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Halorubrum depositum sp. nov., a Novel Halophilic Archaeon Isolated from a Salt Deposit

Shaoxing Chen[1,2] · Siqi Sun[1] · Yao Xu[1] · Jinting Lv[1] · Linan Chen[1] · Liu Liu[1]

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Abstract
A non-motile, pleomorphic rod-shaped or oval, red-pigmented (nearly scarlet), extremely halophilic archaeon, strain Y78^T, was isolated from a salt deposit of Yunnan salt mine, China. Analysis of the 16S rRNA gene sequence showed that it was phylogenetically related to species of the genus *Halorubrum*, with a close relationship to *Halorubrum rutilum* YJ-18-S1^T (98.6%), *Halorubrum yunnanense* Q85^T (98.3%), and *Halorubrum lipolyticum* 9-3^T (98.1%). The temperature, NaCl, and pH ranges for growth were 25–50 °C, 12–30% (w/v), and 6.5–9.0, respectively. Mg^{2+} was required for growth. The polar lipids of strain Y78^T were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, and a sulfated diglycosyl diether. The DNA G+C content was 66.6 mol%. DNA–DNA hybridization values between strain Y78^T and two closely related species of the genus *Halorubrum* were far below 70%. Based on the data presented in this study, strain Y78^T represents a novel species for which the name *Halorubrum depositum* sp. nov. is proposed; the type strain is Y78^T (=CGMCC 1.15456^T=JCM 31272^T).

Introduction

Species of genus *Halorubrum* established by McGenity and Grant in 1995 [16] have always been found in salt mines [3, 29]. At the time of writing, there are 35 validly named *Halorubrum* species. Many species of halotolerant bacterium or haloarchaea were isolated from the salt mine or salt crystal [22, 27, 28]. Hypersaline environments such as salt lakes, salt mine, and evaporation ponds for the production of commercial salts including edible and industrial salts are found on all continents. They are inhabited by a great diversity of microorganisms adapted to life at high salt concentrations [19]. Salt mine, the fixed hypersaline environment, is one kind of unique habitats for keeping the old microorganisms alive in it [12]. The discovery of many new microbial groups, and their isolation has recently turned our limited view of the hypersaline environment in new directions [26]. In this study, we searched for haloalkaliphiles able to grow at pH 8.0, and isolated a novel slightly alkaliphilic haloarchaeon strain Y78^T that belongs to the genus *Halorubrum*. Based on the phylogenetic and phenotypic features, we aim to propose a novel species within the genus *Halorubrum*.

Materials and Methods

Strain Isolation and Cultivation

The salt deposits were collected from Yunnan salt mine (China) in Sep. 2013 (99°46′42.05″E, 26°06′04.05″N). The sample (100 g) was washed with 100 ml sterile water twice for 1 min for each time, and then the rest of the salt sediment was dissolved in 100 ml of sterile 5% (w/v) NaCl solution. And 500 µl of the sample dissolving liquid was spread on JCM 168 medium agar plates. The JCM 168 medium used for cultivating halophilic bacteria or archaea

The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA, *rpoB′* and *ef-2* sequences of strain Y78^T are KX376712, KX530069, and KX530068, respectively. The protologue has been submitted to the Digital Protologue database (http://imedea.uib-csic.es/dprotologue/) under the Taxon Number TA00336.

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✉ Shaoxing Chen
chensx@ahnu.edu.cn

[1] College of Life Sciences, Anhui Normal University, No.1 Beijing East Road, Wuhu 241000, Anhui, China

[2] College of Life Sciences, Honghe University, No.1 Xuefu Road, Mengzi 661100, Yunnan, China

constituted of (per liter): casamino acids (BD-Difco, 5.0 g), yeast extract (BD-Difco, 5.0 g), sodium glutamate (1.0 g), trisodium citrate (3.0 g), $MgSO_4 \cdot 7H_2O$ (20.0 g), KCl (2.0 g), NaCl (200.0 g), $FeCl_2 \cdot 4H_2O$ (36.0 mg), and $MnCl_2 \cdot 4H_2O$ (0.36 mg) (http://jcm.brc.riken.jp/en/). The pH of the medium was adjusted to 8.0 with 1 M KOH.

