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Halorientalis brevis sp. nov., Isolated from an Inland Salt Lake of China

Pan-Pan Yuan[1] · Shuai Yin[1] · Dong Han[1] · Wen-Jiao Zhang[1] · Heng-Lin Cui[1]

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Abstract Halophilic archaeal strain YC89^T was isolated from Yuncheng salt lake in Shanxi, China. Cells from strain YC89^T were short rods, lysed in distilled water, stained Gram-negative and formed red-pigmented colonies on agar plate. Strain YC89^T was able to grow at 25–50 °C (optimum 37 °C), at 1.4–4.8 M NaCl (optimum 2.6–3.1 M), at 0–1.0 M $MgCl_2$ (optimum 0.3 M) and at pH 6.0–9.5 (optimum pH 7.5). The major polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, sulfated mannosyl glucosyl diether and two unknown glycolipids. 16S rRNA gene analysis revealed that strain YC89^T was phylogenetically related to *Halorientalis persicus* D108^T (95.6 % nucleotide identity) and *H. regularis* TNN28^T (95.3 % nucleotide identity). The *rpoB′* gene similarities between strain YC89^T and *H. persicus* IBRC-M 10043^T and *H. regularis* TNN28^T were 88.1 and 88.0 %, respectively. The DNA G+C content of strain YC89^T was determined to be 61.3 mol%. The phenotypic, chemotaxonomic and phylogenetic properties suggested that strain YC89^T (=CGMCC 1.12125^T = JCM 18366^T) represents a new species of *Halorientalis*, for which the name *H. brevis* sp. nov. is proposed.

Introduction

Salt lakes are natural athalassohaline environments hosting diverse halophilic archaea, members of the family *Halobacteriaceae* within the order *Halobacteriales* [1, 7, 12, 15, 16]. The family *Halobacteriaceae* encompasses 47 genera containing over 165 species as of February 2014 [13, 14]. During our survey on halophilic archaeal diversity of an inland salt lake of China, we obtained a halophilic archaeal strain YC89^T, which was most closely related to the members of *Halorientalis*, as judged from 16S rRNA gene sequences. The genus *Halorientalis* was first proposed to accommodate the species *Halorientalis regularis* based on two strains isolated from an artificial marine solar saltern in Eastern China [4]. Recently, *H. persicus* was described based on the strain isolated from an inland salt lake of China [1]. The current two members of *Halorientalis* are aerobic and chemoorganotrophic, using sugars as sources of carbon and energy. The major polar lipids of *Halorientalis* species are phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), sulfated mannosyl glucosyl diether (S-DGD-1) and several unknown glycolipids. In this study, we characterize strain YC89^T as a new species of the genus *Halorientalis*, for which the name *H. brevis* sp. nov. is proposed.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *rpoB′* gene sequences of strain YC89^T are JQ237120 and KJ913076, respectively.
Phase-contrast micrograph of strain YC89^T, thin-layer chromatograms of strain YC89^T and related members, neighbour-joining phylogenetic tree reconstructions based on 16S rRNA gene and *rpoB′* gene sequences showing the relationships between strain YC89^T and related members within the family *Halobacteriaceae* are available as supplementary materials.

Electronic supplementary material The online version of this article (doi:10.1007/s00284-015-0861-3) contains supplementary material, which is available to authorized users.

✉ Heng-Lin Cui
cuihenglin@ujs.edu.cn

[1] School of Food and Biological Engineering, Jiangsu University, 301 Xuefu Road, Jingkou District, Zhenjiang 212013, People's Republic of China

Materials and Methods

Isolation and Cultivation of Halophilic Archaeal Strain

Strain YC89^T was isolated from the brine sampled 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, 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 3 months at 37 °C. After this initial cultivation, colonies were successively re-streaked on NHM agar plates at least three times to obtain pure colonies. The strains were routinely grown aerobically at 37 °C for 7 days in NHM and preserved at −20 °C as a suspension in NHM broth supplemented with glycerol (15 %, w/v).

