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ORIGINAL PAPER

Halorussus salinus sp. nov., isolated from a marine solar saltern

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Abstract A halophilic archaeal strain YJ-37-H^T was isolated from Yangjiang marine solar saltern, China. Cells were pleomorphic rods, stained Gram negative and formed red-pigmented colonies on agar plate. Strain YJ-37-H^T was able to grow at 20–50 °C (optimum 37 °C), at 0.9–4.8 M NaCl (optimum 2.6 M NaCl), at 0–1.0 M $MgCl_2$ (optimum 0.3 $MgCl_2$) and at pH 6.5–9.0 (optimum pH 7.0). The cells lysed in distilled water, and the minimal NaCl concentration to prevent cell lysis was found to be 5 % (w/v). The 16S rRNA gene and *rpoB′* gene of strain YJ-37-H^T were phylogenetically related to the corresponding genes of *Halorussus* members (93.2–95.8 % and 90.1–93.9 % similarities, respectively). The major polar lipids of the strain were phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and five glycolipids, sulfated galactosyl mannosyl glucosyl diether (S-TGD-1), galactosyl mannosyl glucosyl diether (TGD-1), sulfated mannosyl glucosyl diether (S-DGD-1), mannosyl glucosyl diether (DGD-1) and diglycosyl diether (DGD-2). The DNA G+C content of strain YJ-37-H^T was 64.9 mol%. The phenotypic, chemotaxonomic and phylogenetic properties suggested that strain YJ-37-H^T (=CGMCC 1.12571^T = JCM 30032^T) represents a new species of *Halorussus*, for which the name *Halorussus salinus* sp. nov. is proposed.

Keywords *Halorussus salinus* sp. nov. · Halophilic archaeon · Marine solar saltern

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Introduction

Extremely halophilic archaea, members of the class *Halobacteria*, thrive in diverse hypersaline environments such as salt lakes, marine solar salterns, saline soils and salt-preserved and fermented foods (Henriet et al. 2014; Liu et al. 2015; Sorokin et al. 2015; Viver et al. 2015). Diverse halophilic archaea cause spoilage of salt-treated foods and endow the hypersaline brines with characteristic reddish color. Halophilic archaea can produce diverse enzymes and some byproducts which may have the potential applications to various industrial processes (Litchfield 2011). In the course of an isolation of enzyme-producing halophilic archaea, a halophilic archaeal strain YJ-37-H^T was recovered which is most closely related to members of the genus *Halorussus*.

This genus is taxonomically placed within the family *Halobacteriaceae* of the order *Halobacteriales*, class *Halobacteria*. The genus was proposed to accommodate the species *Halorussus rarus* based on two strains isolated from a Chinese marine solar saltern (Cui et al. 2010). At present, the genus *Halorussus* (*Hrs*) consists of three species: *Hrs. rarus*, *Hrs. amylolyticus* and *Hrs. ruber* (Xu et al. 2015; Yuan et al. 2015). Both *Hrs. amylolyticus* YC93^T and *Hrs. ruber* YC25^T have two dissimilar 16S rRNA gene sequences, while *Halorussus rarus* TBN4^T has one kind of 16S rRNA gene sequence. The members of *Halorussus*

have the similar and distinctive polar lipids. In this study, strain YJ-37-H^T was characterized as a new member of the genus *Halorussus*, for which the name *Halorussus salinus* sp. nov. is proposed.

Materials and methods

Isolation and cultivation of halophilic archaeal strain

Strain YJ-37-H^T was isolated from the sediment sampling from Yangjiang marine solar saltern in the southern region of China (21°31′48′ N, 111°28′5′ E; elevation, sea level) in 2012. 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, $MgSO_4·7H_2O$ 26.8, $MgCl_2·6H_2O$ 23.0 and NaCl 184.0 (pH adjusted to 7.0–7.2 with 1 M NaOH solution). One gram of the sediment sample was suspended in 9 mL of liquid NHM, was serially diluted in liquid NHM and then was spread on NHM agar plates. The inoculated plates were incubated for three months at 37 °C. The reddish colonies were picked and were successively restreaked on NHM agar plates at least three times to obtain pure colonies. The isolated strain was preserved at −20 °C as a suspension in NHM broth supplemented with glycerol (150 g/L).

