**Halorubrum yunnanense** sp. nov., isolated from a subterranean salt mine

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Two halophilic archaeal strains, Q85T and Q86, were isolated from a subterranean salt mine in Yunnan, China. Cells were rod-shaped, Gram-stain-negative and motile. Colonies were red, smooth, convex and round (1.0–2.0 mm in diameter). The orthologous 16S rRNA and rpoB' gene sequences of these two strains were almost identical (99.5 and 99.7 % similarities). Their closest relatives were *Halorubrum kocurii* BG-1T (98.0–98.1 % 16S rRNA gene sequence similarity), *Halorubrum aidingense* 31-hongT (97.6–97.7 %) and *Halorubrum lipolyticum* 9-3T (97.5–97.6 %). The level of DNA–DNA relatedness between strains Q85T and Q86 was 90 %, while that between Q85T and other related *Halorubrum* strains was less than 30 % (29 % for *H. kocurii* BG-1T, 25 % for *H. aidingense* 31-hongT and 22 % for *H. lipolyticum* 9-3T). Optimal growth of the two novel strains was observed with 20 % (w/v) NaCl and at 42–45 °C under aerobic conditions, with a slight difference in optimum Mg2⁺ concentration (0.7 M for Q85T, 0.5 M for Q86) and a notable difference in optimum pH (pH 7.5 for Q85T, pH 6.6 for Q86). Anaerobic growth occurred with nitrate, but not with L-arginine or DMSO. The major polar lipids of the two strains were identical, including phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and sulfated diglycosyl diether, which are the major lipids of the genus *Halorubrum*. The G+C contents of strains Q85T and Q86 were 66.3 and 66.8 %, respectively. Based on the phenotypic, chemotaxonomic and phylogenetic properties of strains Q85T and Q86, a novel species, *Halorubrum yunnanense* sp. nov., is proposed. The type strain is Q85T (=CGMCC 1.15057T=JCM 30665T).

Several halophilic archaeal strains have been isolated from ancient rock salt deposits worldwide (Vreeland et al., 2007; Schubert et al., 2010; Jaakkola et al., 2014). Culture-dependent and culture-independent approaches have revealed the long-term survival of species of the genus *Halorubrum* in ancient halite (Park et al., 2009; Sankaranarayanan et al., 2014). The genus *Halorubrum* was established with the transfer of *Halobacterium saccharovorum*, *Halobacterium sodomense*, *Halobacterium trapanicum* and *Halobacterium lacusprofundi* to the new established genus *Halorubrum* (McGenity & Grant, 1995). At the time of writing, the genus *Halorubrum*, containing more than 28 recognized species, is the largest genus within the family *Halobacteriaceae* (Han & Cui, 2015). Many species in the genus *Halorubrum*, as well as other species in other genera in the family *Halobacteriaceae*, have been isolated from solar salterns or salt lakes. Salt mines (halite deposits), another important hypersaline environment, are widely distributed in Yunnan (China), and many of them have been exploited for table salt for human use and salt for domestic animals (Bi, 1996). However, the haloarchaeal species diversity in these subterranean salt mines in Yunnan remains unclear. In this study, we isolated and characterized two novel haloarchaeal strains, Q85T and Q86. Based on the phylogenetic and phenotypic features of these strains, we propose a novel species of the genus *Halorubrum*.

Salt rock was collected at 70 m depth from Xiangyan Salt Mine (99° 05’ 34” E 36° 45’ 6” N) in Yunnan, China.
(September 2013). The rock sample was washed with sterilized 5 % (w/v) NaCl three times (2 min each), then dissolved in sterilized distilled water. The insoluble residue was discarded by filtration (Whatman 3MM, sterilized). The filtrates were spread on to solid high-salinity modified growth medium (MGM) for incubation (Dyall-Smith, 2009). The culture dishes were sealed with plastic film and incubated at 37 °C in a plastic bag. Red colonies appeared on the MGM agar plates after 2 or 3 weeks. Pure cultures were obtained by repeated streaking of the colonies on the agar plates. Two haloarchaean strains, Q85T and Q86, associated with this halite sample from the Yunnan Mine were isolated.