The inoculated agar plates were sealed by plastic preservative film and incubated in a sealed plastic bags at 38 °C for 2 weeks. Red-colored (nearly scarlet) colonies occurred on the agar plates. The colonies that occurred on the agar plates were transferred to fresh agar plates, and pure cultures were obtained after successive streaking. All the colonies were purified for the further searches.

Preliminary Identification by 16S rRNA Gene

The pure cultures obtained via successive streaking were re-suspended in sterile water and the lysates were taken as the PCR template for preliminary identification. PCR amplification of the 16S rRNA gene was performed by using the primer pair F8 (5′-TTGATCCTGCCGGAGGCCATTG-3′) and R1462 (5′-ATCCAGCGCAGATTCCCCTAC-3′) [13]. The PCR products verified by electrophoresis were sent to a biotechnological company for direct DNA sequencing.

Phenotypic and Chemotaxonomic Characterization

Colony morphology was observed on the agar plate after incubation for 2–3 weeks at 38 °C. Gram-staining was performed according to method described by Dussault [8]. Cell morphology and motility were examined using a phase contrast microscope (OLYMPUS BX51 equipped with OLYMPUS DP72) and scanning electron microscope (HITACHI SU8010). The range of salinity for growth was determined by using the JCM 168 medium containing various concentrations of NaCl 5–30% (w/v) with intervals of 5% (w/v). The pH range for growth was assayed from pH 5.0–9.5 at intervals of 0.5 in liquid media containing 50 mM pH buffers (MES for pH 5.0–6.0, PIPES for pH 6.5–7.0, HEPES for pH 7.5–8.0, tricine for pH 8.5, or CHES for pH 9.0–9.5). The temperature range for the growth was determined at 10, 15, 20, 25, 30, 35, 38, 40, 42, 45, 50, and 55 °C in a medium of pH 8.0 with 20% (w/v) NaCl. The concentrations of Mg^{2+} in the media were set to 0, 0.005, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, 0.7, and 1.0 M, for detecting the requirement of Mg^{2+} for the growth. In this test, the $MgSO_4 \cdot 7H_2O$ was replaced by $MgCl_2 \cdot 6H_2O$ to avoid the impacts of SO_4^{2-}. Cell lysis was performed by re-suspending the cell pellets in distilled water and centrifugation.

The similarity search of 16S rRNA gene sequence showed that strain $Y78^T$ was affiliated to the genus *Halorubrum*. The following phenotypic tests were performed under the optimal growth condition, according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* [20]. Tests for catalase and oxidase activities and for the hydrolysis of starch, gelatin, skim milk, Tween 80, Tween 60, Tween 40, and Tween 20 were performed as described by González et al. [9]. H_2S formation from L-cysteine was detected using a filter-paper strip impregnated with lead acetate (10%, w/v) [5]. Indole production from tryptophan was assessed as described by Oren et al. [20]. To determine the utilization of different organic substrates such as carbohydrates, alcohols, amino acids, and organic acids as the only source of carbon, nitrogen, and energy, a medium containing 0.01% (w/v) yeast extract and supplemented with 1% (w/v) of the tested substrate (filtration sterilization) was assessed as described by Oren et al. [20]. Reduction of nitrate and nitrite was detected by using the sulfanilic acid and α-naphthylamine reagent [23]. Anaerobic growth was tested with L-arginine, KNO_3 and DMSO with the final concentration of 5 g l^{-1} in screw-topped sealed vials.

Susceptibility to Antibiotics

Antibiotic sensitivity tests were performed by spreading cell suspensions on culture plates and then placing discs impregnated with antibiotics. Antibiotics and amounts (μg per disc, unless indicated) were shown as follows; ampicillin (10), bacitracin (0.04 IU), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), kanamycin (10), neomycin (30), norfloxacin (10), novobiocin (30), penicillin G (10 IU), rifampicin (5), streptomycin (10), tetracycline (30), and vancomycin (30).

