L-arabitol, cellobiose, N-acetyl-D-glucosamine, adonitol, L-alanine, i-erythritol, D-fructose, D-fucose, D-galactose, gentiobiose, α-D-glucose, myo-inositol, α-D-lactose, maltose, D-mannitol, D-mannose, melibiose, succinic acid, citric acid, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, methyl pyruvate, D-gluconic acid, monomethyl succinate, D-galacturonic acid, D-glucuronic acid, β-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, quinic acid, D-saccharic acid, bromosuccinic acid, L-alanyl glycine, glycerol, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-proline, hydroxy-L-proline, L-serine, L-threonine, γ-aminobutyric acid, urocanic acid, uridine, D-xylose, glucose 1-phosphate, glucose 6-phosphate, dulcitol, L-malate, D-ribose, xylose,

sorbitol and D-tagatose. The G+C content of genomic DNA of the type strain is 64.1 ± 0.4 mol%.

The type strain, strain Alt 505^T (=LMG 24253^T =CGMCC 1.7048^T), and seven other strains were isolated from the wild rice species *Oryza alta*.

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