

***Rhizobium lacunae* sp. nov., Isolated From a Freshwater Pond**

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Abstract

Bacterial strain designated CSW-27^T was isolated from a freshwater pond in Taiwan. Cells were Gram-stain-negative, aerobic, oxidase-positive, catalase-negative, rod-shaped and motile by flagella. Strain CSW-27^T grew at 20-40 °C (optimum, 30-37 °C), at pH 5-9 (optimum, pH 6-7) and in the presence of 0-4% NaCl (optimum, 0%). Phylogenetic analyses based on 16S rRNA gene sequences and an up-to-date bacterial core gene set revealed that strain CSW-27^T was affiliated with species in the genus *Rhizobium*. Analysis of 16S rRNA gene sequences showed that strain CSW-27^T had the highest similarity to *Rhizobium straminoryzae* CC-LY845^T (98.5%) followed by *Rhizobium capsici* CC-SKC2^T (96.9%). The average nucleotide identity, average amino acid identity and digital DNA-DNA hybridization values between strain CSW-27^T and the closely related *Rhizobium* species were 73.4-86.5, 66.0-88.8 and 13.3-22.1%, respectively. The principal fatty acid was summed feature 8 (C18:1 ω7c and/or C18:1 ω6c). The main polar lipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylmethylethanolamine, phosphatidylcholine, one uncharacterized aminophospholipid, three uncharacterized aminolipids and two uncharacterized lipids. The predominant polyamine was spermidine. The major isoprenoid quinone was Q-10. Genomic DNA G+C content of strain CSW-27^T was 63.3%. These polyphasic taxonomic data indicated that strain CSW-27^T should be considered as representing a novel species in the genus *Rhizobium*, for which the name *Rhizobium lacunae* sp. nov. is proposed with strain CSW-27^T (=BCRC 81244^T =LMG 31684^T) as the type strain.

Introduction

The genus *Rhizobium* (type species, *Rhizobium leguminosarum*), first described by Frank (1889) and emended by Young et al. (2001), is a member of the family *Rhizobiaceae* within the class *Alphaproteobacteria* (2005). The genus *Rhizobium* comprises 91 species with validly published names so far stated on the List of Prokaryotic Names with Standing in Nomenclature (<https://lpsn.dsmz.de/genus/rhizobium>). Within the genus are free-living members as well as species that are capable of inducing the formation of plant hypertrophies, either as symbiotic nitrogen-fixing nodules or as pathogenic tumors (Young et al. 2001; Kuykendall 2005). Members of the genus *Rhizobium* are characterized as Gram-stain-negative, non-spore-forming, rod-shaped, aerobic and chemoorganotrophic. Chemotaxononomically, members of the genus are characterized by summed feature 8 (C18:1 ω7c and/or C18:1 ω6c) as predominant fatty acid, Q-10 as the major ubiquinone and the DNA G+C content between 57 to 66 mol% (Gao et al. 2020; Kuykendall 2005; Lin et al. 2014, 2015; Tighe et al. 2000; Young et al. 2001). The present study was carried out to clarify the taxonomic position of strain CSW-27^T phylogenetically close to *Rhizobium* by a polyphasic taxonomic approach.

Materials And Methods

Bacterial isolation and culture conditions

During a survey on cultivable bacterial resources from freshwater environment, a water sample (27 °C, pH 7, 0% NaCl) was collected from the freshwater pond in the Xingang National Primary School (GPS location: 23°33'22" N 120°20'42" E), Xingang Township in Chiayi County, Taiwan on 8 August 2017 (Supplementary Fig. S1). The water sample was plated on R2A agar medium (BD Difco) through serial dilution technique. After incubation at 25 °C for 3 days, strain CSW-27^T was isolated as a single cream colony and subjected to detailed taxonomy analyses. Strain CSW-27^T was sub-cultured on R2A agar and stored at -80 °C in R2A broth with 20% (v/v) glycerol or stored following lyophilization. The phylogenetically related strains, *Rhizobium straminoryzae* CC-LY845^T and *Rhizobium capsici* CC-SKC2^T were maintained in our laboratory, and both strains were used as reference strains and evaluated together under identical experimental conditions to those for strain CSW-27^T.

Morphological, physiological, and biochemical characterizations

The cell morphology was observed by a phase-contrast microscopy (DM 2000; Leica) and a transmission electron microscopy (H-7500; Hitachi) using cells grown on R2A agar at 30 °C for 2 days. The Gram reaction was tested by Gram Stain Set S kit (BD Difco) and Ryu non-staining KOH method. Motility was tested by the hanging drop method (Beveridge et al. 2007), and the Spot Test Flagella Stain (BD Difco) was used for flagellum staining. Poly-β-hydroxybutyrate granule accumulation was examined under light microscopy after staining of the cells with Sudan black (Schlegel et al. 1970) and visualized by UV illumination after directly staining growing bacteria on plates containing Nile red (Spiekermann et al. 1999). Colony morphology was observed on R2A agar under a stereoscopic microscope (SMZ 800; Nikon).

The pH range for growth was determined by measuring the optical densities (absorbance at 600 nm) of R2A broth cultures. The pH of the medium was adjusted prior to sterilization to pH 4-9 (at intervals of 0.5 pH unit) using the following biological buffers (Breznak and Costilow 2007): citrate/Na₂HPO₄ (pH 4-5.5); phosphate (pH 6-7.5); and Tris (pH 8-9). The temperature range for growth was determined on R2A agar at 4-50 °C (4, 10, 15, 20, 25, 30, 35, 37, 40, 45 and 50 °C). To investigate the tolerance to NaCl, R2A broth was prepared according to the formula of the BD Difco medium with NaCl concentration adjusted to 0-6% (w/v, at intervals of 1%). Growth under anaerobic condition was determined after incubating strain CSW-27^T on R2A agar and on R2A agar supplemented with nitrate (KNO₃ 0.1%, w/v) in anaerobic jars by using AnaeroGen anaerobic system envelopes (Oxoid) at 30 °C for 15 days. Bacterial growth was studied on R2A, nutrient, Luria-Bertani and trypticase soy agars (all from Difco) under aerobic condition at 30 °C for 15 days.