Phenotypic Determination

Phenotypic tests were performed according to the proposed minimal standards for description of novel taxa in the order *Halobacteriales* [11]. Determination of morphology and growth characteristics, nutrition, miscellaneous biochemical tests and sensitivity to antimicrobial agents was performed as described and cited previously [3]. The halophilic archaeal strains *H. regularis* CGMCC 1.10123^T and *H. persicus* IBRC-M 10043^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 analysed using one- and two-dimensional TLC, as described previously [3]. Merck silica gel 60 F_{254} aluminium-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 for 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, sulphuric 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 Genomic Analysis

Genomic DNA from halophilic archaeal strain was prepared as described previously for determination of the DNA base composition and PCR-mediated amplification experiments [5]. The 16S rRNA genes were amplified, cloned and sequenced according to the previous protocol [2]. PCR-mediated amplification and sequencing of the *rpoB′* genes were performed according to Minegishi et al. [10]. Multiple sequence alignments were performed using the ClustalW program integrated in the MEGA 5 software [17]. Phylogenetic trees were reconstructed using the Maximum-Likelihood algorithm in the MEGA 5 software. 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 mid-point value (T_m) of the thermal denaturation method [8] at 260 nm with a Beckman-Coulter DU800^{TM} spectrophotometer equipped with a high-performance temperature controller.

Results and Discussion

Morphological, Physiological and Biochemical Characteristics

Cells of strain YC89^T were observed to be motile and short rod-shaped (0.5–1.0 × 1.0–2.5) when grown in NHM liquid medium (Supplementary Fig. S1). They stained Gram-negative, and the colonies were observed to be red-pigmented. Strain YC89^T was able to grow at 25–50 °C (optimum 37 °C), at 1.4–4.8 M NaCl (optimum 2.6–3.1 M), at 0–1.0 M $MgCl_2$ (optimum 0.3 M) and at pH 6.0–9.5 (optimum pH 7.5). 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, DMSO and L-arginine. It was found to be positive for H_2S formation and negative for indole formation. Strain YC89^T did not hydrolyse starch, casein, gelatin or Tween 80. Strain YC89^T was sensitive to the following antimicrobial compounds (μg per disc, unless otherwise indicated): novobiocin (30), bacitracin (0.04 IU per disc) and nitrofurantoin (300). It was resistant to the following antimicrobial compounds: rifampin (5), mycostatin (100), trimethoprim (5), erythromycin (15), penicillin

G (10 IU per disc), 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 main phenotypic characteristics differentiating strain YC89^T from *H. regularis* CGMCC 1.10123^T and *H. persicus* IBRC-M 10043^T are Mg^{2+} requirement, anaerobic growth with nitrate, arginine and DMSO, gas formation from nitrate, utilization of specific carbon sources, indole formation, hydrolysis of Tween 80, H_2S formation (Table 1). More detailed results of phenotypic features of strain YC89^T are given in the species description.

Chemotaxonomic Characteristics

The major polar lipids of strain YC89^T are PG, PGP-Me, one glycolipids (GL2) chromatographically identical to S-DGD-1 and two unknown glycolipids (GL1 and GL3) (Supplementary Fig. S2). The phospholipid profile of strain YC89^T is identical to that of *H. regularis* CGMCC 1.10123^T. The main glycolipid composition of strain YC89^T (GL1 and GL2) is chromatographically identical to the two glycolipids of *H. regularis* CGMCC 1.10123^T. The major polar lipid composition supported classification of strain YC89^T in the genus *Halorientalis*.

