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ORIGINAL PAPER

Rhizobium rhizosphaerae sp. nov., a novel species isolated from rice rhizosphere

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Abstract Two novel, Gram-negative, motile, rod-shaped, aerobic bacterial strains, MH17^T and RD15, were isolated from the sterilized root and rhizosphere soil of rice, respectively. Phylogenetic analysis based on 16S rRNA gene sequences showed that the similarity between strains MH17^T and RD15 was 100%. The isolates exhibit high sequence similarities to *Rhizobium oryzae* CGMCC 1.7048^T (98.7%) and *Rhizobium petrolearium* SL-1^T (97.0% and 97.1%), which supports that they belong to a novel species in the genus *Rhizobium*. Strains MH17^T and RD15 exhibited growth at 15–45 °C, pH 5.0–11.0, 0–2.0% sodium chloride (w/v). Sequence analysis of housekeeping genes *gyrB*, *recA*, *atpD*, *ropB*, *gltA* showed that these two novel strains had less than 94% similarity with the known species, indicating the distinct position of MH17^T and RD15 in the genus *Rhizobium*. The major cellular fatty acids were identified as summed feature 8 ($C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$). Type strain MH17^T had 87.5% DNA–DNA relatedness with RD15 by using the initial renaturation rate method. Based on draft genome sequences, strain MH17^T showed 30.1% DNA–DNA hybridization values to *R. oryzae* CGMCC 1.7048^T, the closely related strain, which supported that MH17^T represents a novel species in the genus *Rhizobium*. Average nucleotide identity (ANI) between strains MH17^T and RD15 were 97.8%, and strain MH17^T showed 82.2% ANI value with *R. oryzae* CGMCC 1.7048^T. The DNA G+C content was 60.4 mol% (T_m). Based on physiological, biochemical characteristic, genotypic data, strains MH17^T and RD15 are concluded to represent a new species within the genus *Rhizobium*, for which the name *Rhizobium rhizosphaerae* sp. nov. is proposed. The type strain is MH17^T (=ACCC 19963^T = KCTC 52414^T).

Keywords *Rhizobium rhizosphaerae* sp. nov. · Polyphasic taxonomy · Root of rice

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB*, *recA*, *atpD*, *ropB*, *gltA* gene sequences of strain MH17^T are KX129902, KX434874, KX434876, KY321909, KY321911, KY321915, respectively.

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Introduction

The genus *Rhizobium* was first proposed by Frank (1889) and revised by Young et al. (2001) and belongs to the bacterial phylum *Proteobacteria*. Up to now, it

has more than 80 identified species, many of which have been isolated from the nodules on leguminous plants with the function of symbiotic nitrogen-fixing. However, there are a growing number of novel species that have been isolated from distinct environments, for example, *Rhizobium alvei* NR-22^T isolated from a freshwater river (Sheu et al. 2015), *Rhizobium capsici* CC-SKC2^T from the root tumor of a green bell pepper (Lin et al. 2015), *Rhizobium flavum* YW14^T from soil (Gu et al. 2014), *Rhizobium tarimense* PL-41^T isolated from *Populus euphratica* forest soil (Turdahon et al. 2013), *Rhizobium pseudoryzae* ACCC 10380^T, *Rhizobium rhizoryzae* ACCC 05916^T, *Rhizobium oryzicola* ACCC 05753^T from the roots of rice (Zhang et al. 2011, 2014, 2015). The genus *Rhizobium* are usually characterized as rod-shaped, aerobic, Gram-negative bacteria, with a DNA G+C content between 57 and 66 mol% (Tighe et al. 2000; Young et al. 2001) and $C_{18:1}$ $\omega 7c$ as the major cellular fatty acids. In this study, we report two novel strains MH17^T and RD15 of the genus *Rhizobium* according to their phenotypic and genotypic characteristics.

Materials and methods

Strains and culture conditions

Endophytic strain MH17^T was isolated from surface sterilized rice root collected from Beijing, and RD15 was from the rhizosphere of rice sampled from Hunan Province. Surface disinfection of rice roots was performed according to the methods of Zhang et al. (2015), and the rhizosphere soil collection was as described by Zhang et al. (2011). Both of bacteria grew on yeast extract mannitol agar medium (Vincent. 1970) at 30 °C. Strains were preserved as glycerol suspension (20%, v/v) at −80 °C and at −4 °C in freeze-dried ampoules for further characterization.