Polar Lipids Profile

Polar lipids were extracted using the widely used chloroform–methanol system and detected using two-dimensional thin layer chromatography (2D-TLC) on an aluminum-backed silica gel 60 plates (20 × 20 cm, Merck) [18]. Glycolipids and phospholipids were detected by spraying with 0.5% (w/v) α-naphthol in methanol:water (1:1, by vol), after drying, with sulfuric acid and ethanol (1:1, by vol), and followed by heating at 120 °C for 10 min. The plate was scanned immediately for recording the results.

Phylogenetic Analysis

An single colony of strain $Y78^T$ was picked with sterile toothpick from the agar plate (2–3 weeks incubation) and re-suspended in 40 μl sterile distilled water. Cells lysed in distilled water were taken as the PCR template. The 16S rRNA gene was amplified by using the primer pair F8 and R1462 [13]. The PCR products were purified with a DNA gel extraction kit (Axygen) and inserted into the cloning

vector pMD-18T (TaKaRa) for transformation. At least five PCR-verified recombinants were picked for the DNA sequencing. The almost complete 16S rRNA gene sequence was taken as the query to search the public database via the online BLAST searching tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the EzBioCloud, a public and professional website focusing on the prokaryotic taxonomy [31].

The *rpoB'* gene was amplified with the pair of degenerated primers 1420F (5′-TGTGGGCTNGTGAAGAACTT-3′) and 153R (5′-GGGTCCATCAGCCCCATGTC-3′) [17], while the *ef*-2 gene was amplified with another pair of degenerated primers EF-2f (5′-ATGGGYMGACGHAAGAA-3′) and EF-2r (5′-GCBGGRCCRCGGTGGAT-3′) [11]. The procedure of the cloning and sequencing of the *rpoB'* and *ef*-2 genes was identical to that of 16S rRNA gene.

Multiple sequence alignments were performed using the Clustal W program implemented in the BioEdit software [10] prior to the phylogenetic analysis. DNA sequences used for the reconstruction of phylogenetic trees were retrieved from the public database. Phylogenetic trees were reconstructed using maximum-likelihood algorithms and neighbor-joining algorithms in the MEGA 5.0 software [25]. Multiple sequence alignments were created after taking into account the amino acid alignments for the *rpoB′* protein-encoding gene generated by translating this gene to protein sequences. To evaluate the robustness of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed.

Determination of the DNA G+C Content and DNA–DNA Relatedness

Genomic DNA of strain Y78^T was obtained by the method of Marmur [15]. The G+C content of genomic DNA was inferred from the mid-point (T_m) of the thermal denaturation profile [14] using the equation of Owen and Hill [21]. DNA–DNA hybridization was performed between strain Y78^T and its closely related species (*Halorubrum rutilum* YJ-18-S1^T and *Halorubrum yunnanense* Q85^T) on a Perkin-Elmer Lambda 35 spectrophotometer equipped with a high-performance temperature controller (PTP-6 Peltier system, Perkin-Elmer, USA) in accordance with the thermal denaturation and renaturation approach [7].

Results and Discussion

A red-pigmented (nearly scarlet) colony, entitled strain Y78^T, was isolated from the deposit sample of Yunnan Salt Mine (China) for further analysis. Similarity search of the almost complete 16S rRNA gene (KX376712, 1439 bp) sequence showed that strain Y78^T was closely related to *Halorubrum rutilum* YJ-18-S1^T, *Halorubrum yunnanense* Q85^T, and *Halorubrum lipolyticum* 9-3^T with the similarities of 98.6, 98.3, and 98.1%, respectively. Lower similarities were obtained with the type strains of other species of genus *Halorubrum* and other haloarchaeal genera. Based on the preliminary identification of the 16S rRNA gene, strain Y78^T was closely related to species of the genus *Halorubrum* in family *Halorubraceae* [1].

