

Rhizobium alarensis sp. nov., and Rhizobium halophilensis sp. nov., isolated from the nodule and rhizosphere of Lotus japonicus

Chuanxing Wan (✉ wanchuanxing@163.com)

Tarim University

Hongling Shen

Tarim University

Xiaoxia Luo

Tarim University

Zhanfeng Xia

Tarim University

Research Article

Keywords: Lotus japonicus, Rhizobium alarensis sp. nov., Rhizobium halophilensis sp. nov.

Posted Date: April 25th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1568627/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Two strains (TRM95111^T and TRM95001^T) of Gram-stain-negative, aerobic, rod-shaped mycobacteria were isolated from the nodule and rhizosphere of *Lotus japonicus* grown in the campus of Tarim University in Alar, Xinjiang, China. Strain TRM95111^T and strain TRM95001^T shared 93.1% 16S rRNA gene sequences similarity with each other and had 98.2% and 97.9% 16S rRNA gene sequence similarity to the closest species *Rhizobium subbarae* JC85^T and *Rhizobium halotolerans* AB21^T, respectively. Phylogenetic analyses based on 16S rRNA gene sequences, two housekeeping gene sequences (*recA*, *atpD*) and core genes showed that two strains belonged to the genus *Rhizobium*. The values of digital DNA-DNA hybridization (dDDH) between strain TRM95111^T and the closest strains *R. subbarae* JC85^T and *R. azooxidifex* Po 20/26^T were 21.8% and 21.4%, respectively. The dDDH values between strain TRM95001^T and the closest strains *R. halotolerans* AB21^T and *R. flavum* YW14^T were 22.6% and 26.7%, respectively. Whole genome average nucleotide identity (ANI) values of the strain TRM95111^T were 75.6%-77.5% and strain TRM95001^T were 79.2%-79.6%, compared to their closely related strains. The G + C content values of strain TRM95111^T and TRM95001^T were 65.1 mol% and 60.7 mol%, respectively. Two isolates contained predominant quinone of Q-10 and the major fatty acids was C_{18:1} ω7c and they were sensitive to 1 μg of amikacin and kanamycin. The polar lipids of strain TRM95111^T included unidentified aminophospho lipids (APL1-3), unidentified phospholipids (PL1-2), phosphatidylcholine (PC), unidentified lipids (L1-5) and phospholipids of phospholipids of unknown structure containing glucosamine (NPG), compared to the polar lipids of strain TRM95001^T including unidentified aminophospho lipids (APL1-3), unidentified phospholipids (PL1-2), phosphatidylcholine (PC), unidentified lipids (L2-5), hydroxy phosphatidyl ethanolamine (OH-PE) and phospholipids of phospholipids of unknown structure containing glucosamine (NPG). Nodulation tests showed that two strains could induce nodules formation in *L. japonicus*. Based on genomic, phenotypic and phylogenetic analyses, strain TRM95111^T and strain TRM95001^T are suggested to represent two new species of the genus *Rhizobium*, whose names are proposed as *Rhizobium alarensis* sp. nov. and *Rhizobium halophilensis* sp. nov. The type strains are TRM95111^T (= CCTCC AB 2021116^T = JCM34826^T) and TRM95001^T (= CCTCC AB 2021095^T = JCM34967^T), respectively.

Introduction

Rhizobia have great environmental and agricultural importance because their symbioses with legumes are responsible for most of the atmospheric nitrogen fixation on earth. *Rhizobium* species usually fix nitrogen after colonization inside the root nodules of legumes and are usually isolated from the root nodules of various leguminous plants (Rahi et al. 2020). However, some *Rhizobium* species have also been isolated from soil (Su et al. 2021), rhizosphere soil (Hang et al. 2019) and non-leguminous plants, such as rice (Zhao et al. 2017), maize (Celador et al. 2017) and grasses (Zhang et al. 2014). At the time of writing the genus contains more than 140 species (<https://lpsn.dsmz.de/genus/rhizobium>) including the recently described *Rhizobium dioscoreae* (Ouyabe et al. 2020), *Rhizobium ruizarguesonis* (Jorin et al.

2020), *Rhizobium rhizophylum* (Gao et al. 2020) and *Rhizobium changzhiense* (Zhang et al. 2021). *Lotus* species has strong root sprouting ability, deep taproot, which can adapt to a variety of habitat conditions and plays an important role in soil fixation, wind prevention and sand fixation, vegetation restoration and water and soil conservation. It can establish highly specific symbiotic relationships with N-fixing soil bacteria collectively known as rhizobia. Research have found that strains *Mesorhizobium loti*, *M. jarvisii*, *M. erdmanii* and *M. japonicum* isolated from *L. japonicus* and *L. corniculatus*, respectively (Martínez-Hidalgo et al. 2015; 2016). During analysis of the endophytic bacterial diversity of *L. japonicus*, two new *Rhizobium* strains can form nodule

s, *Rhizobium alarensis* sp. nov., TRM95111^T and *Rhizobium halophilensis* sp. nov., TRM95001^T, isolated from the root nodule and rhizosphere of *Lotus japonicus*.