Phenotypic determination

Phenotypic tests were performed according to the proposed minimal standards for description of new taxa in the order *Halobacteriales* (Oren et al. 1997) and as described previously by Cui et al. (2010). The analyses were conducted using NHM at 37 °C unless otherwise noted. The type strains *Halorussus rarus* TBN4^T (CGMCC 1.10122^T), *Halorussus amylolyticus* YC93^T (CGMCC 1.12126^T) and *Halorussus ruber* YC25^T (CGMCC 1.12122^T) were selected as reference strains in phenotypic tests. These reference strains were also routinely grown aerobically at 37 °C in NHM. Colony morphology was observed on NHM agar plate. Gram staining was performed according to the method described by Dussault (1955). Cell morphology and motility in exponentially growing liquid cultures were examined using a phase-contrast light microscope. The NaCl range for growth was determined by incubating the strain at NaCl concentrations of 0.9, 1.4, 1.7, 2.1, 2.6, 3.1, 3.4, 3.9, 4.3, 4.8 and 5.1 M. The salt requirement for maintaining cell stability was determined by suspending cells in decreasing concentrations of a salt solution with the salt composition of the optimal culture medium, and the stability of the cells was detected by light microscopic examination. The temperature range for growth was determined by incubating each strain at 10, 15, 20, 25, 30, 37, 40, 42, 45, 50, 55 and 60 °C. Anaerobic growth on nitrate and formation of gas from nitrate were tested in 9-mL screw-topped tubes (with Durham tubes) completely filled with liquid NHM added with $NaNO_3$ (5 g/L). The formation of nitrite was monitored by using Griess reagent (Ivanov 2004), and the formation of gas from nitrate was detected by the presence of gas bubbles in the Durham tubes. Anaerobic growth in the presence of L-arginine or DMSO (5 g/L) was tested in completely filled 9-mL screw-topped tubes. Starch hydrolysis was determined on NHM agar plates supplemented with 2 g/L soluble starch and detected by flooding the surface of agar plate with Lugol's iodine solution. Gelatin hydrolysis was assessed by growing colonies on NHM agar plate added with 5 g/L gelatin and detected by flooding the plate with Frazier's reagent (McDade and Weaver 1959). The catalase and oxidase activities were determined as described by Gonzalez et al. (1978). Hydrolysis of Tween 80 was tested by using the procedure adapted for halophilic archaea by Gutiérrez and González (1972). Production of H_2S was determined by growing the isolate and reference strains in a tube containing liquid NHM supplemented with 5 g/L sodium thiosulfate and detected using a filter paper strip impregnated with lead acetate (Cui et al. 2007). The range of substrates used as carbon and energy sources was assessed in liquid NHM in which fish peptone and sodium pyruvate were omitted. The compound to be tested was added at a concentration of 5 g/L. Antimicrobial sensitivity tests were performed by spreading cell suspensions on NHM agar plates and then applying disks impregnated with antimicrobial agents.

Chemotaxonomic characterization

Halophilic archaeal polar lipids were extracted using a chloroform–methanol system and analyzed using one- and two-dimensional TLC, as described previously (Cui et al. 2010). 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. The presence of phospholipids and glycolipids on the two-dimensional TLC was confirmed by comparing with one-dimensional TLC on which the polar lipid profile of reference strains was developed.