Table 1 Characteristics that distinguish strain YC89^T from *Halorientalis regularis* CGMCC 1.10123^T and *Halorientalis persicus* IBRC-M 10043^T

Characteristic	1	2	3
Mg^{2+} requirement	−	+	−
Anaerobic growth with nitrate	+	−	−
Gas formation from nitrate	−	+	−
Anaerobic growth with arginine	+	−	−
Anaerobic growth with DMSO	+	−	−
Utilization of			
D-Galactose	+	+	−
D-Fructose	−	−	+
Maltose	−	−	+
Lactose	−	−	+
Glycerol	+	+	−
D-Mannitol	+	+	−
Indole formation	−	+	−
Tween 80 hydrolysis	−	+	+
H_2S formation	+	+	−

Taxa: *1* YC89^T, *2* *Halorientalis regularis* CGMCC 1.10123^T, *3* *Halorientalis persicus* IBRC-M 10043^T

+ positive, − negative

Phylogenetic Analysis

Eight complete 16S rRNA gene sequences of strain YC89^T were obtained (1472 bp in length), and sequence comparisons indicated that strain YC89^T has one kind of 16S rRNA gene sequence. 16S rRNA gene analysis revealed that strain YC89^T was phylogenetically related to *H. persicus* D108^T (95.6 % nucleotide identity) and *H. regularis* TNN28^T (95.3 % nucleotide identity). These 16S rRNA gene similarities are well below the recently recommended thresholds (98.2–99.0, 98.65 %) to separate two prokaryotic species [6, 8]. Phylogenetic tree reconstructions using the maximum-likelihood (ML) algorithm revealed that strain YC89^T tightly clustered with the current three members of *Halorientalis* (Fig. 1a). The phylogenetic position was also confirmed in the tree generated using the neighbour-joining (NJ) algorithm (Supplementary Fig. S3a).

The *rpoB′* gene of strain YC89^T was closely similar to the corresponding gene of *H. persicus* D108^T (88.1 % nucleotide identity) and *H. regularis* TNN28^T (88.0 % nucleotide identity), and the similarity values are less than the recommended threshold (86.2 %) which is used to distinguish genera [10]. In phylogenetic tree reconstruction using *rpoB′* (Fig. 1b), strain YC89^T tightly clustered with *H. persicus* D108^T and *H. regularis* TNN28^T. The phylogenetic position was also confirmed in the tree generated using the NJ algorithm (Supplementary Fig. S3b).

The 16S rRNA gene and *rpoB′* gene-based phylogenetic analysis results supported the placement of strain YC89^T in the genus *Halorientalis*.

The DNA G+C content of strain YC89^T was determined to be 61.3 mol%, which is lower than those of *H. regularis* CGMCC 1.10123^T (61.9 mol%) and *H. persicus* IBRC-M 10043^T (62.8 mol%).

Based on these phenotypic, chemotaxonomic and phylogenetic properties, a novel species of the genus *Halorientalis*, *H. brevis* sp. nov., is proposed to accommodate strain YC89^T. Characteristics that distinguish strain YC89^T from the current two *Halorientalis* members are shown in Table 1.

Description of *Halorientalis brevis* sp. nov.

Halorientalis brevis (bre'vis. L. fem. adj. brevis, short)

Cells are motile, short rod-shaped (0.5–1.0 × 1.0–2.5) under optimal growth conditions and stain Gram-negative. Colonies on agar plates containing 3.1 M NaCl are red, elevated and round. The strain is chemoorganotrophic and aerobic. Growth occurs at 25–50 °C (optimum 37 °C), in the presence of 1.4–4.8 M NaCl (optimum 3.1 M NaCl), with 0–1.0 M $MgCl_2$ (optimum 0.3 M $MgCl_2$) and at pH 6.0–9.5 (optimum pH 7.5). Cells lyse in distilled water and the