Materials And Methods

Isolation of *Rhizobium* strains and preservation

Strain TRM95111^T was isolated from the root nodules of *Lotus japonicus* grown in the campus of Tarim University in Alar, Xinjiang, China (40°17' N, 81°23' E). The isolation experiments were as described by Ouyabe et al. (2019a; 2019b). Briefly, samples were washed with sterilized distilled water to remove dust and then surface sterilized in a solution of 70% ethanol for 30 s and in 2.5% sodium hypochlorite (NaClO) for 60 s before ten rinses in sterile distilled water. The samples were then macerated using a sterilized mortar and pestle containing 10 mL autoclaved normal saline (0.9% NaCl). One millilitre of plant macerate was streaked onto yeast extract-mannitol agar (YMA). Among the appeared colonies on plates, bacteria with different colony morphologies were picked up and purified by repeated streaking, and these bacteria were preserved as glycerol suspensions (20%, v/v) at -80 °C. The strain TRM95111^T was routinely cultivated on YMA plates at 25 °C for 2-3 days.

Strain TRM95001^T was isolated from the rhizosphere of *L. japonicus* grown in the campus of Tarim University in Alar City, Xinjiang, China (40°17' N, 81°23' E). The rhizosphere soil sample of 1 g was added to 10 mL ddH₂O and mixed evenly, and then diluted with distilled water to 10⁻³ and 10⁻⁴ dilution inoculated them on the nutrient agar (NA). Among the appeared colonies on plates, bacteria with different colony morphologies were picked up and purified by repeated streaking, and these bacteria were preserved as glycerol suspensions (20%, v/v) at -80 °C. The strain TRM95001^T was routinely cultivated on NA plates at 25 °C for 3-4 days.

Morphological and Phenotypic characterisation

Cell morphological and micromorphological properties of strain TRM95111^T and strain TRM95001^T, were observed by scanning electron microscopy (SEM, FEI APREO-S) after incubation on YMA and NA at 25 °C for 3 and 4 days, respectively. The temperature of TRM95111^T and TRM95001^T for growth was assessed

on YMA and NA media at 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C by measuring turbidity at 600 nm using a spectroscopic method (UV-5800 PC), adjust to different temperature 35-40 °C (at intervals of 1.0 temperature unit). Tolerance of NaCl was tested 0-10% NaCl (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%, w/v), adjust to different pH values 4.0-11.0 (at intervals of 0.5 pH unit) using the appropriate biological buffers as described by Xu et al. (2005). The incubation times for temperature, NaCl, and pH range tests were 3 days. The utilization of various materials as sole sources was tested by using GENIII MicroPlates (Biolog) in accordance with the manufacturer's instructions. Antibiotic susceptibility was tested on YMA and NA agar plates inoculated with strains using filter paper discs containing the following compounds (1 µg, 2.5 µg, 5 µg, 10 µg, 30 µg, 50 µg, 100 µg, 300 µg per disc): amikacin, ampicillin, chloramphenicol, erythromycin, kanamycin, nalidixic acid, streptomycin, sulfate, tetracycline and vancomycin. After incubating at 25 °C for 3 days, the inhibition zones were measured according to the method described by (Zhong et al. 2016).

Determination of 16S rRNA gene sequence and phylogenetic analyses

The genomic DNA of strains TRM95111^T and TRM95001^T was extracted by the method of Marmur et al. (1961). 16S rRNA gene was amplified using the universal primers 27F and 1492R set by Lane et al. (1985). The amplicons were ligated into the pMD19-T vector (TaKaRa, China), transformed into *Escherichia coli* DH5α strain and sequenced by Personal Biotechnology Co., Ltd (Shanghai, China) to obtain a complete sequence. The determined 16S rRNA gene sequences of the two strains were BLAST-searched against the GenBank and EzBioCloud (<http://www.ezbiocloud.net>) databases. Housekeeping genes (*recA* and *atpD*) were amplified using primers as described by Gaunt et al. (2001). Related sequences for housekeeping genes were obtained from GenBank and edited with BioEdit program. Multiple alignments were from closely related *Rhizobium* species and sequence similarity was calculated using the EzBioCloud server. Sequences were aligned using the Clustal W program in MEGA X (Kumar et al. 2018). Phylogenetic trees were constructed using MEGA X with the neighbor-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and minimum-evolution (Rzhetsky and Nei 1992) algorithms. Bootstrap analysis (1000 resamplings) was performed to evaluate the tree topology (Felsenstein et al. 1985). *Bradyrhizobium japonicum* USDA 6^T (AP012206) was used as an outgroup.

Genome sequencing and core gene analysis

The whole genomes of strain TRM95111^T and strain TRM95001^T were sequenced by Personal Biotechnology Co., Ltd (Shanghai, China). As representatives of the isolates, the whole-genome sequences of two strains were determined using the Illumina NovaSeq platform. Quality-filtered sequence reads were denovo assembled using SPAdes (<http://cab.spbu.ru/software/spades/>). The genomic features were annotated using the Prokaryotic Genome Annotation Pipeline (PGAP) at GenBank. The predicted coding sequences were translated and used as queries to search the COG database. Genome-

based phylogeny was inferred with the whole genome sequences available for representatives of the family *Rhizobium* using Prokaryotic Genomics and Comparative Genomics Analysis Pipeline (PGCGAP analysis). The reliability of the nodes was evaluated using gene support index. The G+C content of two strains was obtained by whole genomic DNA. Average nucleotide identity (ANI) values were calculated using the orthoANI calculator (<https://www.ezbiocloud.net/tools/ani>; Yoon et al. 2017). Digital DNA-DNA hybridization (dDDH) values were calculated at the Genome-to-Genome Distance Calculator (GGDC) website using formula 2 described by Meier-Kolthoff et al. (2013).