Strain Y78^T was pleomorphic rod-shaped or oval (Fig. S1). It differed from all the closely related species in genus *Halorubrum* on the mobility and the optimum pH for growth. The mobility of strain Y78^T was weak, nearly non-motile, while others were motile (Table 1). The optimum pH for growth of strain Y78^T was pH 8.5, which obviously was higher than others (Table 1). Strain Y78^T was capable of growing in the range of 10–30% (w/v) NaCl, pH 6.5–9.0, and 25–50 °C. Weak growth also observed with the temperature between 15 and 25 °C when the duration of incubation prolonged (> 4 weeks). Strain Y78^T was resistant to bacitracin under the indicated amount (0.04 IU), while other closely related species were sensitive to it.

The profiles of the major glycolipids and phospholipids were similar to those of *Halorubrum trueperi* Y73^T [4]. Strain Y78^T contained sulfated diglycosyl diether (S-DGD-1) as the sole glycolipid and phosphatidylglycerol phosphate methyl ester (PGP-Me) and phosphatidylglycerol sulfate (PGS) as the major phospholipids (Fig. S2).

The sequences of *rpoB'* gene (KX530069, 1830 bp) and *ef*-2 gene (KX530068, 1692 bp) were deposited in the GenBank. The relatives of strain Y78^T deduced from the sequence similarity search of 16S rRNA gene were the

Table 1 Differential characteristics between strain Y78^T and type strains of closely related species of the genus *Halorubrum*

Characteristic	1	2	3	4
Motility	Non-motile	Motile	Motile	Motile
Optimum pH for growth	8.5	7.5	7.0	7.5
D-Mannose	+	−	+	+
D-Galactose	−	+	−	+
D-Fructose	−	−	−	+
L-Sorbose	−	−	+	−
Maltose	+	+	−	+
Lactose	−	+	−	−
D-Sorbitol	+	+	+	−
Acetate	+	−	+	−
Tween 80 hydrolysis	+	−	−	+
H_2S formation	−	+	+	−
Bacitracin (0.04 IU)	R	S	S	S
Rifampin (5)	S	R	S	S
DNA G+C content (mol%)	66.6	66.3	66.2	65.9

Strains: *1* Strain Y78^T (data from this study), *2 Halorubrum yunnanense* Q85^T [2], *3 Halorubrum rutilum* YJ-18-S1^T [30], *4 Halorubrum lipolyticum* 9-3^T [6]. +, positive; −, negative; *R* resistant; *S* sensitive

same as the *rpoB'* gene. The similarity search of *rpoB'* gene sequence showed that strain Y78^T was closely related to *Halorubrum yunnanense* Q85^T, *Halorubrum rutilum* YJ-18-S1^T, and *Halorubrum saccharovorum* JCM 8865^T with the similarities of 96.5, 96.5, and 96.0%, respectively. However, the relatives deduced from the *ef-2* gene sequence were different from 16S rRNA gene and *rpoB'* gene. The similarity search of *ef-2* gene sequence showed that strain Y78^T was closely related *Halorubrum rubrum* YC87^T, *Halorubrum xinjiangense* CGMCC 1.3527^T, and *Halorubrum trapanicum* JCM 10477^T with the similarities of 95.4, 95.4, and 95.3%.

The phylogenetic tree based on the 16S rRNA gene reconstructed by the maximum-likelihood method confirmed that strain Y78^T was closely related to *Halorubrum rutilum* YJ-18-S1^T and *Halorubrum yunnanense* Q85^T and that they formed a independent lineage (Fig. 1). Topologies of phylogenetic trees inferred by using the neighbor-joining

