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ORIGINAL PAPER

Halorussus ruber sp. nov., isolated from an inland salt lake of China

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Abstract Halophilic archaeal strain YC25^T was isolated from Yuncheng salt lake in Shanxi, China. Cells of strain YC25^T were observed to be pleomorphic rods, stained Gram-negative, and formed red-pigmented colonies on solid media. Strain YC25^T was found to be able to grow at 25–50 °C (optimum 37 °C), at 1.4–4.8 M NaCl (optimum 1.7 M), at 0–1.0 M $MgCl_2$ (optimum 0.01 M), and at pH 5.5–9.0 (optimum pH 6.5). The cells lysed in distilled water, and the minimal NaCl concentration to prevent cell lysis was found to be 8 % (w/v). The major polar lipids of the strain were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), sulfated galactosyl mannosyl glucosyl diether (S-TGD-1), sulfated mannosyl glucosyl diether (S-DGD-1), galactosyl mannosyl glucosyl diether (TGD-1), mannosyl glucosyl diether (DGD-1), and an unknown diglycosyl diether (DGD-2) chromatographically identical to those of *Halorussus rarus* CGMCC 1.10122^T. The 16S rRNA gene and *rpoB′* gene of strain YC25^T were phylogenetically related to the corresponding genes of *Halorussus rarus* CGMCC 1.10122^T (94.3–95.4 and 91.5 % nucleotide identity, respectively). The DNA G+C content of strain YC25^T was determined to be 63.3 mol%. The phenotypic, chemotaxonomic, and phylogenetic properties suggested that strain YC25^T (=CGMCC 1.12122^T = JCM 18363^T) represents a new species of *Halorussus*, for which the name *Halorussus ruber* sp. nov. is proposed.

Keywords *Halorussus ruber* sp. nov. · Halophilic archaeon · Salt lake

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Phase-contrast micrograph of strain YC25^T, thin-layer chromatograms of the polar lipids extracted from strain YC25^T and some related haloarchaea, maximum-parsimony and neighbor-joining phylogenetic tree reconstructions based on 16S rRNA gene and *rpoB′* gene sequences showing the relationships between strain YC25^T and related members within the family *Halobacteriaceae* are available as supplementary materials.

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Introduction

Inland salt lakes, natural athalassohaline environments, harbor diverse halophilic archaea, the members of the family *Halobacteriaceae* (Pagaling et al. 2009; Youssef et al. 2012). Some of these hypersaline environments have been long-term targets for the study of halophilic archaeal resources, and a number of novel strains representing new species have been isolated from these inland salt lakes (Corral et al. 2013; Qiu et al. 2013a, b; Zhang et al. 2013; Amoozegar et al. 2014). The family *Halobacteriaceae* encompasses 47 genera containing over 165 species as of February 2014 (Oren 2012; 2014; Parte 2014). During our

survey on halophilic archaeal diversity of the Yuncheng salt lake in Shanxi, China, we obtained a halophilic archaeal isolate, YC25^T, that was most closely related to the members of *Halorussus*, as judged from 16S rRNA gene sequences.

The genus *Halorussus*, belonging to the family *Halobacteriaceae*, was proposed to accommodate the species *Halorussus rarus* described based on two strains, TBN4^T and TBN5, which were isolated from an artificial marine solar saltern in Eastern China (Cui et al. 2010). The most distinctive characteristic of *Halorussus rarus* was its polar lipid profile. *Halorussus rarus* contained phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), sulfated galactosyl mannosyl glucosyl diether (S-TGD-1), sulfated mannosyl glucosyl diether (S-DGD-1), galactosyl mannosyl glucosyl diether (TGD-1), mannosyl glucosyl diether (DGD-1), and an unknown diglycosyl diether (DGD-2; Cui et al. 2010). In this study, we characterize strain YC25^T as a new species of the genus *Halorussus*, for which the name *Halorussus ruber* sp. nov. is proposed.