Preliminary identification was carried out via amplifying and sequencing of the 16S rRNA gene. The DNA templates for PCR were prepared by picking a single colony with a sterilized toothpick, lysis in 100 µl double distilled H2O and collection by removing the cell debris via centrifugation (1 000 r.p.m., 3 min). Primers 18F and 1518R (Cui et al., 2009) were used in the PCR amplification. The PCR products were purified and inserted into a T-vector (pMD-18T; TaKaRa) for sequencing. Molecular cloning of the 16S rRNA gene (1454 nt) and other genes was performed according to the method described by Sambrook & Russell (2001). The 16S rRNA gene sequence was taken as the query to search the public DNA database via the BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the EzBioCloud (http://www.ezbiocloud.net/eztaxon) (Kim et al., 2012). The results showed that strains Q85T and Q86 shared 99.5 % 16S rRNA gene sequence similarity and were closely related to Halorubrum kocurii BG-1T (98.0–98.1 %), Halorubrum aidingense 31-hongT (97.6–97.7 %) and Halorubrum lipolyticum 9-3T (97.5–97.6 %), suggesting that strains Q85T and Q86 belong to the genus Halorubrum.

Comprehensive characterization was performed in accordance with the minimal standards for description of new taxa in the order Halobacteriales (Oren et al., 1997). Colony morphology was observed on agar plates after incubation for 2–3 weeks at 37 °C. Gram-staining was performed according to Dussault (1955). Cell morphology and motility were examined using phase contrast microscopy (Olympus BX51 equipped with Olympus DP72) and scanning electron microscopy (Hitachi SU8010) (Fig. S1). AS-168 medium (Li et al., 2003) used for the determination of general physiological features contained the following ingredients (per litre): 7.5 g Casamino acids (Difco), 10 g yeast extract (Difco), 3 g sodium citrate, 20 g MgSO4 . 7H2O, 2 g KCl, 0.036 g FeCl2 . 4H2O, 0.0036 g MnCl2 . 4H2O and 200 g NaCl. The pH was adjusted to 7.0–7.2. The medium was solidified with 2.0 % agar. According to requirements, the components or pH of the AS-168 medium were modified. The range of salinity for growth was determined by using the growth medium AS-168 containing various concentrations of NaCl (5–35 %, w/v) at intervals of 5 % (w/v). The pH range for growth was adjusted from pH 5.0 to 9.0 at intervals of 0.5 pH units in liquid media with pH buffers (MES pH 5.0–6.0, PIPES pH 6.5–7.0, HEPES pH 7.5–8.0, Tricine pH 8.5 or CHES pH 9.0; all at 50 mM). The temperature range for growth was tested at 10, 15, 20, 25, 30, 34, 38, 40, 42, 45, 50 and 55 °C in medium of pH 7.2 with 20 % (w/v) NaCl. The range of Mg2+ for growth was tested using MgCl2 . 6H2O (0, 0.005, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, 0.7 and 1.0 M). Cells of strains Q85T and Q86 were motile, short rod- or oval-shaped (0.7–1.2 × 0.8–1.4 µm) and Gram-stain-negative. Colonies on solid medium after incubation at 37 °C for 2 weeks were red or pink, smooth, convex, round and 1.0–2.0 mm in diameter. Cell lysis occurred in distilled water. The ranges of NaCl, pH and temperature for growth were 10–30 % (w/v), 6.0–8.5 and 25–50 °C, respectively. Optimal growth occurred with 20 % (w/v) (or 3.4 M) NaCl, at pH 7.5 (for strain Q85T) or pH 6.6 (for strain Q86) and at 42–45 °C. Mg2+ was required for growth, and the Mg2+ optimum was 0.7 M for Q85T and 0.5 M for Q86, the highest Mg2+ requirement thus far observed in the genus Halorubrum.