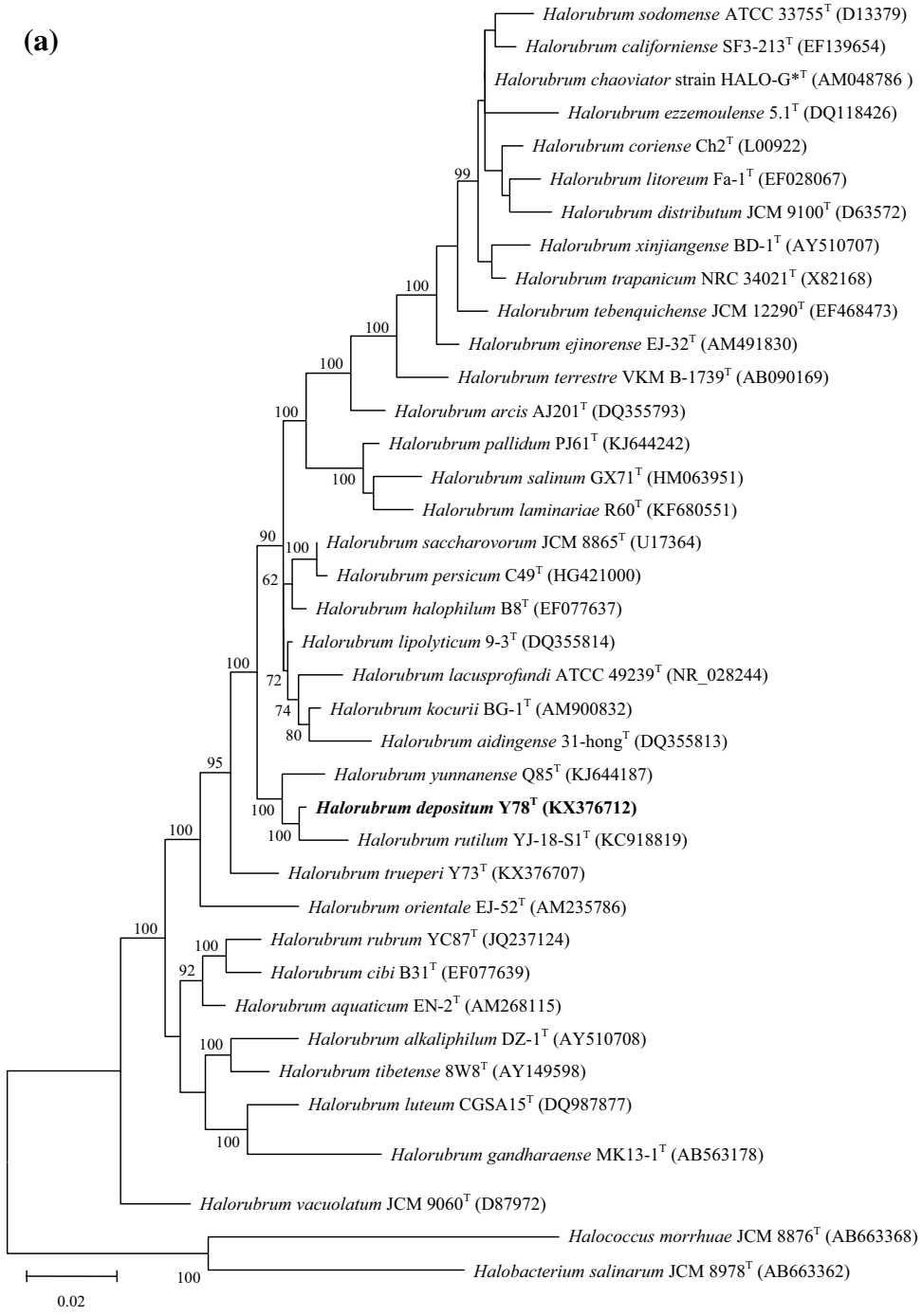