Catalase activity was determined by bubble production in 3% (v/v) hydrogen peroxide and oxidase activity was assessed colorimetrically using tetramethyl *p*-phenylenediamine. DNA hydrolysis was investigated on DNase test agar (BD Difco). Hydrolyses of casein (2% skimmed milk, w/v), starch (2.5% soluble starch, w/v), lecithin (10%, w/v), corn oil (3%, w/v) and Tweens 20, 40, 60 and 80 (1%, w/v) was

determined according to the methods described by Tindall et al. (2007). Chitin hydrolysis was assessed on chitinase-detection agar as described by Wen et al. (2002) and hydrolysis of carboxymethyl cellulose (CM-cellulose) was tested as the method described by Bowman using R2A agar as the basal medium (2000). Utilization of carbon sources was investigated in a basal medium containing (l^{-1}): 0.4 g KH_2PO_4 , 0.53 g Na_2HPO_4 , 0.3 g NH_4Cl , 0.3 g NaCl, 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.11 g CaCl_2 and 1 ml trace element solution, pH 7 as described by Chang et al. (2004). Substrates were added at a concentration of 0.1% (w/v) and the tubes incubated under aerobic conditions at 30 °C for 15 days. Additional biochemical tests were performed using API ZYM and API 20NE kits (both from bioMérieux) according to the manufacturers' recommendations.

Determination of cellular fatty acids, polar lipids, polyamines and isoprenoid quinones

The fatty acid profiles of strain CSW-27^T and the two phylogenetic related strains were determined using cells grown on R2A agar at 30 °C for 3 days. The fatty acid methyl esters were prepared and separated according to the instructions of the Microbial Identification System (MIDI), analyzed by GC (Hewlett-Packard 5890 Series II) and identified by using the Aerobe (RTSBA6) database of the MIDI System (Sherlock version 6.0) (Sasser 1990). The polar lipid profile of strain CSW-27^T was determined using cells grown on R2A agar at 30 °C for 3 days, and polar lipids were extracted and analyzed by two-dimensional TLC according to Embley and Wait (1994). Molybdophosphoric acid was used for the detection of total polar lipids, ninhydrin for amino lipids, Zinzadze reagent for phospholipids, Dragendorff reagent for choline-containing lipids and α-naphthol reagent for glycolipids. Polyamines were extracted from strain CSW-27^T and analysis was carried out as described by Busse and Auling (1988) and Busse et al. (1997). After bacterial cells were cultivated in R2-PYE medium as described by Kämpfer et al. (2007) at 30 °C for 3 days, the polyamines were extracted and analyzed by using an HPLC with UV-VIS detector. The isoprenoid quinones of strain CSW-27^T were extracted and purified according to the method of Collins and analyzed by HPLC with a Spherisorb ODS column (1994).

Determination 16S rRNA gene sequence and phylogenetic analysis

Genomic DNA was isolated and the 16S rRNA gene was amplified using the universal primer set (27F, 5'-AGAGTTGATCCTGGCTCAG-3' and 1541R, 5'-AAGGAGGTGATCCAGCC-3') (Anzai et al. 1997; Weisburg et al. 1991). PCR products were purified and then sequenced using four primers (27F, 520F, 800R and 1541R) (Anzai et al. 1997; Weisburg et al. 1991) with an ABI Prism 3730xl automated DNA analyzer (Applied Biosystems). The sequence obtained was compared with those available from EzBioCloud (Yoon et al. 2017). Multiple sequence alignments were performed with clustal W (Larkin et al. 2007) in BioEdit software (Hall 1999). Phylogenetic trees were reconstructed by the neighbour-joining (NJ) (Saitou and Nei 1987), maximum-likelihood (ML) (Felsenstein 1981) and maximum-parsimony (MP) (Kluge and Farris 1969) methods in the program MEGA 7 (Kumar et al. 2016). In each case bootstrap values were calculated based on 1000 resamplings.

Genome sequencing, analysis and comparison

A whole genome sequence was prepared by the Genomics BioSci & Tech. Co., Ltd. (Taipei, Taiwan, ROC) using the Illumina NextSeq sequencer platform and using MultiQC v1.2 for evaluating read quality (Ewels et al. 2016). The sequence was assembled using SPAdes (version 3.10.1) (Bankevich et al. 2012). The authenticity and contamination of the whole genome sequence were checked as indicated by Chun et al. (2018) and executed using EzBioCloud Contamination Estimator by 16S (ContEst16S) as indicated by Lee et al. (2017). Gene prediction and annotation by Prokka pipeline (Seemann 2014). Genome features were visualized using the CGView server (<https://cgview.ca/>). The protein encoding genes were classified into functional categories based on eggNOG (evolutionary genealogy of genes: Nonsupervised Orthologous Groups)-Mapper using precomputed cluster and phylogenies from the eggNOG database according to Huerta-Cepas et al. (2016, 2017).

Digital DNA-DNA hybridization (dDDH) analysis was performed on the DSMZ Genome-to-Genome Distance Calculator platform as described by Meier-Kolthoff et al. (2013). Average nucleotide identity (ANI) values were calculated by OrthoANI analysis (Lee et al. 2016). Average amino acid identity (AAI) calculations were performed (<http://enve-omics.ce.gatech.edu/>). A genome-based phylogenetic tree was reconstructed using an up-to-date bacterial core gene set (UBCG, concatenated alignment of 92 core genes) as described by Na et al. (2018). For genome comparison, the genome sequences of strain CSW-27^T and four closely related *Rhizobium* species were annotated by the NCBI Prokaryotic Genome Annotation Pipeline and Rapid Annotation of microbial genomes using Subsystem Technology (RAST) server as described by Overbeek et al. (2014). The examining the prevalence of total phage in the host genome by using the PHASTER server (<http://phaster.ca/>). Comparative gene contents were analyzed using an enhanced software platform EDGAR 2.0 according to the method described by Blom et al. (2016).