Tests for catalase and oxidase activities and for hydrolysis of starch, gelatin, casein, and Tweens 80, 60, 40 and 20 were performed as described by Gonzalez et al. (1978). H2S formation was determined based on black sulfide precipitate in the soft agar medium containing 0.5 % (w/v) cysteine. Indole production from tryptophan and the utilization of sugars and organic acids were assessed as described by Oren et al. (1997). Reduction of nitrate and nitrite were detected by using sulfanilic acid and naphthyleamine reagent (Smibert & Krieg, 1994). Anaerobic growth was tested with L-arginine (5 %, w/v), KN03 (5 %, w/v) and DMSO (5 %, v/v) in screw-topped sealed vials. To assess the utilization of sole carbon and energy sources, Casamino acids were omitted, yeast extract was reduced to 0.1 % and 1 % of each of the following substrates was added individually: acetate, L-alanine, L-arginine, L-aspartate, citrate, D-fructose, fumarate, D-galactose, D-glucose, L-glutamate, glycerol, glycine, DL-lactate, lactose, L-lysine, L-malate, maltose, mannitol, D-mannose, L-ornithine, pyruvate, D-ribose, sorbitol, L-sorbitose, starch, succinate, sucrose or D-xylose (Zhang & Cui, 2014). Results of these physiological tests are given in the species description. The differential features between strains Q85T and Q86 and other closely related species are highlighted in Table 1.

To test antibiotic sensitivity, cells of strains Q85T and Q86 were spread onto agar plates. Antibiotic discs containing the following compounds were stuck onto the agar plates (Han & Cui, 2015) (μg per disc, unless indicated otherwise): 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). Strain Q85T was sensitive to novobiocin and bacitracin, but resistant to vancomycin, norfloxacin, ampicillin, neomycin, penicillin G, ciprofloxacin, tetracycline, erythromycin, kanamycin, chloramphenicol, rifampicin and streptomycin. Strain Q86 showed the opposite sensitivity to bacitracin and rifampicin.

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Table 1. Differential characteristics between strain Q85<sup>T</sup>, strain Q86 and closely related species of the genus *Halorubrum*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
<th>Strain 5</th>
<th>Strain 6</th>
<th>Strain 7</th>
<th>Strain 8</th>
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<tr>
<td>Cell size (µm)</td>
<td>1.0–1.2 × 1.3–1.4</td>
<td>0.7–1.0 × 0.8–1.2</td>
<td>0.9–1.0 × 3.6–6.4</td>
<td>&gt;12</td>
<td>0.6–0.6 × 0.9–1.1</td>
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<td>0.9–1.2 × 1.8–5.5</td>
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<td>Short rods or oval</td>
<td>Rods</td>
<td>Pleomorphic rods</td>
<td>Pleomorphic, short rods or oval</td>
<td>Rods</td>
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<td>Motility</td>
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<td>Succinate</td>
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<td>Casein</td>
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<td>Tween 80</td>
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<td>H₂S formation</td>
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<td>Rifampicin (5)</td>
<td>R</td>
<td>H</td>
<td>S</td>
<td>S</td>
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<td>R</td>
<td>S</td>
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<td>Erythromycin (15)</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
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<td>DNA G+C content (mol%)</td>
<td>66.3</td>
<td>66.8</td>
<td>64.2</td>
<td>69.4</td>
<td>65.3</td>
<td>64.6</td>
<td>71.2</td>
<td>65.9</td>
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*Determined in this study.
Polar lipids were extracted using a chloroform-methanol system and detected using two-dimensional TLC on silica gel 60 F254 aluminium-backed thin-layer plates (Merck). The first dimension solvent was chloroform/methanol/water (65 : 25 : 4, v/v), and the second dimension solvent was chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, v/v). The compositions of the polar lipid spots were determined by spraying each plate with specific detection reagents as follows: ethanolic molybdophosphoric acid (5 %, v/v) for total lipids, molybdenum blue for phospholipids and x-naphthol-sulfuric acid for glycolipids (Zhang & Cui, 2014). The polar lipid profiles of strains Q85T and Q86 were identical (Fig. S2), both of them possessing the following major lipids: phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, phosphatidylglycerol and sulfated diglycosyl dietherglycolipids (Fig. S3). The lipid profiles of strains Q85T and Q86 were simple, containing only those basic major lipids described for neutrophilic species of the genus *Halorubrum* (McGenity & Grant, 1995). No new polar lipids were detected, and several polar lipids observed in the type strains of other species of the genus *Halorubrum* were absent from strains Q85T and Q86.