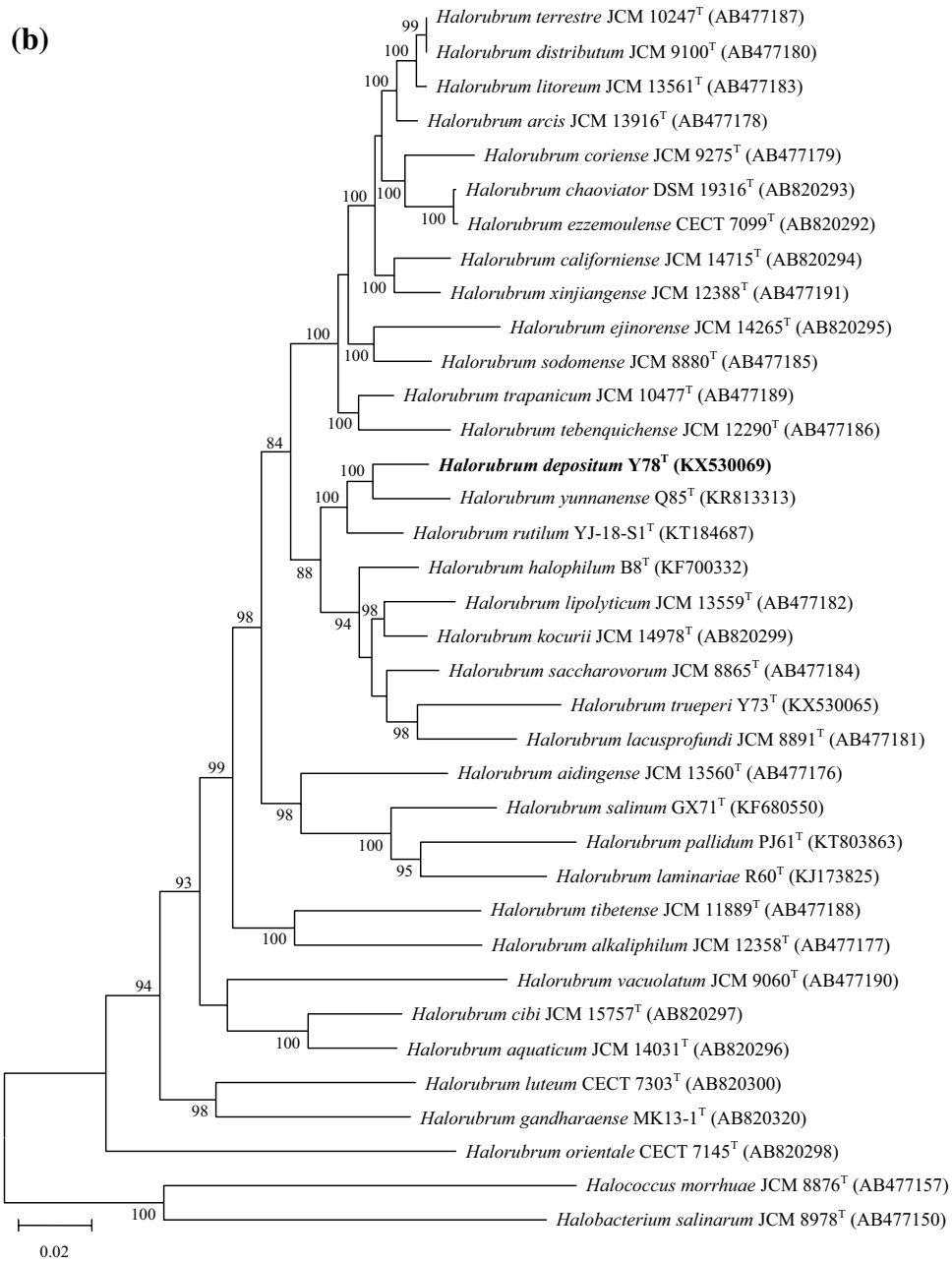