Materials and methods

Isolation and cultivation of halophilic archaeal strain

Strain YC25^T was isolated from the brine sample taken from Yuncheng salt lake at Yuncheng, Shanxi Province, China (35°00′14″N, 111°00′19″E; elevation 323 m above sea level) and stored at 4 °C during transport to the laboratory in 2010. The pH of the brine was 7.9 and the salinity 235.6 g/L. The neutral haloarchaeal medium (NHM) was used for the isolation procedure and contained the following ingredients (g/L): yeast extract (Oxoid) 0.05, fish peptone (Sinopharm Chemical Reagent Co., Ltd.) 0.25, sodium pyruvate 1.0, KCl 5.4, K_2HPO_4 0.3, $CaCl_2$ 0.29, NH_4Cl 0.27, $MgSO_4{\cdot}7H_2O$ 26.8, $MgCl_2{\cdot}6H_2O$ 23.0, and NaCl 184.0 (pH adjusted to 7.0–7.2 with 1 M NaOH solution). The brine was serially diluted in liquid NHM medium and spread onto NHM agar plates. The inoculated plates were incubated for 1 month at 37 °C. After this initial cultivation, colonies were successively restreaked on NHM agar plates at least three times to obtain pure colonies. The strain was routinely grown aerobically at 37 °C in NHM medium.

Phenotypic determination

Phenotypic tests were performed according to the proposed minimal standards for the description of novel taxa in the order *Halobacteriales* (Oren et al. 1997). Determination of morphology and growth characteristics, nutrition, miscellaneous biochemical tests, and sensitivity to antimicrobial agents was performed as described and cited previously (Cui et al. 2010). The halophilic archaeal strains *Halorussus rarus* CGMCC 1.10122^T, *Haladaptatus paucihalophilus* JCM 13897^T, and *Haloarchaeobius litoreus* CGMCC 1.10390^T were selected as reference strains. These reference strains were routinely grown aerobically at 37 °C in NHM medium.

Chemotaxonomic characterization

Polar lipids were extracted using a chloroform/methanol system and analyzed using one- and two-dimensional TLC, as described previously (Cui et al. 2010). Merck silica gel 60 F_{254} aluminum-backed thin-layer plates were used for TLC analyses. In two-dimensional TLC, the first solvent was chloroform–methanol–water (65:25:4, by vol.) and the second solvent was chloroform–methanol–acetic acid–water (80:12:15:4, by vol.). The latter solvent mixture was also used in one-dimensional TLC. Two specific detection spray reagents, phosphate stain reagent for phospholipids and α-naphthol stain for glycolipids, were used. The general detection reagent, sulfuric acid–ethanol (1:2, by vol.), was also used to detect total polar lipids.

Phylogenetic and genomic analysis

Genomic DNA from halophilic archaeal strains was prepared as described previously (Cui et al. 2011). The 16S rRNA genes were amplified, cloned, and sequenced according to the previous protocol (Cui et al. 2009). PCR-mediated amplification and sequencing of the *rpoB′* genes were performed as described previously (Minegishi et al. 2010). Multiple sequence alignments were performed using the ClustalW program integrated in the MEGA 5 software (http://www.megasoftware.net/). Phylogenetic trees were reconstructed using maximum-likelihood (ML), maximum-parsimony (MP), and neighbor-joining (NJ) algorithms in the MEGA 5 software (Tamura et al. 2011). Gene sequence similarity among halophilic archaea was calculated using the Pairwise-Distance computing function of MEGA 5. The DNA G+C content was determined from the midpoint value (T_m) of the thermal denaturation method (Marmur and Doty 1962) at 260 nm with a Beckman-Coulter DU800™ spectrophotometer equipped with a high-performance temperature controller.

Results and discussion

Cells of strain YC25^T were observed to be motile and pleomorphic rods when grown in NHM liquid medium (Supplementary Fig. S1). They stained Gram-negative, and the colonies were observed to be red-pigmented. Strain

Table 1 Characteristics that distinguish strain YC25^T from *Halorussus rarus* CGMCC 1.10122^T

Characteristic	1	2
Optimum NaCl (M)	1.7	2.1
Optimum Mg^{2+}(M)	0.01	0.005
Optimum pH	6.5	7.0
Anaerobic growth with nitrate	+	−
Anaerobic growth with arginine	+	−
Anaerobic growth with DMSO	+	−
Utilization of		
Maltose	−	+
Glycerol	−	+
Pyruvate	−	+
L-alanine	−	+
L-aspartate	−	+
Gelatin hydrolysis	−	+
Casein hydrolysis	−	+
H_2S formation	−	+
G+C content (mol%)	63.3	66.1

Taxa: 1, strain YC25^T ; 2, *Halorussus rarus* CGMCC 1.10122^T. Symbols: + positive; − negative