To study the chemotaxonomic and molecular characteristics, biomass was collected from YMA medium at 30 °C for 2 days. The reference strains *Rhizobium oryzae* CGMCC 1.7048^T, *R. petrolearium* SL-1^T were obtained from China General Microbiological Culture Collection Center (CGMCC; China) and Agricultural Culture Collection of China (ACCC; China), respectively. The two reference strains were cultured for comparative analysis under the same conditions as strains MH17^T and RD15.

Morphological, physiological and biochemical characteristics

Cell morphology was examined using light microscope (CX21; Olympus). Gram-staining was carried out using the Gram-Stain Kit (HiMedia). Growth features were tested in different temperatures at 4, 15, 25, 30, 37, 40, 45, 50 °C and pH 3.0–12.0 (at 1.0 unit intervals) on YMA medium. Salt tolerance was tested on YMA with NaCl concentration of 0, 1.0, 3.0, 5.0, 7.0, 10.0% (w/v). Oxidase and catalase activities were determined by using 1%(w/v) tetramethyl-p-phenylenediamine and 3% (v/v) H_2O_2, respectively. Motility was observed by semisolid culture-medium (0.4% agar added). Mean generation time was estimated based on the growth curve from YMA medium. The basic biochemical characteristics were investigated on Biolog GN2 Microplates (Hay-ward, CA) and API-20NE test strips (bioMérieux, France).

Molecular study

Genomic DNA was extracted from pure cultures using TIANamp Bacteria DNA Kit (Tiangen) based on the manufacture's protocol. The 16S rRNA gene was amplified using the universal primers 27F and 1492R according to Lane et al. (1991). The housekeeping genes *gyrB*, *recA*, *atpD*, *ropB*, *gltA* were amplified using the methods of Martens et al. (2007, 2008). The 16S rRNA gene sequences similarity and multiple sequences alignment were analysed using EzTaxon-e Service (Kim et al. 2012) and CLUSTALW (Thompson et al. 1994), respectively. Similarities of housekeeping genes were performed by NCBI BLAST program (http://www.ncbi.nlm.nih.gov) and multiple sequence alignment was carried out by CLUSTALW. Phylogenetic trees were constructed using MEGA 7.0 software (Kumar et al. 2016) with neighbor-joining (Saitou and Nei 1987), minimum evolution (Rzhetsky and Nei 1992) and maximum likelihood (Felsenstein 1981) methods.

To determine the genomic DNA G+C content, Beckman DU 800 Spectrophotometer (Beckman collulter, brea, CA, USA) was used according to the thermal denaturation methods (Marmur and Doty 1962). *Escherichia coli* K12 was used as the reference strain. The initial renaturation rate method (De ley 1970) was used to determine DNA relatedness.

Draft genome sequences were determined using Hiseq Technology by Zeta Biosciences (Shanghai) Co., Ltd. The genomic sequences were assembled with

SOAP denovo version 2.04 software and annotated with Prokaryotic Genome Annotation Pipeline (PGAP) by NCBI. The genome sequences were submitted to NCBI. The DDH (DNA-DNA hybridization) estimates were using GGDC (Genome-to-Genome Distance Calculator; http://ggdc.dsmz.de) with the BLAST+ (recommended) method (Meier-Kolthoff et al. 2012). The ANI values were estimated by using ANI Calculator in the EZBioCloud (http://www.ezbiocloud.net).

Fatty acid analysis

For cellular fatty acids analysis, strains MH17^T, RD15 and *R. oryzae* CGMCC 1.7048^T, *R. petrolearium* SL-1^T were grown on YMA for 2 days at 28 °C. Cultures were harvested and fatty acid methyl esters were prepared and separated using methods described by Sasser (1990) and were identified with the MIDI Sherlock Microbial Identification System.