Chemotaxonomic Analysis

The cells were collected by centrifugation, washed with distilled water, and freeze-dried. Analysis of the diaminopimelic acid isomers was performed as described by Hasegawa et al. (1983). Amino acids and sugars of whole-cell hydrolysates were analyzed following the method of Staneck and Roberts. (1974). Polar lipids were extracted and separated by two-dimensional thin-layer chromatography (TLC) and identified following the method of Minnikin et al. (1984). Quinones were extracted and analyzed by HPLC according to the method of Collins and Jones. (1981). The profile of the cellular fatty acids were determined for strains grown on YMA and NA media for 4 days at 30 °C. Cellular fatty acids were assayed using the Microbial Identification System (MIDI Sherlock version 6.0) (Sasser et al. 1990) .

Nodulation test and functional genes analysis

To confirm the symbiotic properties of strain TRM95111^T and strain TRM95001^T with *L. japonicus*, nodulation test was performed. Seeds of *L. japonicus* were surface sterilized, pregerminated and inoculated according to the standard method of (Vincent et al. 1970). After germination on solid nitrogen-free Murashige and Skoog (MS) salt medium (Gibco-BRL) which included 0.8% (w/v) agar and 1.5% (w/v) sucrose, 4 day-old seedlings were inoculated by dripping 200 µl bacterial suspension at 10⁸ c.f.u. ml⁻¹ onto the root tip. Nodulation was observed after 45 days of growth. Non-inoculated plants were used as negative controls.

Results And Discussion

Morphological, cultural and physiological characteristics

The colonies of strain TRM95111^T were white coloured, circular, convex, aerobic, with entire margins cultivated on YMA agar plates for 3 days at 25 °C. The scanning electron microscopy (SEM) image showed that the strain was rod-shaped (1.05-1.10 µm in length and 0.26-0.30 µm in width) without flagella (Supplementary Fig. 1(left)) incubated at 25 °C, 1% NaCl (w/v) and pH 7.0 for 3 days. Strain TRM95111^T was able to grow at 12-37 °C (optimum, 25 °C) , pH 6.0-9.0 (optimum, 7.0) and NaCl < 3% (w/v). Based on the results of GENIII MicroPlates, strain TRM95111^T was positive for utilization of *i*-

erythritol, D-fructose, α-D-glucose, gentiobiose, *myo*-inositol, D-maltose, D-mannitol and sucrose, but negative for glycogen, formic acid and α-hydroxy-butyric acid in consistent with the closest strains. Strain TRM95111^T was positive for utilizing inosine, α-D-lactose and acetic acid, while the reference strains are negative. The nitrate reduction, oxidase, starch hydrolysis and urease were positive. Cellulose and Tween 20, 40, 60 and 80 were hydrolysed. The differential characteristics between strain TRM95111^T and its reference strains were summarized in Table 1, and all the negative traits are shown in Table S1.

Strain TRM95001^T was a Gram-negative, aerobic, short rods, 1.12-1.20 μm long and 0.45-0.5 μm wide, without flagella (Supplementary Fig. 1(right)) incubated at 25 °C, 1% NaCl (w/v) and pH 7.0 for 4 days. Colonies on NA medium were circular, raised, pale yellow and opaque within 4 days at 25 °C. The growth of strain TRM95001^T was observed at 12-37 °C, (optimum, 25 °C), at pH 6.0-9.0 (optimum, 7.0) and 0-7.0% NaCl (w/v) (optimum, 1%). Strain TRM95001^T was positive for utilization of *D*-erythritol, D-fructose, α-D-glucose, gentiobiose, *myo*-inositol, inosine, D-maltose, D-melibiose, but negative for utilization of D-arabitol, dextrin, D-fructose, gentiobiose, glycogen, α-D-lactose, D-maltose, sucrose, D-sorbitol, D-trehalose, L-histidine and N-acetyl-D-glucosamine. Strain TRM95001^T was positive for propionic acid, α-hydroxy-butyric acid, while the reference strains are negative. Positive for catalase, lipase, oxidase, starch hydrolysis, urease, milk coagulation and peptonisation, and hydrogen peroxide. The other differential characteristics of strain TRM95001^T with its reference strains were summarized in Table 1, and all the negative traits are shown in Table S1.

Table 1

Differential phenotypic characteristics of strain TRM95111^T, strain TRM95001^T and their closely related species of the genus *Rhizobium*