YC25^T was found to be able to grow at 25–50 °C (optimum 37 °C), at 1.4–4.8 M NaCl (optimum 1.7 M), at 0–1.0 M $MgCl_2$ (optimum 0.01 M), and at pH 5.5–9.0 (optimum pH 6.5). The cells lysed in distilled water, and the minimal NaCl concentration to prevent cell lysis was found to be 8 % (w/v). The strain was able to grow under anaerobic conditions using nitrate, L-arginine, and DMSO. It was found to be positive for indole formation while negative for H_2S formation. Strain YC25^T hydrolyzed Tween 80 but did not hydrolyze starch, gelatin, or casein. Strain YC25^T was sensitive to the following antimicrobial compounds (μg per disk, unless otherwise indicated): Anisomycin (5), aphidicolin (5), novobiocin (30), bacitracin (0.04 IU per disk), rifampin (5), ciprofloxacin (5), mycostatin (100), and nitrofurantoin (300). It was resistant to the following antimicrobial compounds: trimethoprim (5), erythromycin (15), penicillin G (10 IU per disk), ampicillin (10), chloramphenicol (30), neomycin (30), norfloxacin (10), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10), and nalidixic acid (30). The main phenotypic characteristics differentiating strain YC25^T from *Halorussus rarus* CGMCC 1.10122^T are optimum NaCl, optimum Mg^{2+}, optimum pH, anaerobic growth with nitrate, arginine, and DMSO, utilization of specific carbon sources, gelatin and casein hydrolysis, and H_2S formation (Table 1). More detailed results of phenotypic features of strain YC25^T are given in the species description.

The major polar lipids of strain YC25^T were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), sulfated galactosyl mannosyl glucosyl diether (S-TGD-1), sulfated mannosyl glucosyl diether (S-DGD-1), galactosyl mannosyl glucosyl diether (TGD-1), mannosyl glucosyl diether (DGD-1), and an unknown diglycosyl diether (DGD-2) chromatographically identical to those of *Halorussus rarus* CGMCC 1.10122^T (Supplementary Fig. S2). The major polar lipid compositions supported classification of strain YC25^T in the genus *Halorussus*.

Sequence comparisons indicated that strain YC25^T possessed two different 16S rRNA genes (denoted *rrnA* and *rrnB*) that differed in sequence by 4.4 %. The *rrnA* and *rrnB* genes of strain YC25^T were phylogenetically related to that of *Halorussus rarus* CGMCC 1.10122^T (94.3 and 95.4 % nucleotide identity, respectively), 16S rRNA gene similarities that are well below the recently recommended thresholds (98.2–99.0 %) to separate two prokaryotic species (Meier-Kolthoff et al. 2013). Phylogenetic tree reconstructions using the ML algorithm revealed that strain YC25^T tightly clustered with *Halorussus rarus* (Fig. 1a). The phylogenetic position was also confirmed in other trees generated using the MP and NJ algorithms (Supplementary Fig. S3a & Fig. S4a).

The *rpoB′* gene of strain YC25^T was closely similar to the corresponding gene of *Halorussus rarus* (91.5 % nucleotide identity). In phylogenetic tree reconstructions using *rpoB′* (Fig. 1b), strain YC25^T tightly clustered with *Halorussus rarus*. The phylogenetic position was also confirmed in trees generated using the MP and NJ algorithms (Supplementary Fig. S3b & Fig. S4b).

The 16S rRNA gene and *rpoB′* gene-based phylogenetic analysis results supported the placement of strain YC25^T in the genus *Halorussus*.

The DNA G+C content of strain YC25^T was determined to be 63.3 mol%, which is lower than that of *Halorussus rarus* CGMCC 1.10122^T (66.1 mol%).

Based on these phenotypic, chemotaxonomic, and phylogenetic properties, a novel species of the genus *Halorussus*, *Halorussus ruber* sp. nov., is proposed to accommodate strain YC25^T. Characteristics that distinguish strain YC25^T from *Halorussus rarus* CGMCC 1.10122^T are shown in Table 1.

Description of *Halorussus ruber* sp. nov

Halorussus ruber (ru.ber. L. adj. ruber red-colored, red)

Cells are motile, pleomorphic rods under optimal growth conditions and stain Gram-negative. Colonies on agar plates containing 1.7 M NaCl are red, elevated, and round. Chemoorganotrophic and aerobic. Growth occurs at 25–50 °C (optimum 37 °C), at 1.4–4.8 M NaCl (optimum 1.7 M), at 0–1.0 M $MgCl_2$ (optimum 0.01 M), and at pH 5.5–9.0