Results And Discussion

Phenotypic characteristics

Strain CSW-27^T was isolated from freshwater environment. Cells grew well on R2A agar, Luria-Bertani agar, trypticase soy agar and nutrient agar. Cells of strain CSW-27^T were Gram-stain-negative, aerobic, oxidase-positive and catalase-negative. Transmission electron microscopy of strain CSW-27^T showed a short rod-shaped bacterium that motile by flagella (Supplementary Fig. S2). Colonies were cream. The growth ranges of temperature, pH and NaCl concentration were at 20-40 °C, pH 5-9 and 0-4% NaCl, respectively. Detailed results from the phenotypic and biochemical analyses of strain CSW-27^T are provided in the species description and Supplementary Table S1. Differential features between strain CSW-27^T and two phylogenetically related strains, *Rhizobium straminoryzae* CC-LY845^T and *Rhizobium capsici* CC-SKC2^T were provided in Table 1.

Table 1

Differential characteristics of *Rhizobium lacunae* CSW-27^T and the two phylogenetic related *Rhizobium* species

Characteristic	1	2	3
Isolation source	freshwater pond	surface of rice straw	root tumor of a green bell pepper
Motility	+	-	+
Temperature range for growth (°C)	20-40	25-40	25-37
pH range for growth	5-9	5-9	4-9
Glucose fermentation	+	-	-
Hydrolysis of :			
Urea	+	-	-
Esculin	+	-	-
Tween 20	-	-	+
Enzymatic activities :			
Catalase	-	+	+
Cystine arylamidase	+	+	-
N-Acetyl-β-glucosaminidase	-	+	+
Assimilation :			
N-Acetyl-glucosamine	-	+	+
Adipate	+	-	-
Strains: 1, CSW-27 ^T ; 2, <i>Rhizobium straminoryzae</i> CC-LY845 ^T ; 3, <i>Rhizobium capsici</i> CC-SKC2 ^T .			
All data from this study except the G+C content of <i>Rhizobium straminoryzae</i> CC-LY845 ^T (Lin et al. 2014) and <i>Rhizobium capsici</i> CC-SKC2 ^T (Lin et al. 2015). +, Positive reaction; -, negative reaction. All strains are aerobic, rod-shaped and formed cream-colored colonies, and positive for oxidase, alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase activities, hydrolysis of DNA, CM-cellulose and Tween 40, assimilation of glucose, arabinose, mannose, mannitol, maltose, gluconate and malate, utilization of D-glucose, D-fructose, D-galactose, D-cellobiose, N-acetyl-glucosamine, D-sorbitol as carbon sources. All strains are negative for: Gram staining; nitrate reduction; indole production; hydrolyses of gelatin, casein, starch, chitin, corn oil, lecithin and Tweens 60 and 80; arginine dihydrolase, C14 lipase, trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase and α-fucosidase activities; assimilation of caprate and phenylacetate; utilization of L-phenylalanine and Tween 80 as carbon sources.			

Characteristic	1	2	3
Citrate	-	+	-
Carbon source utilization :			
L-Leucine and L-aspartic acid	+	-	+
Tween 40	+	-	-
L-Arabinose, maltose, sucrose, D-mannose, L-rhamnose, D-raffinose, D-trehalose, dextrin, glycerol, D-adonitol, D-mannitol, acetate, gluconate, L-alanine, L-histidine, L-asparagine, L-ornithine, L-glutamic acid and L-proline	-	+	+
Citrate	-	+	-
L-Serine and L-threonine	-	-	+
DNA G+C content (% or mol%)	63.3	68.3	60.5
Strains: 1, CSW-27 ^T ; 2, <i>Rhizobium straminoryzae</i> CC-LY845 ^T ; 3, <i>Rhizobium capsici</i> CC-SKC2 ^T .			
All data from this study except the G+C content of <i>Rhizobium straminoryzae</i> CC-LY845 ^T (Lin et al. 2014) and <i>Rhizobium capsici</i> CC-SKC2 ^T (Lin et al. 2015). +, Positive reaction; -, negative reaction. All strains are aerobic, rod-shaped and formed cream-colored colonies, and positive for oxidase, alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase and β -glucosidase activities, hydrolysis of DNA, CM-cellulose and Tween 40, assimilation of glucose, arabinose, mannose, mannitol, maltose, gluconate and malate, utilization of D-glucose, D-fructose, D-galactose, D-cellobiose, N-acetyl-glucosamine, D-sorbitol as carbon sources. All strains are negative for: Gram staining; nitrate reduction; indole production; hydrolyses of gelatin, casein, starch, chitin, corn oil, lecithin and Tweens 60 and 80; arginine dihydrolase, C14 lipase, trypsin, α -chymotrypsin, β -glucuronidase, α -mannosidase and α -fucosidase activities; assimilation of caprate and phenylacetate; utilization of L-phenylalanine and Tween 80 as carbon sources.			

Chemotaxonomic characterizations

The fatty acid profiles of strain CSW-27^T and two phylogenetically related strains were present in Table 2, and their profiles were similar. Their major fatty acid contained summed feature 8, which constitutes 55–70% of the total fatty acids, and they all had C_{16:0} 3-OH as the predominant hydroxyl fatty acid. The predominant cellular fatty acid (> 50% of the total fatty acids) of strain CSW-27^T was summed feature 8 (C18:1 ω 7c and/or C18:1 ω 6c; 57.3%).

Table 2

Cellular fatty acid composition of *Rhizobium lacunae* CSW-27^T and the two phylogenetic related *Rhizobium* species

Fatty acid	1	2	3
Saturated			
C _{14:0}	0.8	tr	tr
C _{16:0}	9.2	7.4	6.8
C _{18:0}	1.6	1.1	1.4
Unsaturated			
C _{17:0} cyclo	0.8	0.9	tr
C _{18:1} $\omega 7c$ 11-methyl	4.0	1.4	4.3
C _{19:0} cyclo $\omega 8c$	6.9	3.2	5.2
Hydroxy			
C _{16:0} 3-OH	1.4	2.1	2.2
Summed features*			
2	8.4	9.3	8.9
3	6.6	5.9	1.4
8	57.3	65.8	66.9
Strains: 1, CSW-27 ^T ; 2, <i>Rhizobium straminoryzae</i> CC-LY845 ^T ; 3, <i>Rhizobium capsici</i> CC-SKC2 ^T .			
All strains were grown on R2A agar at 30 °C for 3 days. Data are expressed as percentages of the total fatty acids. Only fatty acids representing more than 0.5% of the total fatty acids of at least one of the strains are shown. tr, traces (less than 0.5% of total); -, not detected.			
For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. <i>cis</i> isomer is indicated by the suffix <i>c</i> . *Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total. Summed feature 2 comprises C _{14:0} 3-OH and/or iso-C _{16:1} I. Summed feature 3 comprises C _{16:1} $\omega 7c$ and/or C _{16:1} $\omega 6c$. Summed feature 8 comprises C _{18:1} $\omega 7c$ and/or C _{18:1} $\omega 6c$.			