Phylogenetic and genotypic analysis

Halophilic archaeal genomic DNA was extracted and purified using a genomic DNA extraction kit (CW0552, Beijing ComWin Biotech Co., Ltd.), and the 16S rRNA gene

was amplified with the forward primer 0018F and reverse primer 1518R, then cloned and sequenced according to a previous protocol (Cui et al. 2009). The *rpoB′* gene was amplified using the primer pair HrpoB2 1420F and HrpoA 153R (Minegishi et al. 2010), and the PCR product was sequenced using the following primers: HrpoB2 1420F, HrpoA 153R and B1-628F (5′-CCNGCNGSVCAGAACTTC-3′). These sequences were aligned using the ClustalW program integrated in the MEGA 6 software (Tamura et al. 2013), and the phylogenetic trees were reconstructed using maximum-likelihood (ML) (Felsenstein 1981), maximum-parsimony (MP) (Fitch 1971) and neighbor-joining (NJ) (Saitou and Nei 1987) algorithms in the MEGA 6 software. Sequence similarity was analyzed by comparing the 16S rRNA gene sequence of strain YJ-37-H^T with known sequences from the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon) (Kim et al. 2012). 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

The main phenotypic characteristics differentiating strain YJ-37-H^T from the related members of the genus *Halorussus* are anaerobic growth with nitrate, arginine and DMSO, utilization of specific carbon sources, indole formation, hydrolysis of casein, gelatin, starch and Tween 80, and H_2S formation (Table 1). More detailed results of phenotypic features of strain YJ-37-H^T are given in the species description.

The major polar lipids of strain YJ-37-H^T were phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and five glycolipids, sulfated galactosyl mannosyl glucosyl diether (S-TGD-1), galactosyl mannosyl glucosyl diether (TGD-1), sulfated mannosyl glucosyl diether (S-DGD-1), mannosyl glucosyl diether (DGD-1) and diglycosyl diether (DGD-2) (Supplementary Fig. S2). Since the polar lipid profile of strain YJ-37-H^T was identical to those of the current members of the genus *Halorussus* (Cui et al. 2010; Xu et al. 2015; Yuan et al. 2015), the major polar lipid composition supports the classification of strain YJ-37-H^T in the genus *Halorussus*.

Complete 16S rRNA gene sequence comparisons indicated that strain YJ-37-H^T has one kind of 16S rRNA gene sequence (1471 bp in length). The 16S rRNA gene of the strain was phylogenetically related to *Halorussus amylolyticus* YC93^T (93.2–95.2 % similarities), *Halorussus rarus* TBN4^T (95.2 % similarity) and *Halorussus ruber* YC25^T (94.2–95.8 % similarities). These 16S rRNA gene similarities are well lower than the recently recommended thresholds (98.7–99.0 %) to separate two prokaryotic species (Stackebrandt and Ebers 2006). Phylogenetic tree reconstructions using the maximum-likelihood (ML) algorithm revealed that strain YJ-37-H^T tightly clustered with *Halorussus* members (Fig. 1a). The phylogenetic position was also confirmed in other trees generated using the maximum-parsimony (MP) and neighbor-joining (NJ) algorithms (Supplementary Fig. S3a & Fig. S4a).

The *rpoB′* gene of strain YJ-37-H^T was closely similar to the corresponding gene of *Halorussus amylolyticus* YC93^T (90.1 % similarity), *Halorussus rarus* TBN4^T (91.1 % similarity) and *Halorussus ruber* YC25^T (93.9 % similarity). In phylogenetic tree reconstructions using *rpoB′* (Fig. 1b),

Table 1 Differential characteristics among strain YJ-37-H^T and the current members of *Halorussus*

Characteristic	1	2	3	4
Anaerobic growth with nitrate	−	+	−	+
Anaerobic growth with arginine	−	+	−	+
Anaerobic growth with DMSO	−	+	−	+
Utilization of				
D-Galactose	−	+	+	+
Maltose	−	−	+	−
Lactose	−	+	+	+
Starch	+	+	+	−
Glycerol	+	+	+	−
D-Mannitol	+	−	+	+
D-Sorbitol	−	+	−	+
Acetate	−	+	+	+
Pyruvate	+	+	+	−
DL-Lactate	−	+	+	+
Succinate	−	−	+	−
Fumarate	+	+	−	+
Citrate	−	+	+	+
L-Alanine	−	+	+	−
L-Arginine	−	+	−	−
L-Aspartate	+	−	+	−
L-Ornithine	−	+	+	+
Indole formation	+	−	+	+
Starch hydrolysis	+	+	w	−
Gelatin hydrolysis	+	+	+	−
Casein hydrolysis	−	+	+	−
Tween 80 hydrolysis	−	−	+	+
H_2S formation	+	+	+	−
G+C content (mol%)	64.9	64.6	66.1	63.3