Characteristics	1	2	3	4	5	6
Temperature for growth (°C)	12-37	25-30	25-30	12-37	25-37	10-40
	(25)	(25)	(25-30)	(25)	(30)	(30)
pH for growth	6-9(7)	6.0-7.5(7)	6-8(8)	6-9(7)	6-9(8)	6-10(7)
NaCl tolerance for growth (% w/v)	0-3	0-3	0-3	0-7	0-3	0-4
D-Arabitol	+	ND	+	-	+	-
Dextrin	-	ND	ND	-	ND	ND
<i>D</i> -Erythritol	+	+	+	+	ND	ND
D-Fructose	+	+	+	-	+	+
L-Fructose	+	+	-	+	+	-
α -D-Glucose	+	+	+	+	+	+
Gentiobiose	+	+	+	-	ND	ND
Glycogen	-	-	-	-	-	ND
<i>myo</i> -Inositol	+	+	+	+	+	+
Inosine	+	-	-	+	ND	ND
α -D-Lactose	+	-	-	-	+	ND
D-Maltose	+	+	+	-	+	+
D-Mannose	+	ND	+	+	+	-
D-Mannitol	+	+	+	+	+	+
D-Melibiose	+	ND	-	+	-	-
D-Raffinose	+	-	+	+	ND	ND
L-Rhamnose	+	-	+	+	+	-
D-Sorbitol	-	+	+	-	+	-
Sucrose	+	+	+	-	+	+
D-Trehalose	+	ND	+	-	ND	-
Formic acid	-	-	-	+	+	ND
Acetic acid	+	-	-	+	ND	ND
Propionic acid	+	+	-	+	-	+
L-Histidine	+	+	-	-	-	ND

L-Alanine	+	ND	-	+	+	+
L-Serine	+	+	-	+	ND	-
L-Proline	+	+	-	+	+	+
α-Hydroxy-butyric acid	-	-	-	+	-	+
N-Acetyl-D-glucosamine	+	ND	+	-	+	+
γ-Amino-butyric acid	-	-	-	+	ND	ND
DNA G+C (%)	65.1	67.7	62.8	60.7	62.5	62.8

Species/strains: 1, *Rhizobium alarensis* sp. nov. (TRM95111^T) ; 2 , *Rhizobium subbaraois* JC85^T; 3, *Rhizobium azooxidifex* Po 20/26^T; 4, *Rhizobium halophilensis* sp. nov. (TRM95001^T); 5, *Rhizobium halotolerans* AB21^T; 6, *Rhizobium flavum* YW14^T; +, positive -, negative, ND, not detected. Data of 2, 3, 5 and 6 were from Ramana et al. (2013), Behrendt et al. (2016), Diange et al. (2013), Gu et al. (2014), respectively.

Molecular phylogenetic analyses

The almost complete 16S rRNA gene sequences of strain TRM95111^T (1513 bp) and strain TRM95001^T (1449 bp) were obtained through PCR amplification and sequencing and they shared 93.1% similarity with each other. The 16S rRNA gene sequence of strain TRM95111^T was with high sequence similarity to *Rhizobium subbaraois* JC85^T (GenBank accession no. [OBQD01000060](#); 98.2%) and *Rhizobium azooxidifex* Po 20/26^T (GenBank accession no. LN832063; 97.2%). For phylogenetic analyses, the 16S rRNA gene sequences of closely related type strains were obtained from the EZBioCloud database, the phylogenetic study based on neighbor-joining, maximum-likelihood and maximum-parsimony methods also approved that strain TRM95111^T was clustered together with *Rhizobium subbaraois* JC85^T and *Rhizobium azooxidifex* Po 20/26^T (Fig. 2, Fig. S1, Fig. S2). The 16S rRNA gene sequence of strain TRM95001^T had high sequence similarity to *Rhizobium halotolerans* AB21^T (GenBank accession no. JX307098, 97.9%) and *Rhizobium flavum* YW14^T (GenBank accession no. MUX001000043, 97.5%). The phylogenetic study (based on the neighbor-joining, maximum-likelihood and maximum-parsimony methods) also approved that strain TRM95001^T formed a clade with *Rhizobium halotolerans* AB21^T and *Rhizobium flavum* YW14^T (Fig. 2, Fig. S1, Fig. S2).

Genome sequencing and phylogenetic analysis

The genome of strain TRM95111^T was 5.19 Mbp in length and consisted of 306 contigs. The depth of coverage was 389 × and the genomic DNA G + C content was 65.1 mol%, which was lower than the G + C content of *Rhizobium subbaraois* JC85^T (67.7 mol%), but higher than the G + C content of *Rhizobium*

azooxidifex Po 20/26^T (52.8 mol%). The distribution of genes into COG functional categories revealed that the percentage of genes was assigned to inorganic ion transport and metabolism (5.5%), carbohydrate transport and metabolism (5.9%), transcription (7.2%), amino acid transport and metabolism (8.8%) and function unknown (23.7%) (Table S2). The dDDH values between strain TRM95111^T and the most closely related strain *Rhizobium subbaraois* JC85^T and *Rhizobium album* NS-104^T were calculated to be 21.7% and 21.4%, whereas the ANI values between them were 77.5% and 75.6%, respectively, which were lower than DDH of 70% and ANI of 95-96% for species delineation (Goris et al. 2007; Chun et al. 2018). Strain TRM95001^T contained genome sequence of 4.36 Mbp distributed to 41 contigs, with the coverage of 323 × and DNA G + C content of 60.7 mol%, which was lower than the G + C contents of *Rhizobium halotolerans* AB21^T and *Rhizobium flavum* YW14^T (62.5 and 62.8 mol%). The distribution of genes into COG functional categories revealed that the percentage of genes was assigned to carbohydrate transport and metabolism (5.4%), transcription (5.9%), inorganic ion transport and metabolism (6.1%), amino acid transport and metabolism (7.9%) and function unknown (23.8%) (Table S2). The dDDH values between strain TRM95111^T and the most closely related strain *Rhizobium halotolerans* AB21^T and *Rhizobium flavum* YW14^T were calculated to be 21.7% and 26.7%, whereas ANI values between them were 79.6% and 79.2%, respectively. The dDDH and OrthoANI values between strain strain TRM95111^T, TRM95001^T and their closely related type strains of the genus *Rhizobium* were given in Table S3. The genomes of strain TRM95111^T and TRM95001^T contained 589 core genes by the PGCGAP analysis. Neighbour-joining phylogenetic tree (Fig. 3) based on 589 core genes showed that the position of strain strain TRM95111^T were tightly associated with *Rhizobium subbaraois* JC85^T. Strain TRM95001^T was closely clustered with *Rhizobium halotolerans* AB21^T and *Rhizobium flavum* YW14^T.