The polar lipids of strain CSW-27^T consisted of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylmonomethylethanolamine (PME), phosphatidylmethylethanolamine (PDE), phosphatidylcholine (PC), one uncharacterized aminophospholipid (APL1), three uncharacterized aminolipids (AL1-AL3) and two uncharacterized lipids

(L1 and L2) (Supplementary Fig. S3). Strain CSW-27^T contained spermidine (SPD, 99.5%) as the major polyamine and small amounts of putrescine (PUT, 0.5%) (Supplementary Fig. S4). The sole respiratory quinone detected in strain CSW-27^T was ubiquinone-10 (Q-10) (Supplementary Fig. S5), as in all known members of the genus *Rhizobium*.

16S rRNA gene similarities and phylogenetic analysis

The almost complete 16S rRNA gene sequence of strain CSW-27^T was obtained (1532 bp, GenBank accession number MH729077). Based on the 16S rRNA gene sequence similarities, strain CSW-27^T was closely related to the species of the genus *Rhizobium* and showed high sequence similarity with *Rhizobium straminoryzae* CC-LY845^T (98.5%) followed by *Rhizobium capsici* CC-SKC2^T (96.9%), *Rhizobium helianthi* Xi19^T (96.8%), *Rhizobium rhizoryzae* J3-AN59^T (96.4%), *Rhizobium endolithicum* JC140^T (95.7%) and *Rhizobium petrolearium* SL-1^T (95.2%). Sequence similarities < 95.1% were observed with the type strains of all other *Rhizobium* species. The phylogenetic trees manifested that strain CSW-27^T formed a separate phylogenetic branch clustered with *Rhizobium straminoryzae* CC-LY845^T, *Rhizobium capsici* CC-SKC2^T and *Rhizobium oryzicola* ZYY136^T within the genus *Rhizobium* in the neighbour-joining tree in Fig. 1. Similar tree topologies were obtained in the maximum-likelihood and maximum-parsimony trees.

Genomic features

The genome of strain CSW-27^T comprised a total size of 5.66 Mb (GenBank accession number NZ_JAHHZ000000000000) with 101 contigs, and G+C content was 63.3% (Supplementary Fig. S6). It had an average coverage of 158x and a N50 size of 323865 bp, and was confirmed to be free of contamination. The sequence of the 16S rRNA gene from the genome and that of PCR determined sequence is very close but not identical with several nucleotides different, and the original sequence determined by PCR has been corrected. The genome harbored 5244 protein encoding genes, 6 rRNA genes and 49 tRNA genes. The protein encoding genes were classified into 21 functional categories (Supplementary Table S2), and most of coding sequences were classified as functional unknown (S, 22.2% of all assigned eggNOG), amino acid transport and metabolism (E, 12.1%), transcription (K, 9.7%) and inorganic ion transport and metabolism (P, 7.7%).

The dDDH values between strain CSW-27^T and the strains of other related *Rhizobium* species were 13.3–22.1% (Supplementary Table S3), which are below the cut-off (70%) for species delineation (Goris et al. 2007). ANI values obtained from the comparison with available genomes were 73.4–86.5%. These values are lower than the threshold (95–96%) for species definition as described by Richter and Rosselló-Móra (2009). AAI values obtained were 66.0–88.8%, which is below the threshold of 90% for species boundary and above the threshold of 60% for genus boundary proposed by Rodriguez-R and Konstantinidis (2014). Therefore, strain CSW-27^T was considered a new bacterial species.

UBCG was utilized for construction of a genome-based phylogenetic tree. The phylogenetic tree based on the coding sequences of 92 protein clusters showed that strain CSW-27^T formed a distinct phylogenetic

lineage cluster with *Rhizobium straminoryzae* SM12, *Rhizobium rhizoryzae* J3-AN59^T and *Rhizobium ipomoeae* shin9-1^T in the genus *Rhizobium* (Fig. 2), which supported that strain CSW-27^T should be assigned to a novel species of the genus *Rhizobium*.

Genome comparative analysis

For further comparative analyses, the genome sequences of strain CSW-27^T and four genome sequences from the genus *Rhizobium* were used, including *Rhizobium straminoryzae* SM12, *Rhizobium rhizoryzae* J3-AN59^T, *Rhizobium ipomoeae* shin9-1^T and *Rhizobium petrolearium* SL-1^T. Genome characteristics of these strains is provided in Supplementary Table S4. Results from RAST showed that all five strains had very important characteristics for the gene compositions, and some genes are shared and some genes are different (Table 3).

Table 3

Comparison of the presence and absence of selected genes among *Rhizobium lacunae* CSW-27^T and the four strains of the genus *Rhizobium*

Genes putatively encoding	1	2	3	4	5
Cofactors and vitamins					
Ubiquinone biosynthesis	+	-	-	+	+
Chlorophyll biosynthesis	-	-	-	+	-
Coenzyme B12 biosynthesis	-	-	-	+	+
Cell wall and capsule					
dTDP-rhamnose synthesis, rhamnose containing glycans	+	+	+	-	+
Capsular polysaccharide biosynthesis and assembly	-	-	-	-	+
Teichoic and lipoteichoic acids biosynthesis	+	-	-	-	+
Virulence, disease and defense					
Adaptation to d-cysteine	-	+	+	+	+
Mercuric resistance	-	-	+	+	+
Zinc resistance	-	-	-	+	-
Fosfomycin resistance	-	-	+	-	-
Bile hydrolysis	-	-	-	-	+
<i>Mycobacterium</i> virulence operon possibly involved in quinolinate biosynthesis	-	-	+	+	+
Photosynthesis					
Bacterial light-harvesting proteins	-	-	-	+	-
Photosystem II-type photosynthetic reaction center	-	-	-	+	-
Miscellaneous					
Niacin-choline transport and metabolism	-	-	-	-	+
Dioxygenases (EC 1.14.12.-)	+	+	+	-	-
Muconate lactonizing enzyme family	+	-	+	+	+
Aromatic dioxygenase mess	+	-	+	-	+
<i>Bacillus subtilis</i> scratch	-	-	+	+	+