Taxa: 1, YJ-37-H^T; 2, *Halorussus amylolyticus* YC93^T (CGMCC 1.12126^T); 3, *Halorussus rarus* TBN4^T (CGMCC 1.10122^T); 4, *Halorussus ruber* YC25^T (CGMCC 1.12122^T). Symbols: +, positive; −, negative; w, weak

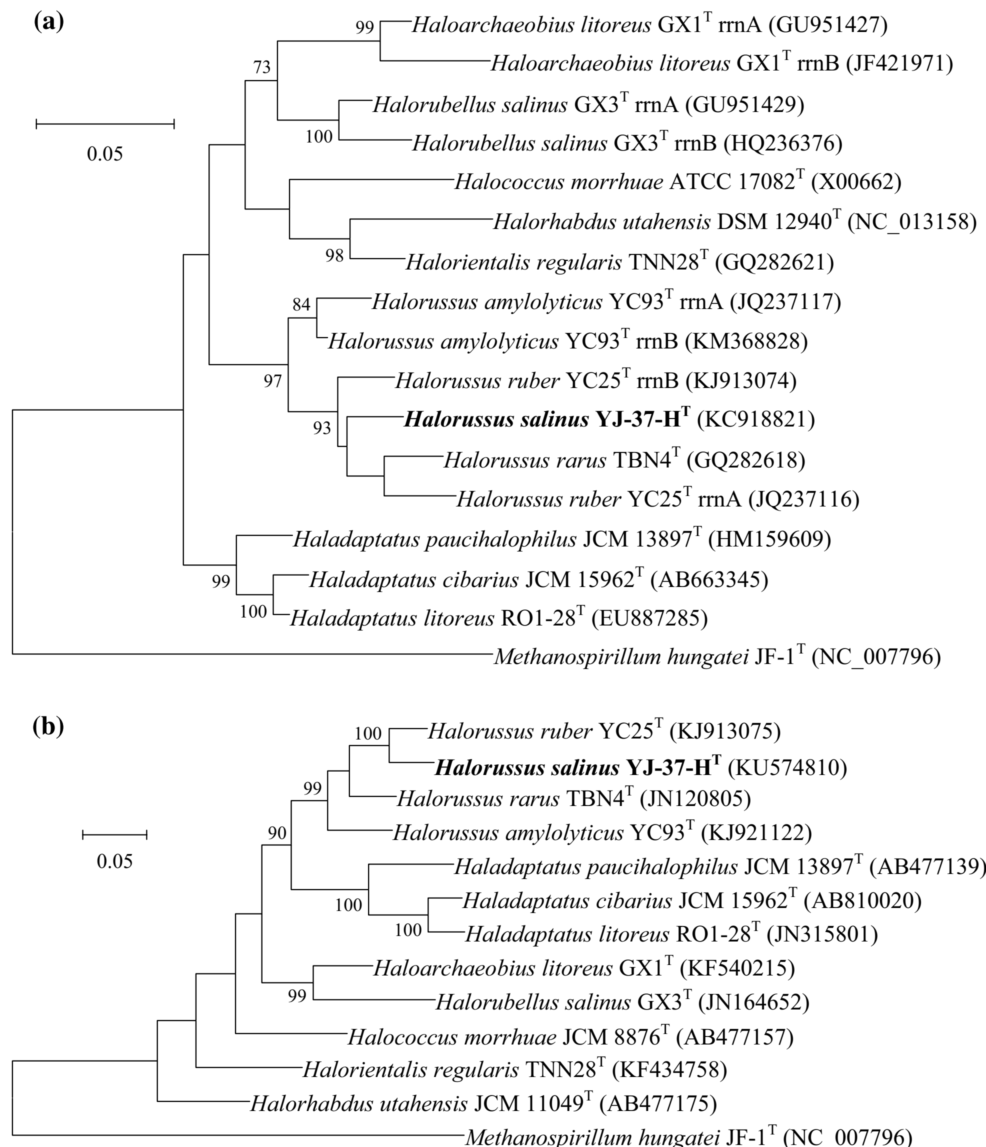

Fig. 1 Maximum-likelihood phylogenetic tree reconstructions based on 16S rRNA gene (**a**) and *rpoB′* gene (**b**) sequences, showing the relationships between strain YJ-37-H^T and related members within the class *Halobacteria*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more 70 % bootstrap support. Bar represents expected substitutions per nucleotide position

strain YJ-37-H^T tightly clustered with the members of *Halorussus*. The phylogenetic position was also confirmed in trees generated using the maximum-parsimony (MP) and neighbor-joining (NJ) algorithms (Supplementary Fig. S3b & Fig. S4b).

The 16S rRNA gene and *rpoB′* gene-based phylogenetic analysis results supported the placement of strain YJ-37-H^T in the genus *Halorussus*.

The DNA G+C content of strain YJ-37-H^T was 64.9 mol%, well within the range of values reported for other members of the genus (63.3–66.1 mol%).

Based on these phenotypic, chemotaxonomic and phylogenetic properties, a novel species of the genus *Halorussus* is proposed to accommodate the strain, *Halorussus salinus* sp. nov.

Description of *Halorussus salinus* sp. nov

Halorussus salinus (sa.li'nus. L. masc. adj. salinus of or belonging to salt.)

Cells are motile, pleomorphic rods (0.5–2.0 × 2.0–5.0 μm) under optimal growth conditions and stain Gram negative. Colonies on agar plates containing 2.6 M NaCl are red, elevated and round. Optimal growth is obtained at 2.6 M NaCl (range 0.9–4.8 M), 0.3 M $MgCl_2$ (range 0–1.0 M),