Results and discussion

Morphological, physiological and biochemical characteristics

Strains MH17^T and RD15 were observed to be rod-shaped aerobic, motile, Gram-stain negative bacteria. Colonies were circular and pearl white on YMA medium at 30 °C. Growth occurred at 15–45 °C (optimum 30 °C) and the pH range for growth was 5.0–11.0. The tolerance of NaCl was up to 2.0% (w/v). Strains MH17^T and RD15 were observed to be catalase and oxidase positive. Mean generation time of MH17^T was 2.0 hours in YMA medium. Other physiological and biochemical characteristics of the novel isolates and reference strains are given in Table 1. Strains MH17^T and RD15 could be distinguished from *R. oryzae* CGMCC 1.7048^T in utilization of dextrin, i-erythritol, lactulose, α-keto glutaric acid. In contrast to *R. petrolearium* SL-1^T, both of them could utilize adonitol, i-erythritol, lactulose, D-raffinose, succinic acid as carbon source.

Table 1 Different phenotypic characteristics of strains MH17^T and RD15 and closely related strains: 1, MH17^T; 2, RD15; 3, *R. oryzae* CGMCC 1.7048^T; 4, *R. petrolearium* SL-1^T. +, positive; −, negative

	1	2	3	4
Growth in/at				
45 °C	−	+	−	−
NaCl (3.0%)	−	+	−	+
pH range for growth	5–11	5–11	5–11	6–11
Urease	+	−	+	+
Utilization as sole carbon source of				
Dextrin	+	+	−	+
Adonitol	+	+	+	−
i-Erythritol	+	+	−	−
m-Inositol	+	−	+	+
Lactulose	+	+	−	−
D-Psicose	−	−	−	+
D-Raffinose	+	+	+	−
Citric acid	−	+	+	−
γ-Hydroxybutyric acid	+	−	−	−
α-Keto glutaric acid	+	+	−	+
Malonic acid	−	−	+	−
Propionic acid	−	−	−	+
Succinic acid	+	+	+	−
Glycyl-L-aspartic acid	+	−	−	+
Glycyl-L-glutamic acid	+	−	−	+
Hydroxy L-proline	+	−	+	−
L-pyroglutamic acid	−	+	−	+
L-Threonine	+	−	−	−
D, L-Camitine	+	−	−	−
Inosine	+	−	+	−
Glucose-1-phosphate	+	−	+	−
Glucose-6-phosphate	+	−	+	−

Data for the reference species are from the present study

Molecular results

The almost complete 16S rRNA (1444 bp) gene sequences of strains MH17^T and RD15 were determined and subjected to phylogenetic analysis. Similarity search in EzTaxon-e revealed that strains MH17^T and RD15 are closely related to members of the genus *Rhizobium,* showing high 16S rRNA gene sequence similarity to *R. oryzae* CGMCC 1.7048^T (98.7%), followed by *R. petrolearium* SL-1^T (97.0% and 97.1%). In the phylogenetic tree based on the neighbor-joining method, and also recovered using maximum-likelihood and maximum-evolution methods, the two strains were clustered into a novel branch within the genus *Rhizobium* (Fig. 1).

To further explore these phylogenetic relationships, five housekeeping genes, *gyrB* (684 bp), *recA* (570 bp), *atpD* (521 bp), *ropB* (935 bp), *gltA* (674 bp), were also sequenced. Sequence similarities of these housekeeping genes between strains MH17^T and RD15 are 98–99%. Strain MH17^T shared high *gyrB* gene sequence similarity with *R. oryzae* CGMCC 1.7048^T (88%) and no more than 83% with other species of the genus *Rhizobium*. The *recA* and *ropB* gene sequences of strain MH17^T showed a high degree of similarity to that of *R. oryzae* CGMCC 1.7048^T (93%) and less than 89% sequence similarity to other members of the genus *Rhizobium*. The *atpD* gene sequence similarity between strain MH17^T and *R. oryzae* CGMCC 1.7048^T was 94%, and no more than 91% between MH17^T and other species of the genus *Rhizobium*. Strain MH17^T showed no more than 92% *gltA* gene sequence similarity with members of the genus *Rhizobium.* In addition, the phylogenetic tree reconstructed based on the concatenated housekeeping genes demonstrated that strains MH17^T and RD15 formed a phylogenetic cluster with *R. oryzae* CGMCC 1.7048^T (Fig. 2), indicating that the novel strains belonged to the genus of *Rhizobium* but represent a new species.