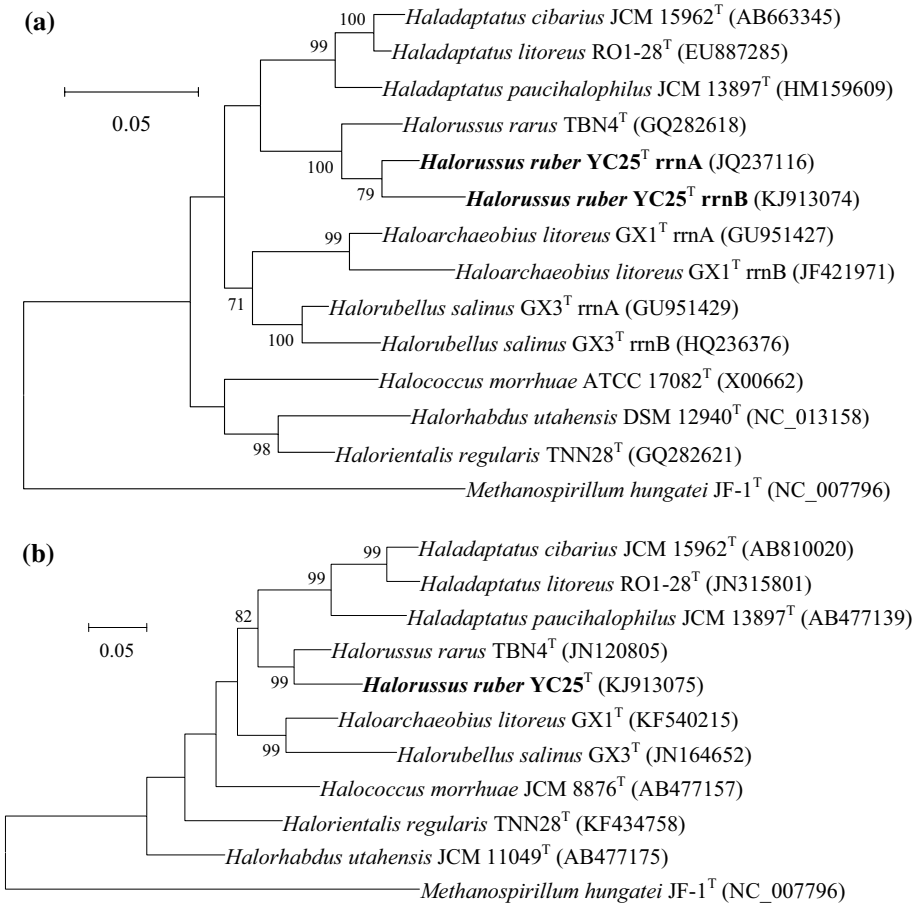

Fig. 1 Maximum-likelihood phylogenetic tree reconstructions based on 16S rRNA gene (**a**) and *rpoB′* gene (**b**) sequences, showing the relationships between strain YC25^T and related members within the family *Halobacteriaceae*. Bootstrap values (%) are based on 1,000 replicates and are shown for branches with more 70 % bootstrap support. *Bar* represents expected changes per site

(optimum pH 6.5). Cells lyse in distilled water, and the minimal NaCl concentration to prevent cell lysis is 8 % w/v. Catalase and oxidase positive. Grow anaerobically in the presence of nitrate, arginine, and DMSO. Nitrate reduction to nitrite is observed, but no gas formed from nitrate. Indole formation is positive, but H_2S formation is negative. Hydrolyze Tween 80 but does not hydrolyze starch, gelatin, or casein. The following substrates are utilized as single carbon and energy sources for growth: acetate, citrate, fumarate, D-galactose, D-glucose, DL-lactate, lactose, L-malate, D-mannitol, D-mannose, D-sorbitol, and sucrose. The following substrates are utilized as single carbon, nitrogen, or energy sources for growth: L-glutamate and L-ornithine. No growth occurs on L-alanine, L-arginine, L-aspartate, D-fructose, glycerol, glycine, L-lysine, maltose, pyruvate, D-ribose, L-sorbose, starch, succinate, or D-xylose. The major polar lipids are phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), sulfated galactosyl mannosyl glucosyl diether (S-TGD-1), sulfated mannosyl glucosyl diether (S-DGD-1), galactosyl mannosyl glucosyl diether (TGD-1), mannosyl glucosyl diether (DGD-1), and an unknown diglycosyl diether (DGD-2). The DNA G+C content of strain YC25^T was 63.3 mol% (T_m). The type strain is YC25^T (=CGMCC 1.12122^T = JCM 18363^T). The GenBank/EMBL/DDBJ accession numbers for the *rrnA* and *rrnB* 16S rRNA gene and *rpoB′* gene sequences of strain YC25^T are JQ237116, KJ913074 and KJ913075, respectively.

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