Chemotaxonomic characteristics

The whole cell sugars of strain TRM95111^T were identified as arabinose, glucose and ribose. Cells of the strain were found to contain *meso*-diaminopimelic acid as the diagnostic amino acid. The detected polar lipids were unidentified aminophospho lipids (APL1-3), unidentified phospholipids (PL1-2), phosphatidylcholine (PC), unidentified lipids (L1-5) and phospholipids of unknown structure containing glucosamine (NPG) (Fig. S3). The predominant menaquinones were identified as Q-10. The major fatty acids of strain TRM95111^T (> 5% of the total amounts) were C_{18:1} ω7c (63.6%), C_{19:0} cyclo ω8c (10.8%), C_{16:0} (8.3%), C_{18:1} ω7c 11-methyl (5%), summed feature 2 (5.0%). This profile was consistent with other species in the genus *Rhizobium*. The minor differences between strain TRM95111^T and its closely relatives (*Rhizobium subbaraois* JC85^T and *Rhizobium azooxidifex* Po 20/26^T) were shown in Table 2.

Table 2

Cellular fatty acid composition of TRM95111^T, TRM95001^T and the related species of the genus *Rhizobium*

Fatty acids	1	2	3	4	5	6
Saturated fatty acids						
C _{16:0}	8.3	6.9	3.4	8.0	13	4.9
C _{17:0}	tr	-	-	tr	1.0	-
C _{18:0}	1.2	1.2	-	1.1	2.2	2.2
Unsaturated fatty acids						
C _{15:1} ω 8c	tr	-	-	tr	-	-
C _{16:1} ω 7c	1.9	-	-	-	-	-
Methylated fatty acids						
C _{18:1} ω 7c 11-methyl	5.0	-	-	tr	tr	-
C _{19:0} 10-methyl	-	-	1.9	-	1.3	1.2
Hydroxy fatty acids						
C _{16:0} 3-OH	tr	tr	1	1.3	-	tr
C _{18:0} 3-OH	1.9	1.1	1.5	-	2.2	-
Cyclopropane acids						
C _{19:0} cyclo ω 8c	10.8	8.9	13.6	12.2	10.9	tr
Summed Feature *						
Summed Feature 2	5.0	4.3	7.1	4.5	3.2	4.3
Summed Feature 3	2.0	2.0	1.9	2.1	1.3	tr
Summed Feature 8	63.6	69.2	69.3	68.4	62.8	79.5
Species/strains: 1, <i>Rhizobium alarensis</i> sp.nov. TRM95111^T; 2, <i>Rhizobium subbaraonis</i> JC85^T; 3, <i>Rhizobium azooxidifex</i> Po 20/26^T; 4, <i>Rhizonium halophilensis</i> sp. nov. (TRM95001^T); 5, <i>Rhizobium halotolerans</i> AB21^T; 6, <i>Rhizobium flavum</i> YW14^T; +, positive -, negative. Data were expressed as percentages of total fatty acids. tr, <1.0 % of total fatty acids; -, not detected. Data of 2, 3, 5 and 6 were from Ramana et al. (2013), Behrendt et al. (2016), Diange et al. (2013), Gu et al. (2014), respectively. *Summed features consist of two or more fatty acids that could not be separated by gas chromatography. Summed feature 2 (one or more of C_{14:0} 3-OH, iso-C_{16:1} l); summed feature 3 (C_{16:1} ω7c and /or C_{16:1} ω6c); summed feature 8 (composed of C_{18:1} ω7c and/or C_{18:1} ω6c)						

The whole cell sugars of strain TRM95001^T were identified as arabinose, galactose, glucose and ribose. Cells of strain TRM95001^T were found to contain D, D-diaminopimelic acid as the diagnostic amino acid. The detected polar lipids were unidentified aminophospho lipids (APL1-3), unidentified phospholipids (PL1-2), phosphatidylcholine (PC), unidentified lipids (L2-5), hydroxy phosphatidyl ethanolamine (OH-PE) and phospholipids of phospholipids of unknown structure containing glucosamine (NPG) (Fig. S4). The predominant menaquinones were identified as Q-10. The predominant fatty acids of strain TRM95001^T comprised C_{18:1} ω 7c (68.4%), C_{19:0} cyclo ω 8c (12.2%), and C_{16:0} (8.0%). This profile was consistent with other species in the genus *Rhizobium*. The minor differences between strain TRM95001^T and its closely relatives (*Rhizobium halotolerans* AB21^T and *Rhizobium flavum* YW14^T) were shown in Table 4.