The DNA G+C content of strain MH17^T was determined to be 60.4 mol% (T_m), which was within the range values reported for members of the genus *Rhizobium* (57–66 mol%; Jordan. 1984). The DNA-DNA relatedness between strains MH17^T and RD15 was 87.5% by using the initial renaturation rate method, which was higher than the species threshold of 70%. The genome sequences were submitted to NCBI and the BioProject IDs for MH17^T and *R. oryzae* CGMCC 1.7048^T are PRJNA344723 and PRJNA344729. The level of DNA-DNA relatedness between strains MH17^T and *R. oryzae* CGMCC 1.7048^T, a closely related strain, was 30.1%, according to the draft genome sequences. The ANI value between strains MH17^T and RD15 was 97.8%, and strain MH17^T showed 82.2% ANI value with *R. oryzae* CGMCC 1.7048^T. These values indicate that strain MH17^T represents a novel species in *Rhizobium* genus.

Fatty acid analysis

The major cellular fatty acids of strain MH17^T were summed feature 8 ($C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$). The detailed fatty acid compositions of strains MH17^T, RD15 and *R. oryzae* CGMCC 1.7048^T, *R. petrolearium* SL-1^T are shown in Table 2. Strains MH17^T and RD15 contained the same fatty acids in similar proportions but in comparison with the close relative

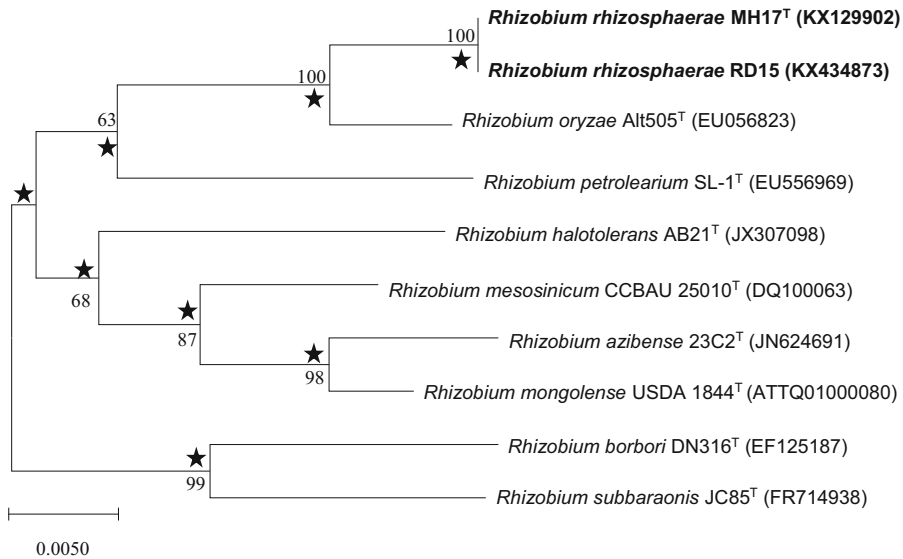

Fig. 1 Neighbor-joining method reconstructed from a comparative analysis of 16S rRNA gene sequences, showing the relationships between strains RD15^T and MH17 and closely related species. *Asterisk* indicates branches that are also recovered using the ML and ME methods. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. Genbank accession numbers are given in parentheses. *Bar*, 0.005 substitutions per nucleotide position