Strains: 1, CSW-27^T; 2, *Rhizobium straminoryzae* SM12; 3, *Rhizobium rhizoryzae* J3-AN59^T; 4, *Rhizobium ipomoeae* shin9-1^T; 5, *Rhizobium petrolearium* SL-1^T. +, Present; -, absent.

Genes putatively encoding	1	2	3	4	5
Dioxygenases (EC 1.13.11.-)	+	-	+	-	+
Phage, prophage, transposable element and plasmid					
Transposable elements : CBSS-203122.12.peg.188	-	-	+	-	+
Phage replication	+	+	+	-	+
Phage tail fiber proteins	+	-	-	-	+
Phage lysis modules	+	-	+	-	-
Membrane transport					
Protein secretion system, type I (for aggregation)	-	-	-	+	-
Protein and nucleoprotein secretion system, type IV (conjugative transfer)	+	+	-	+	+
Protein secretion system, type VI	+	+	-	-	-
TRAP transporter unknown substrate 5 and substrate 9	+	-	-	-	+
TRAP transporter unknown substrate 4	+	-	-	-	-
Regulation and cell signaling					
DNA-binding regulatory proteins, strays	+	-	+	-	+
HPr catabolite repression system	-	-	+	-	-
MazEF toxin-antitoxing (programmed cell death) system	+	-	+	-	-
Murein hydrolase regulation and cell death	+	-	-	-	-
Dormancy and sporulation					
Persister cells	+	-	-	-	-
Respiration					
Terminal cytochrome O ubiquinol oxidase	+	+	+	-	+
Carbon monoxide oxidation	-	-	-	+	-
Formate dehydrogenase	-	-	-	-	+
Stress response					
Osmoregulation	+	-	-	-	+
Cluster containing glutathione synthetase	-	-	-	+	-

Strains: 1, CSW-27^T; 2, *Rhizobium straminoryzae* SM12; 3, *Rhizobium rhizoryzae* J3-AN59^T; 4, *Rhizobium ipomoeae* shin9-1^T; 5, *Rhizobium petrolearium* SL-1^T. +, Present; -, absent.

Genes putatively encoding	1	2	3	4	5
Heat shock dnak gene cluster extended	+	-	+	+	+
Flavohaemoglobin	-	-	+	-	-
SigmaB stress response regulation	-	-	-	-	+
Dimethylarginine metabolism	+	+	-	-	-
Iron acquisition and metabolism					
Iron siderophore sensor and receptor system	+	-	+	+	+
Siderophore enterobactin	+	-	-	-	-
Siderophore anthraachelin	-	+	-	+	-
Siderophore assembly kit	-	+	-	+	-
ABC transporter [iron.B12.siderophore.hemin]	-	+	+	+	+
Nitrogen metabolism					
Allantoin utilization	-	-	-	-	+
Denitrification	+	-	+	+	+
Phosphorus metabolism					
Phosphoenolpyruvate phosphomutase	-	-	+	-	-
Sulfur metabolism					
L-cystine uptake and metabolism	-	-	-	+	+
Utilization of glutathione as a sulfur source	-	-	-	-	+
Sulfur oxidation	-	-	-	-	+
RNA metabolism					
tRNA modification position 34	-	-	-	+	-
Group II intron-associated genes	+	+	-	-	+
Protein metabolism					
Protein chaperones	+	-	+	+	+
Selenocysteine metabolism	-	-	-	-	+
N-linked glycosylation in <i>Bacteria</i>	+	+	+	+	-

Strains: 1, CSW-27^T; 2, *Rhizobium straminoryzae* SM12; 3, *Rhizobium rhizoryzae* J3-AN59^T; 4, *Rhizobium ipomoeae* shin9-1^T; 5, *Rhizobium petrolearium* SL-1^T. +, Present; -, absent.

Genes putatively encoding	1	2	3	4	5
DNA metabolism					
Nonhomologous end-joining in <i>Bacteria</i>	-	-	+	+	+
CRISPRs	-	+	-	-	-
Restriction-modification system	+	+	+	-	-
Metabolism of amino acids and derivatives					
Glutamate and aspartate uptake in <i>Bacteria</i>	+	+	-	-	-
Histidine degradation	+	+	+	-	+
Cyanophycin metabolism	+	+	+	+	-
S-methylmethionine	+	+	+	-	-
Isoleucine, leucine, valine degradation and HMG-CoA metabolism	-	-	+	+	+
HMG-CoA synthesis	-	-	+	-	+
Aromatic amino acid interconversions with aryl acids	-	+	-	-	+
Indole-pyruvate oxidoreductase complex	-	-	-	-	+
Metabolism of aromatic compounds					
Central meta-cleavage pathway of aromatic compound degradation	+	-	-	-	-
Homogentisate pathway of aromatic compound degradation	-	-	-	-	+
Biphenyl degradation	-	+	-	-	-
Carbohydrate metabolism					
One-carbon metabolism: methanogenesis	-	-	-	-	+
CO ₂ fixation: CO ₂ uptake, carboxysome, photorespiration (oxidative C2 cycle), Calvin-Benson cycle	-	-	-	-	+
Di- and oligosaccharide: lactose and galactose uptake and utilization	+	+	-	+	+
Di- and oligosaccharide: maltose and maltodextrin utilization	+	+	+	-	+
Sugar alcohol: erythritol utilization	-	-	-	+	-
Fermentation: acetoin and butanediol metabolism	+	+	+	+	-
Monosaccharide: D-galacturonate and D-glucuronate utilization	+	+	+	+	-

Strains: 1, CSW-27^T; 2, *Rhizobium straminoryzae* SM12; 3, *Rhizobium rhizoryzae* J3-AN59^T; 4, *Rhizobium ipomoeae* shin9-1^T; 5, *Rhizobium petrolearum* SL-1^T. +, Present; -, absent.