Nodulation test and nodule genes analysis

Strain TRM95111^T and TRM95001^T could form nodules in *L. japonicus* (Fig.S5). By analyzing the whole genome of TRM95111^T and TRM95001^T, both of them contained *nodG*, *nodQ*, *nodN*, *nodT*, *nodV*, *nodW*, *nolG*, *nolF*, *nolR*, *noeK*, *noeJ* and *nifS* gene. These genes were mainly used for the metabolism of nitrogen and biological nitrogen fixation.

Description of *Rhizobium alarensis* sp. nov. and *Rhizobium halophilensis* sp. nov.

Rhizobium alarensis (a.lar.en'sis N.L.neut. adj. *Alarensis*, representing where the type strain was isolated).

Strain TRM95111^T grown on YMA agar plates for 3 days at 25 °C and cells were circular, smooth, convex, white coloured and rod shaped (1.05-1.10 μ m in length and 0.26-0.30 μ m in width). Strain TRM95111^T occurred at 12-37 °C (optimum, 25 °C), pH 6.0-9.0 (optimum, pH 7.0) and 0-3% (w/v) NaCl (optimum, 1%). The following substrates were utilized on Biolog GENIII microplates: D-arabitol, D-cellobiose, D-erythritol, D-fructose, L-fructose, D-fructose-6-PO₄, D-galactose, α -D-glucose, D-glucose-6-PO₄, gentiobiose, glycerol, 3-methyl-glucose, myo-inositol, inosine, α -D-lactose, D-maltose, D-mannose, D-mannitol, D-melibiose, D-raffinose, L-rhamnose, stachyose, sucrose, D-trehalose, D-turanose, pectin, acetic acid, N-acetylneuraminic acid, D-aspartic acid, L-aspartic acid, D-gluconic Acid, D-glucuronic, L-glutamic acid, α -keto-glutaric acid, D-malic acid, L-malic acid, mucic acid, propionic acid, quinic acid, D-salicin, D-saccharic acid, L-alanine, L-arginine, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl- β -D-mannosamine, β -methyl-D-glucoside, glucuronamide, L-histidine, L-serine, L-pyroglutamic and urea. The nitrate reduction, oxidase, starch hydrolysis and urease were positive. H₂S production, lipase, catalase production, melanin production, milk coagulation and peptonisation were negative. Cellulose and Tween 20, 40, 60 and 80 were hydrolysed. The whole cell sugars consisted mainly of ribose, glucose and arabinose. Contained *meso*-diaminopimelic acid as the diagnostic cell wall amino acid. The detected polar lipids were unidentified aminophospho lipids, unidentified phospholipids, phosphatidylcholine,

unidentified lipids and phospholipids of unknown structure. The predominant menaquinones was identified as Q-10. Major fatty acids of strain TRM95111^T (> 5% of the total amounts) were Summed Feature 8 (C_{18:1} ω 7c and/or C_{18:1} ω 6c), C_{19:0} cyclo ω 8c, C_{16:0} and C_{18:1} ω 7c 11-methyl. Resistant to ampicillin, chloramphenicol, erythromycin, gentamicin, nalidixic acid, tetracycline and vancomycin, but sensitive to amikacin, kanamycin and streptomycin.

The type strain TRM95111^T (= CCTCC AB 2021116^T = JCM 34826^T) was isolated from the nodules of *L. japonicus* in Alar, Xinjiang, China. The G+C content of type strain was 65.1 mol%. The GenBank accession numbers for the genome and 16S rRNA gene sequence of strain TRM 95111^T were JAKGBU000000000 and MZ366481, respectively.

Rhizobium halophilensis (ha.lo.philensis'. Gr. n. hals halos, salt; L. part. adj. philensis, tolerating; N.L. part. adj. halophilensis, referring to the salt-tolerating ability of this microorganism).

Colonies of strain TRM95001^T grown on NA agar plates for 3 days at 30 °C were circular, convex, light yellow colored and rod shaped (1.12-1.20 μ m in length and 0.45-0.50 μ m in width). Strain TRM95001^T occurred at 12-37 °C (optimum, 25 °C), pH 6.0-9.0 (optimum, pH 6.5-7.0) and 0-7% (w/v) NaCl (optimum, 1%). The following substrates were utilized on Biolog GENIII microplates D-arabitol, i-erythritol, D-fructose, L-fructose, α -D-glucose, inosine, myo-inositol, α -D-lactose, D-mannose, D-mannitol, D-maltose, D-melibiose, D-raffinose, L-rhamnose, sorbitol, sucrose, D-trehalose, D-cellobiose, D-galactose, gentiobiose, 3-methyl-glucose, glycogen, glycerol, D-turanose, stachyose, D-malic acid, L-malic acid, propionic acid, D-salicin, β -methyl-D-glucoside, N-acetyl-D-glucosamine, N-acetyl- β -D-mannosamine, D-serine, N-acetyl-D-galactosamine, N-acetylneuraminic acid, D-glucose-6-PO₄, D-fructose-6-PO₄, D-aspartic acid, gelatin, glycyl-L-proline, L-alanine, L-arginine, L-aspartic Acid, L-glutamic acid, L-histidine, L-pyroglutamic, L-serine, pectin, D-gluconic Acid, D-glucuronic glucuronamide, mucic acid, quinic acid, α -keto-glutaric acid and urea. Positive for catalase, oxidase, urease, lipase, starch hydrolysis, milk coagulation and hydrogen peroxide, negative for H₂S production, reduction of nitrate to nitrite and melanin production. The whole cell sugars consisted mainly of arabinose, galactose, glucose and ribose. Contained D, D-diaminopimelic acid as the diagnostic cell wall amino acid. The detected polar lipids were unidentified aminophospho lipids, unidentified phospholipids, phosphatidylcholine, unidentified lipids, hydroxy phosphatidyl ethanolamine and phospholipids of unknown structure containing glucosamine. Major fatty acids of strain TRM95001^T were Summed Feature 8 (68.4%), C_{19:0} cyclo ω 8c, and C_{16:0}. Q-10 was the major the quinone. Resistant to ampicillin, chloramphenicol, erythromycin (10), gentamicin, nalidixic acid, streptomycin, tetracycline and vancomycin, but sensitive to amikacin and kanamycin.