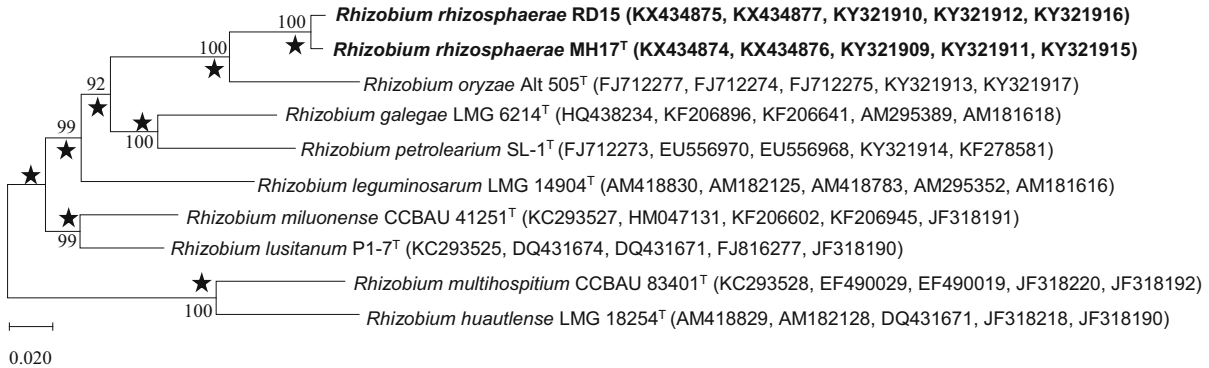

Fig. 2 Neighbor-joining phylogenetic tree based on the comparision of the concatenated housekeeping genes (*gyrB*, *recA*, *atpD*, *ropB*, *gltA*) using MEGA 7.0, showing the phylogenetic relationship between novel strains and recognized species of the *Rhizobium*. *Asterisk* means branches that are also recovered using the ML and ME methods. Bootstrap values (%) based on 1000 replicates, >50%. *Bar*, 2% sequence divergence

R. oryzae CGMCC 1.7048^T, the two novel strains differed in presence of $C_{18:1}$ $\omega 9c$. Strain MH17^T could also be distinguished from *R. petrolearium* SL-1^T by containing $C_{18:1}$ $\omega 7c$ 11-methyl, summed feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$) and lacking $C_{19:0}$ cyclo $\omega 8c$.

Taxonomic conclusion

By means of biochemical, physiological and morphological characteristics, DNA-DNA hybridization and genotypic comparison of 16S rRNA and housekeeping genes, strains MH17^T and RD15 are proposed to represent a novel species within the genus *Rhizobium* for which the name *Rhizobium rhizosphaerae* is proposed.

Table 2 Cellular fatty acid content of strains MH17^T and RD15 and closely related species

	1	2	3	4
$C_{16:0}$	9.2	9.2	12.7	11.5
$C_{16:0}$ 3OH	1.6	1.4	1.3	3.0
$C_{18:0}$	1.6	4.0	4.7	4.3
$C_{18:1}$ $\omega 9c$	–	–	1.7	–
$C_{18:1}$ $\omega 7c$ 11-methyl	3.7	3.9	3.4	–
$C_{19:0}$ cyclo $\omega 8c$	–	–	–	4.7
Summed feature 2[a]	4.5	5.0	4.1	3.6
Summed feature 3[a]	6.6	6.1	4.2	–
Summed feature 8[a]	69.8	65.0	62.6	63.3

Strains: 1, MH17^T; 2, RD15; 3, *R. oryzae* CGMCC 1.7048^T; 4, *R. petrolearium* SL-1^T. All data were obtained in this study. A dash indicates <1% or not detected

[a] Summed features consist of two or more fatty acids that could not be separated by the Microbial Identification System. Summed feature 2 is comprised of aldehyde-$C_{12:0}$ and/or unknown ECL10.9525;summed feature 3 is comprised of $C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$; summed feature 8 is comprised of $C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$

Description of *Rhizobium rhizosphaerae* sp. nov.

Rhizobium rhizosphaerae (rhi.zo.sphae'rae. Gr. n. rhiza, a root; L. n. sphaera, ball, sphere; N.L. fem. n. *rhizosphaera*, the rhizosphere; N.L. gen. n. *rhizosphaerae*, of the rhizosphere).

Cells are Gram-negative, motile rods and aerobic. Colonies are circular, pearl white, smooth and convex after 2 days incubation on YMA medium at 30 °C. Growth occurs from 15 to 45 °C and pH 5.0–11.0. NaCl is tolerated up to a concentration of 2% (w/v). Catalase and oxidase activities are positive. Reduces potassium nitrate. Hydrolyses esculin and gelatin. Assimilates arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, gluconate, decanoic acid, oxalic acid, malic acid, citric acid and phenylacetic acid. The following substrates are also positive: dextrin, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, a-D-lactose, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, methyl pyruvate, acetic acid, cis aconitic acid, formic acid, D-gluconic acid, α-keto glutaric acid, D, L-

lactic acid, quinic acid, succinic acid, bromo succinic acid, L-alanyl-glycine, L-asparagine, L-glutamic, L-histidine, L-ornithine, L-proline, L-serine, γ-amino butyric acid, glycerol. The major cellular fatty acids are summed feature 8 ($C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$).

The type strain is MH17^T (= ACCC 19963^T = KCTC 52414^T), which was isolated from root of rice collected from Beijing, China. The DNA G+C content of the type strain is 60.4 mol%.

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