Genes putatively encoding	1	2	3	4	5
Monosaccharide: L-rhamnose utilization	+	+	-	+	-
Monosaccharide: L-fucose utilization temp	+	+	+	-	+
Monosaccharide: D-galactarate, D-glucarate and D-glycerate catabolism	+	+	+	+	-
Strains: 1, CSW-27 ^T ; 2, <i>Rhizobium straminoryzae</i> SM12; 3, <i>Rhizobium rhizoryzae</i> J3-AN59 ^T ; 4, <i>Rhizobium ipomoeae</i> shin9-1 ^T ; 5, <i>Rhizobium petrolearium</i> SL-1 ^T . +, Present; -, absent.					

The primary differences are that only strain CSW-27^T had genes related to membrane transport e.g. TRAP transporter unknown substrate 4, related to programmed cell death and toxin-antitoxin systems (regulation and cell signaling) e.g. murein hydrolase regulation and cell death, related to dormancy and sporulation e.g. persister cells, related to iron acquisition and metabolism e.g. siderophore enterobactin, related to metabolism of central aromatic intermediates e.g. central meta-cleavage pathway of aromatic compound degradation, but the other four strains had not these genes (Supplementary Table S5). Other features are that only *Rhizobium straminoryzae* SM12, *Rhizobium rhizoryzae* J3-AN59^T, *Rhizobium ipomoeae* shin9-1^T and *Rhizobium petrolearium* SL-1^T existed genes putatively encoding for adaptation to d-cysteine e.g. cystine ABC transporter, permease protein related to virulence, disease and defense; genes putatively encoding for ABC transporter (iron.B12.siderophore.hemin) e.g. ATP-binding component, periplasmic substrate-binding component, permease component related to iron acquisition and metabolism, but the novel strain had not the related genes.

Regarding the protein secretion systems of membrane transport, all five strains had Type II but none Types III, V and VII. And, the five strains showed different patterns for Types I, IV and VI. Only *Rhizobium ipomoeae* shin9-1^T possessed genes encoding proteins associated with Type I, only strain CSW-27^T and *Rhizobium straminoryzae* SM12 existed genes encoding proteins associated with Type VI, and except *Rhizobium rhizoryzae* J3-AN59^T the other four strains had genes encoding proteins associated with Type IV (Supplementary Table S6). Concerning the nitrogen metabolism, all five strains had most related enzymes. Furthermore, only *Rhizobium petrolearium* SL-1^T existed genes encoding methanol dehydrogenase, formate dehydrogenase and carbamate kinase, and only *Rhizobium rhizoryzae* J3-AN59^T had formamidase related gene. And, only both strains, *Rhizobium petrolearium* SL-1^T and *Rhizobium rhizoryzae* J3-AN59^T, possessed gene encoding aspartate ammonia-lyase (Supplementary Table S7). When examining the prevalence of total phage in genomes of strain CWS-27^T, *Rhizobium straminoryzae* SM12, *Rhizobium rhizoryzae* J3-AN59^T, *Rhizobium ipomoeae* shin9-1^T and *Rhizobium petrolearium* SL-1^T, it is found that it is about 0.5 to 2.6 percent in the five *Rhizobium* strains (Supplementary Table S8). However, only strain CSW-27^T existed the three phage types including intact, questionable and incomplete which sorted by the completeness. And, only strain CSW-27^T, *Rhizobium straminoryzae* SM12 and *Rhizobium rhizoryzae* J3-AN59^T had intact prophage e.g. *Siphoviridae*, *Rhodobacter* phage RcapNL in

strain CSW-27^T; e.g. *Siphoviridae*, *Rhodobacter* phage RC1 and *Myoviridae*, *Aurantimonas* phage AmM-1 in *Rhizobium straminoryzae* SM12; e.g. *Myoviridae*, *Aurantimonas* phage AmM-1 in *Rhizobium rhizoryzae* J3-AN59^T.

Homology analysis of gene contents were performed between strain CSW-27^T and *Rhizobium straminoryzae* SM12, *Rhizobium rhizoryzae* J3-AN59^T, *Rhizobium ipomoeae* shin9-1^T and *Rhizobium petrolearium* SL-1^T. As a result, a total of 2265 genes common are shared among the five strains (Supplementary Fig. S7), and there are 724 genes present as specific genes in strain CSW-27^T. In summary, by comparing genomic information, we can gain insights into how these rhizobia strains metabolize various nutrients, their resistance to pathogens or harmful substances, and their ability to adapt to environmental changes, which provides a basic theory. These capabilities may give various rhizobia a competitive advantage to adapt to diverse environments in a complex microbial ecosystem.

Taxonomic conclusion

Phenotypic examination revealed many common traits between the novel strain and *Rhizobium straminoryzae* CC-LY845^T and *Rhizobium capsici* CC-SKC2^T. However, strain CSW-27^T could be clearly differentiated from these two phylogenetic related strains by its ability to grow at lower temperature (< 25 °C), by its ability to ferment glucose, by its ability to hydrolyze urea and esculin, by the absence of catalase and *N*-acetyl-β-glucosaminidase activities, by its inability to assimilate *N*-acetyl-glucosamine, by its ability to assimilate adipate, by the ability to utilize Tween 40 as carbon sources and by the inability to utilize L-arabinose, maltose, sucrose, D-mannose, L-rhamnose, D-raffinose, D-trehalose, dextrin, glycerol, D-adonitol, D-mannitol, acetate, gluconate, L-alanine, L-histidine, L-asparagine, L-ornithine, L-glutamic acid and L-proline as carbon sources (Table 1).