Fig. 1 Maximum-likelihood phylogenetic trees based on 16S rRNA gene (**a**), *rpoB'* gene (**b**) sequences, showed the relationships between strain Y78^T and other members of the genus *Halorubrum* and other related groups. Species *Halococcus morrhuae* JCM 8876^T and *Halobacterium salinarum* JCM 8978^T were taken as outgroups for the 16S rRNA gene and the *rpoB'* gene tree. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown. GenBank accession numbers are shown in parentheses. Bar: 2% substitution

Fig. 1 (continued)



were highly similar to that of the tree reconstructed by the maximum-likelihood method (Fig. S3).

The calculated DNA G+C content of strain Y78^T was 66.6 mol%. The DNA–DNA relatedness between strain Y78^T and *Halorubrum rutilum* YJ-18-S1^T was 27.3 ± 0.5%, while 38.6 ± 0.9% for strain Y78^T and *Halorubrum yunnanense* Q85^T, which are far below the threshold value (70%) for the separation of two different species [24].

The phylogeny based on the *rpoB′* gene was assessed after recovering gene sequences from the GenBank database. Topology of phylogenetic tree inferred by *rpoB′* gene was coincident with that inferred by the 16S rRNA gene (Fig. 1), which strengthens the phylogenetic position and relationship of strain Y78^T. However the topology of phylogenetic tree deduced from the *ef-2* gene was different from 16S rRNA gene and *rpoB′* gene, which reflects the divergence of the evolutionary rate (data not shown). The similar results had happened in the *Halorubrum* species, *Halorubrum yunnanense* [2]. Horizontal gene transfer may attribute to the fact that different collinear genes share different evolutionary rates.

Other detailed results of the physiological tests are shown in the species description and Table 1.

Conclusion

A polyphasic approach including phylogenetic analyses using 16S rRNA and *rpoB′* gene sequence comparisons (Fig. 1), polar lipid profiles (Fig. S2), DNA–DNA hybridization, and detailed phenotypic characterization (Table 1) confirms that the strain Y78^T represents a novel species of the genus *Halorubrum*, for which the name *Halorubrum depositum* sp. nov. is proposed.

Description of *Halorubrum depositum* sp. nov

Halorubrum depositum (de.po′si.tum. L. neut. part. adj. *depositum* deposited).

Cells stain Gram-negative. Colonies on agar medium containing 20% (w/v) NaCl are 1–2 mm in diameter, translucent, red-pigmented (nearly scarlet), circular, slightly raised, and smooth. Cells are non-motile and pleomorphic rods or oval-shaped (approximately 0.6–0.8 × 0.8–1.2 μm). Cell lysed in distilled water. Chemo-organotrophic, aerobic growth occurs at 10–30% (w/v) NaCl, pH 6.5–9.0, and 25–50 °C. Optimum NaCl concentration, pH, and temperature for growth are 20% (w/v), pH 8.5, and 38 °C, respectively. Mg^{2+} is required for growth (at least 0.005 M) with an optimum at 0.3 M. They are catalase- and oxidase-positive. Anaerobic growth with nitrate, DMSO, or L-arginine does not occur. Nitrate is not reduced to nitrite. Indole is not produced. Gelatin is not liquefied. Starch, aesculin, Tween 60, Tween 40, and Tween 20 are not hydrolysed; however, Tween 80 is hydrolysed. The following substrates are utilized for growth as sole sources of carbon and energy: D-glucose, D-mannose, maltose, sucrose, glycerol, D-sorbitol, acetate, pyruvate, lactate, succinate, malate, fumarate, and citrate. The strain can produce acid from D-glucose, D-mannose, maltose, and sucrose. D-galactose, D-fructose, L-sorbose, D-ribose, D-xylose, lactose, starch, and D-mannitol are not used as sole sources of carbon and energy. The following amino acids are used as sole sources of carbon, nitrogen, and energy: L-arginine, L-glutamate, L-ornithine. Glycine, L-alanine, L-asparagine, and L-lysine are not used as sole sources of carbon, nitrogen, and energy. They are sensitive to rifampicin and novobiocin, and resistant to ampicillin, bacitracin, chloramphenicol, ciprofloxacin, erythromycin, kanamycin, neomycin, norfloxacin, penicillin G, streptomycin, tetracycline, and vancomycin. The major components of the polar lipids are sulfated diglycosyl diether, phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, and phosphatidylglycerol sulfate. The DNA G+C content is 66.6 mol% (T_m).

The type strain, Y78^T (= CGMCC 1.15456^T = JCM 31272^T), was isolated from a salt deposit of Yunnan salt mine, China (99°46′42.05″E, 26°06′04.05″N).

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Research Involving Animal Participants This article does not contain any studies with animals performed by any of the authors.

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