The type strain TRM95001^T (= CCTCC AB 2021095^T = JCM34967^T) was isolated from the rhizosphere of *L. japonicus* in Alar, Xinjiang, China. The G+C content of type strain was 60.1 mol%. The GenBank accession numbers for the genome and 16S rRNA gene sequence of strain TRM95001^T were JAKJHS000000000 and MZ336521, respectively.

Declarations

Funding

Our research was supported by Bingtuan Science and Technology Program (2021BB007), Microbial Resources Utilization Innovation Team in Key Field of Xin Jiang Production and Construction Corps (2017CB014) and the Postgraduate Research and Innovation Project of Xinjiang (TDBSCX202002).

Author contributions

HLS contributed to performing the experiments, the morphological analyses, and writing the initial draft. XXL contributed to core genes analysis. ZFX contributed to the guide of the experient and phylogenetic analysis. CXW contributed to the guidance of experimental operations, critically revising the manuscript and the financial support for this work. All authors approved the manuscript.

Disclosure statement

All authors declare no conflict of interest.

References

1. Behrendt U, Kaempfer P, Glaeser SP, Jürgen A, Ulrich A (2016) Characterization of the N₂O-producing soil bacterium *Rhizobium azooxidifex* sp. nov. Int J Syst Evol Microbiol 66: 2354-2361
2. Celador LL, Menéndez E, Peix A, Igual JM, Velázquez E, Rivas R (2017) *Rhizobium zeae* sp. nov., isolated from maize (*Zea mays* L.) roots. Int J Syst Evol Microbiol 67: 2306-2311
3. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, da Costa MS, Rooney AP, Yi H, Xu XW, de Meyer S, Trujillo ME (2018) Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int J Syst Evol Microbiol 68: 461-466
4. Collins MD, Jones D (1981) Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. Microbiol Rev 45: 316-354
5. Diange EA, Lee SS (2013) *Rhizobium halotolerans* sp. nov., Isolated from chloroethylenes contaminated soil. Curr Microbiol. 66: 599-605
6. Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17: 368-376
7. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791
8. Gao JL, Wang LW, Xue J, Tong S, Sun JG (2020) *Rhizobium rhizophilum* sp. nov., an indole acetic acid-producing bacterium isolated from rape (*Brassica napus* L.) rhizosphere soil. Int J Syst Evol Microbiol 70: 5019-5025
9. Gaunt MW, Turner SL, Rigottier-Gois L (2001) Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. Int J Syst Evol Microbiol 51: 2037-2048

10. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 57: 81-91
11. Gu T, Sun LN, Zhang J, Sui X, Li SP (2014) *Rhizobium flavum* sp. nov., a triazophos-degrading bacterium isolated from soil under the long-term application of triazophos. *Int J Syst Evol Microbiol*, 64: 2017-2022
12. Hang P, Zhang L, Zhou XY, Hu Q (2019) *Rhizobium album* sp. nov., isolated from a propanil-contaminated soil. *Antonie van Leeuwenhoek* 112: 319-327
13. Hasegawa T, Takizawa M, Tanida S (1983) A rapid analysis for chemical grouping of aerobic actinomycetes. *J Gen Appl Microbiol* 29: 319-322
14. Jorin B, Palacios JM, Peix L, Imperial J (2020) *Rhizobium ruizarguesonis* sp. nov., isolated from nodules of *Pisum sativum* L. *Syst Evol Microbiol* 70: 5019-5025
15. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 35:1547-1549
16. Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A* 82: 6955-6959
17. Marmur J (1961) A Procedure of deoxyribonucleic acid from micro-organism. *J Mol Biol* 3: 208-218
18. Martínez-Hidalgo P, Ramírez-Bahena MH, Flores-Félix JD, Rivas R, Igual JM, Mateos PF, Martínez-Molina E, León-Barrios M, Peix A, Velázquez E. (2015) Revision of the taxonomic status of type strains of *Mesorhizobium loti* and reclassification of strain USDA 3471^T as the type strain of *Mesorhizobium erdmanii* sp. nov. and ATCC 33669^T as the type strain of *Mesorhizobium jarvisii* sp. nov. *Int J Syst Evol Microbiol*. 65, 1703-1708
19. Martínez-Hidalgo P, Ramírez-Bahena MH, Flores-Félix JD, Igual JM, Sanjuán J, León-Barrios M, Peix A, Velázquez E. (2016) Reclassification of strains MAFF 303099^T and R7A into *Mesorhizobium japonicum* sp. nov. *Int J Syst Evol. Microbiol*. 66(12), 4936-4941
20. Meier-Kolthoff PJ, Alexander FA, Klenk HP, Goker M (2013) Genome sequence based species delimitation with confidence intervals and improved distance functions. *BMC Bioinform* 14:60-74
21. Minnikin DE, O'Donnell AG, Goodfellow M, Alderson G, Athalye M, Schaal A, Parlett JH (1984) An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* 2: 233-241
22. Ouyabe M, Kikuno H, Tanaka N, (2019a) Nitrogenfixing endophytic bacteria of water yam (*Dioscorea alata* L.) in relation with fertilization practices. *Trop Agric Develop* 63: 122-130
23. Ouyabe M, Kikuno H, Tanaka N, Babil P, Shiwachi H (2019b) Contribution of biological nitrogen fixation in lesser yam (*Dioscorea esculenta* L.) associated with endophytic diazotrophic bacteria. *Trop Agric Develop* 63: 131-139
24. Ouyabe M, Tanaka N, Shiwa Y, Fujita N, Shiwachi H (2020) *Rhizobium dioscoreae* sp. nov., a plant growth-promoting bacterium isolated from yam (*Dioscorea species*). *Int J Syst Evol Microbiol* 70:

25. Rahi P, Giram P, Chaudhari D, diCenzo GC, Kiran S, Khullar A, Chandel M, Gawari S, Mohan A, Chavan S, Mahajan B (2020) *Rhizobium indicum* sp. nov., isolated from root nodules of pea (*Pisum sativum*) cultivated in the Indian trans-Himalayas. Syst Appl Microbiol 43: 126127-126157
26. Ramana CV, Parag B, Girija KR, Ram BR, Ramana VV, Sasikala C (2013) *Rhizobium subbaraonis* sp. nov., an endolithic bacterium isolated from beach sand. Int J Syst Evol Microbiol 63: 581-585
27. Rzhetsky A, Nei M (1992) A simple method for estimating and testing minimum-evolution trees. Mol Biol Evol 9: 945-967
28. Sasser M (1990) Identification of bacteria by gas chromatography of cellular fatty acids, MIDI technical note 101. MIDI Inc, Newark
29. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425
30. Staneck JL, Roberts GD (1974) Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl Microbiol 28: 226-231
31. Su XJ, Liu GP, Zhang L, Zhou XY, Qiao WJ, Jiang JD (2021) *Rhizobium flavescens* sp. nov., isolated from a Chlorothalonil-Contaminated Soil. Curr Microbiol 78: 2165-2172
32. Vincent, JM (1970) The cultivation, isolation and maintenance of rhizobia. In A Manual for the Practical Study of the Root-Nodule Bacteria, Blackwell Scientific 8: 1-13
33. Xu P, Li WJ, Tang SK, Zhang YQ, Chen G Z, Chen HH, Xu LH, Jiang CL (2005) *Naxibacter alkalitolerans* gen. nov., sp. nov., a novel member of the family 'Oxalobacteraceae' isolated from China. Int J Syst Evol Microbiol 55:1149-1153
34. Yoon SH, Ha SM, Kwon S, Lim J, KimY SH, Chun J (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 67: 1613-1617
35. Zhao JJ, Zhang J, Sun L, Zhang CW, Yin HQ, Zhang XX (2017) *Rhizobium oryzae* sp. nov., isolated from rice roots. Int J Syst Evol Microbiol 67: 963-968
36. Zhang L, Shi X, Si MR, Li CF, Zhu LF, Zhao L, Shen XH, Wang Y (2014) *Rhizobium smilacinae* sp. nov., an endophytic bacterium isolated from the leaf of *Smilacina japonica*. Antonie van Leeuwenhoek 106: 715-723
37. Zhang J, Peng S, Andrews M, Liu C, Zhang X (2021) *Rhizobium changzhiense* sp. nov., isolated from effective nodules of *Vicia sativa* L. in North China. Int J Syst Evol Microbiol 71: 4812
38. Zhong ZP, Liu Y, Wang F, Zhou YG, Liu HC, Liu ZP (2016) *Lacimicrobium alkaliphilum* gen. nov., sp. nov., a member of the family Alteromonadaceae isolated from a salt lake. Int J Syst Evol Microbiol 66: 422-429

Figures

Figure 1

(left) Scanning electron micrograph of strain TRM 95111^T cultivated on YMA medium for 3 days at 25 °C. Bar, 2 µm. **(right)** Scanning electron micrograph of strain TRM 95001^T cultivated on NA medium for 4 days at 25 °C. Bar, 2 µm.

Figure 2

Neighbour-joining phylogenetic tree based on 16S rRNA (1513 bp, 1449 bp) gene sequences showing the relationships of strain TRM95111^T, TRM95001^T and their closely related *Rhizobium* species. The corresponding algorithms were achieved using the MEGA X. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. Only values above 50% were indicated. *Bradyrhizobium japonicum* USDA 6^T (AP012206) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Figure 3

Phylogenetic tree generated with the neighbor-joining algorithm based on the core gene sequences showing the phylogenetic positions of strain TRM95111^T, TRM95001^T and related taxa. Bootstrap values above 50% were shown on the nodes as percentages of 1000 replicates. *Bradyrhizobium japonicum* USDA 6^T was used as an outgroup. The scale bar equaled 0.1 changes per nucleotide position.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryData418.docx](#)
- [FigureS1.tif](#)
- [FigureS2.tif](#)
- [FigureS3.tif](#)
- [FigureS4.tif](#)
- [figureS5.tif](#)