From the comparative genomic analyses for strain CSW-27^T, *Rhizobium straminoryzae* SM12, *Rhizobium rhizoryzae* J3-AN59^T, *Rhizobium ipomoeae* shin9-1^T and *Rhizobium petrolearium* SL-1^T, it is also found that although they have common characteristics, strain CSW-27^T could be obviously discriminated from these strains by its unique abilities. Based on the data obtained from 16S rRNA gene sequence and whole genome sequence comparison, strain CSW-27^T occupies a distinct position within the genus *Rhizobium* that is supported by a unique combination of chemotaxonomic and biochemical characteristics. Strain CSW-27^T represents a novel species of the genus *Rhizobium*, for which the name *Rhizobium lacunae* sp. nov. is proposed.

Description of *Rhizobium lacunae* sp. nov.

Rhizobium lacunae (la.cu'nae. L. gen. n. /acunae of a pond, from where the type strain was isolated).

Cells are Gram-stain-negative, aerobic, motile by flagella, rod-shaped (0.8-1.1 μm wide and 1.7-2.2 μm long) and chemo-heterotrophic. After 48 h of incubation at 30 °C on R2A agar, colonies are cream colored,

convex, round, smooth with entire edges and approximately 0.9-1.5 mm in diameter. Cells grow at 20-40 °C (optimum, 30-37 °C), at pH 5-9 (optimum, pH 6-7) and with 0-4% NaCl (optimum, 0%). Negative for poly-β-hydroxybutyrate accumulation. Positive for oxidase activity and negative for catalase activity. Capable of hydrolyzing DNA, CM-cellulose and Tween 40. Incapable of hydrolyzing casein, starch, chitin, lecithin, corn oil and Tweens 20, 60 and 80. Positive for glucose fermentation, urea and esculin hydrolysis, β-galactosidase (PNPG) activity, and assimilation of glucose, arabinose, mannose, mannitol, maltose, gluconate, adipate and malate. Cells present alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase (ONPG), α-glucosidase and β-glucosidase activities. Positive growth under aerobic condition are observed for D-glucose, D-fructose, D-galactose, D-cellobiose, N-acetyl-glucosamine, D-sorbitol, Tween 40, Tween 60, adipate, L-aspartic acid and L-leucine. The major fatty acid is summed feature 8 (C₁₈:1 ω 7c and/or C₁₈:1 ω 6c). Polar lipids present are phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine, phosphatidylcholine, one uncharacterized aminophospholipid, three uncharacterized aminolipids and two uncharacterized lipids. The predominant polyamine is spermidine. The sole and major isoprenoid quinone is Q-10.

The genome size of strain CSW-27^T is 5.66 Mb with 63.3% G+C content. The type strain CSW-27^T (=BCRC 81244^T =LMG 31684^T) was isolated from a freshwater pond in the Xingang National Primary School, Xingang Township in Chiayi County, Taiwan. The GenBank accession numbers for the 16S rRNA gene sequence and the whole genome of *Sphingomonas lacunae* CSW-27^T are MH729077 and NZ_JAHHZ000000000000.

Abbreviations

eggNOG, evolutionary genealogy of genes: Nonsupervised Orthologous Groups; dDDH, digital DNA-DNA hybridization; ANI, average nucleotide identity; AAI, average amino acid identity; UBCG, up-to-date bacterial core gene set; RAST, Rapid Annotation of microbial genomes using Subsystem Technology; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PME, phosphatidylmonomethylethanolamine; PDE, phosphatidyldimethylethanolamine; PC, phosphatidylcholine; APL, uncharacterized aminophospholipid; AL, uncharacterized aminolipid; L, uncharacterized lipid; SPD, spermidine; PUT, putrescine; Q-10, ubiquinone-10

Declarations

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Compliance with ethical standards