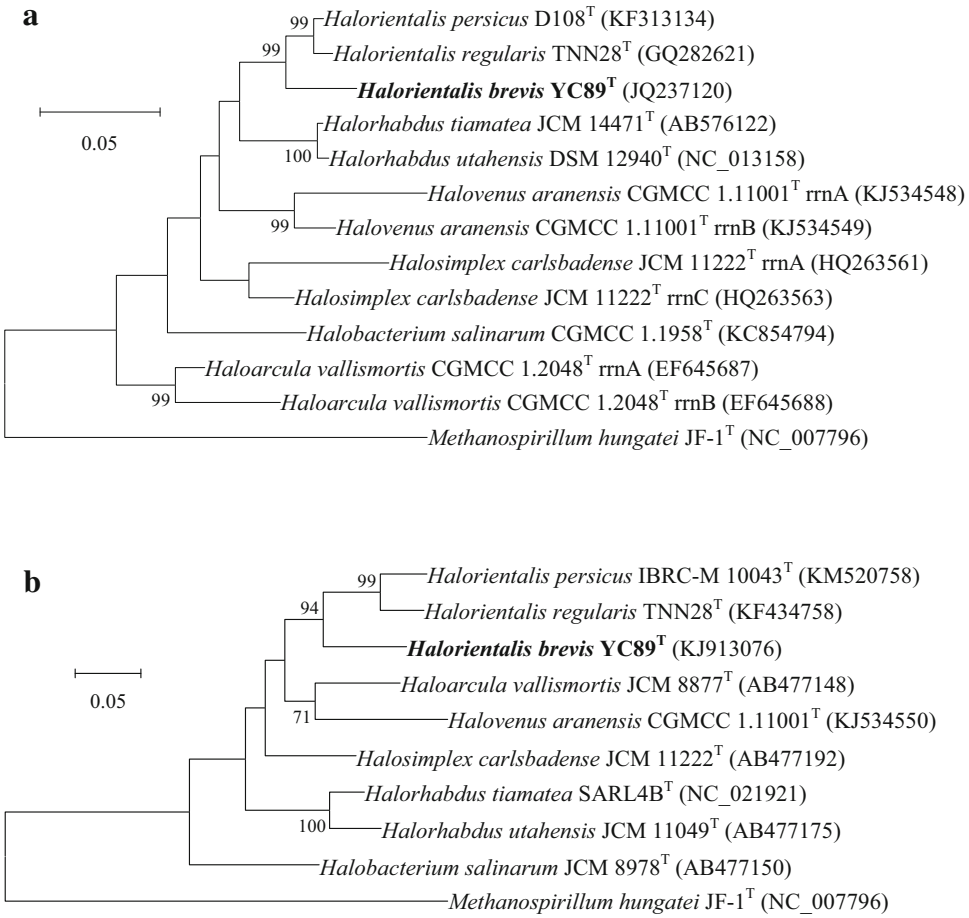

Fig. 1 Maximum-likelihood phylogenetic tree reconstructions based on 16S rRNA gene (**a**) and *rpoB′* gene (**b**) sequences, showing the relationships between strain YC89^T and related members within the family *Halobacteriaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more 70 % bootstrap support. *Bar* represents expected substitutions per nucleotide position

minimal NaCl concentration to prevent cell lysis is 8 % (w/uv). Catalase and oxidase are positive. Anaerobic growth occurs in the presence of nitrate, arginine and DMSO. Nitrate reduction to nitrite is observed, but gas formation from nitrate does not occur. H_2S formation is positive, and indole formation is negative. Does not hydrolyse starch, casein, gelatin or Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, sucrose, glycerol, D-mannitol, D-sorbitol, acetate, pyruvate, DL-lactate, succinate, L-malate, fumarate and citrate. The following substrates are utilized as single carbon, nitrogen or energy sources for growth: L-alanine, L-arginine, L-aspartate, L-glutamate, L-lysine and L-ornithine. No growth occurs on D-fructose, L-sorbose, D-ribose, D-xylose, maltose, lactose or glycine. The major polar lipids are phosphatidic acid (PA), PG, PGP-Me, S-DGD-1 and two unknown glycolipids. The DNA G+C content of the type strain was 61.3 mol% (T_m).

The type strain is YC89^T (=CGMCC 1.12125^T = JCM 18366^T) and was isolated from Yuncheng salt lake in Shanxi, China.

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