37 °C (range 20–50 °C) and pH 7.0 (range 6.5–9.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 5 % w/v. They are catalase and oxidase positive. Nitrate reduction to nitrite is observed, but gas formation from nitrate does not occur. Anaerobic growth is not observed in the presence of nitrate, L-arginine or DMSO. Indole formation and H_2S formation are positive. It hydrolyzes starch and gelatin but does not hydrolyze casein or Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, sucrose, starch, glycerol, D-mannitol, pyruvate, L-malate and fumarate. The following substrates are utilized as single carbon, nitrogen or energy sources for growth: L-aspartate and L-glutamate. No growth occurs on D-galactose, D-fructose, L-sorbose, D-ribose, D-xylose, maltose, lactose, D-sorbitol, acetate, DL-lactate, succinate, citrate, glycine, L-alanine, L-arginine, L-lysine or L-ornithine. The type strain was sensitive to the following antimicrobial compounds (μg per disk, unless otherwise indicated): novobiocin (30), bacitracin (0.04 IU per disk), rifampin (5), nitrofurantoin (300) and nystatin (100). 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), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The major polar lipids are phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and five glycolipids, sulfated galactosyl mannosyl glucosyl diether (S-TGD-1), galactosyl mannosyl glucosyl diether (TGD-1), sulfated mannosyl glucosyl diether (S-DGD-1), mannosyl glucosyl diether (DGD-1) and diglycosyl diether (DGD-2). The DNA G+C content of type strain was 64.9 mol% (T_m). The type strain is YJ-37-H^T (=CGMCC 1.12571^T = JCM 30032^T).

The type strain is strain YJ-37-H^T and was isolated from Yangjiang marine solar saltern, Guangdong Province, China.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *rpoB′* gene sequences of strain YJ-37-H^T are KC918821 and KU574810, respectively.

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