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figures

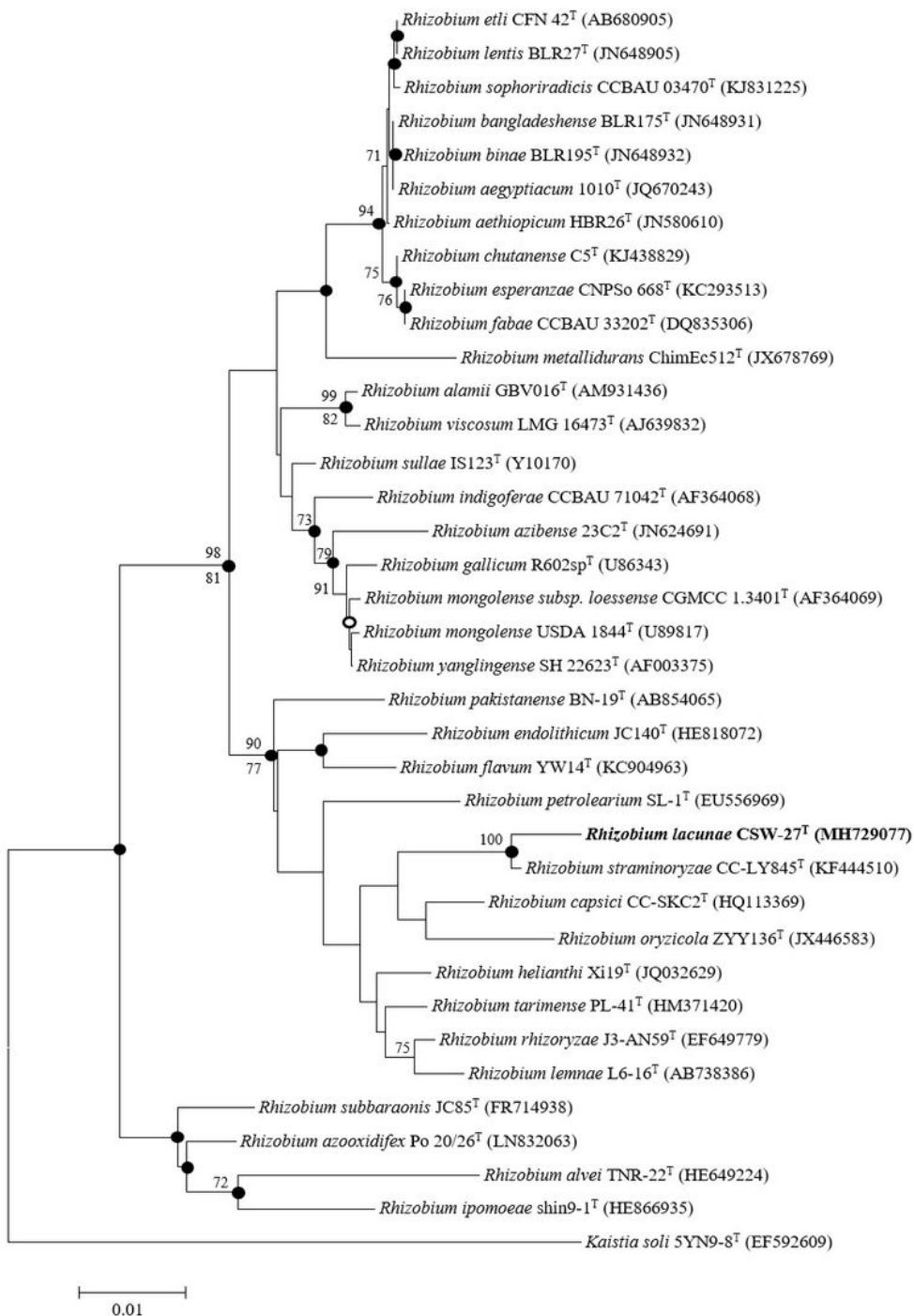


Figure 1

Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of *Rhizobium lacunae* CSW-27^T and type strains of species of the genus *Rhizobium*. Numbers at nodes are bootstrap percentages $\geq 70\%$ based on the neighbour-joining (above nodes) and maximum-parsimony (below nodes) tree-making algorithms. Filled circles indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. Open circles indicate

that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. *Kaistia soli* 5YN9-8^T was used as an out-group. Bar, 0.01 substitutions per nucleotide position.

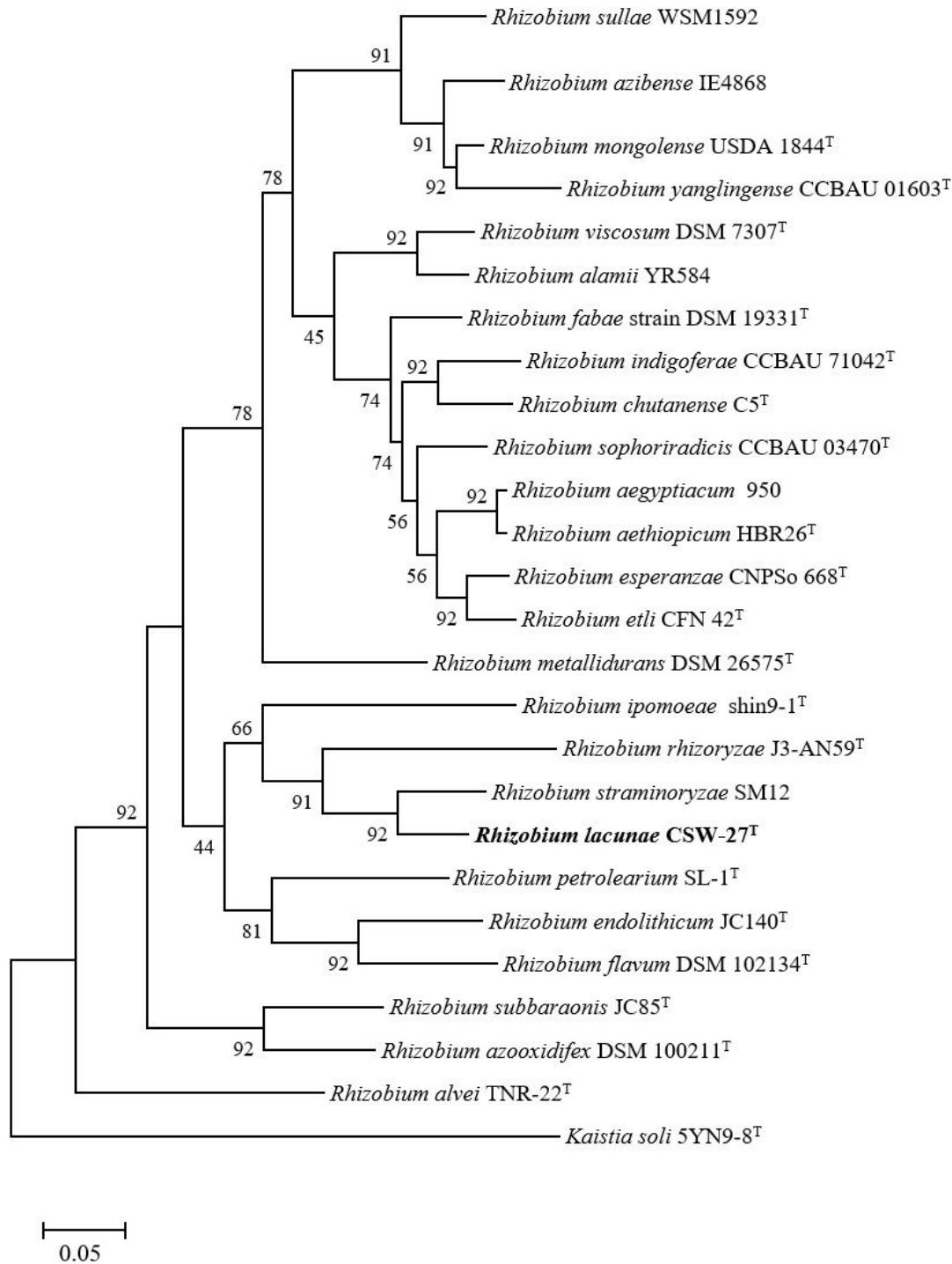


Figure 2

Phylogenetic tree inferred using UBCGs (concatenated alignment of 92 core genes) showing the position of *Rhizobium lacunae* CSW-27^T and type strains of species of the genus *Rhizobium*. The number of single gene trees supporting a branch in a UBCG tree is calculated and designated the Gene Support Index (GSI). The GSIs are given at branching points. *Kaistia soli* 5YN9-8^T was used as an out-group. Bar, 0.05 substitutions per position.

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