Besides the 16S rRNA gene, another two gene markers, *rpoB* and *ef-2*, were cloned and sequenced for phylogenetic analysis. Primer pair HrpoB2 1420F and HrpoA 153R were used to amplify the *rpoB* gene (Minegishi et al., 2010), while EF-2f and EF-2r were used to amplify the *ef-2* gene (Han & Cui, 2015). Nucleotide *Blast* analysis of the *rpoB* gene showed that strains Q85T and Q86 were almost identical (99.7 % sequence similarity), and were closely related to *H. kocurii* JCM 14978 (95 %), *Halorubrum saccharovorum* JCM 8865 (95 %), *H. lipolyticum* JCM 13559 (95 %) and *Halorubrum trapanicum* JCM 10477 (95 %).

The available 16S rRNA and *rpoB* gene sequences of species in the genus *Halorubrum* were then retrieved from NCBI (http://www.ncbi.nlm.nih.gov/nucleotide/). Multiple sequence alignment of 16S rRNA and *rpoB* gene sequences were performed using the CLUSTAL W program integrated in the BioEdit software (Hall, 1999). Phylogenetic analysis of the 16S rRNA and *rpoB* genes was conducted using the MEGA 5 software (Tamura et al., 2011). Phylogenetic trees were reconstructed using the maximum-likelihood (Fig. 1), neighbour-joining (Fig. S4) and maximum-parsimony (Fig. S5) algorithms (bootstrapping, 1000 replicates) in the MEGA 5 software (Saitou & Nei, 1987; Tamura et al., 2011). The results indicated that strains Q85T and Q86 always clustered together in the phylogenetic trees based on 16S rRNA and *rpoB* genes (Fig. 1). Strains Q85T and Q86 also clustered with *H. lipolyticum*, *Halorubrum lacusprofundi*, *H. kocurii*, *H. saccharovorum* and *Halorubrum halophilum* in both the 16S rRNA and the *rpoB* trees, but formed an independent branch separate from these species in the genus *Halorubrum*.

The G+C content of the genomic DNA was determined by thermal denaturation (Marmur & Doty, 1962), and calculated using the equation of Owen & Pitcher (1985). The DNA G+C content of strains Q85T and Q86 was 66.3 and 66.8 mol%, respectively, values that are within the range reported for the genus *Halorubrum* (60.2–71.2 mol%; Oren et al., 2009).

DNA–DNA hybridizations were performed by using the thermal denaturation and renaturation method (De Ley et al., 1970). The results showed that the level of DNA–DNA relatedness between strains Q85T and Q86 was very high (90 %), while that between Q85T and other related *Halorubrum* strains was quite low: *H. kocurii* BG-1T (29 %), *H. aidingense* 31-hongT (25 %) and *H. lipolyticum* 9-3T (22 %), further indicating that Q85T and Q86 may belong to a novel species of the genus *Halorubrum*.

Interestingly, the level of *ef-2* gene sequence similarity between strain Q86 and strain Q85T was only 92 %, which is less than that between strain Q86 and many other *Halorubrum* species (92–95 %) (data not shown). This result may indicate that the evolutionary rate of the *ef-2* gene is different from those of the 16S rRNA and *rpoB* genes in these two strains, or it may have a different explanation via lateral or horizontal gene transfer. Again, strain Q85T formed an independent branch separated from all other species that constitute the genus *Halorubrum* in the *ef-2* gene tree (Fig. S6).

In conclusion, the results of the phenotypic, phylogenetic and chemotaxonomic analyses indicate that strains Q85T and Q86 belong to the genus *Halorubrum*, and exhibit significant differences compared with other closely related type strains in the genus. Based on data from this polyphasic taxonomic study, Q85T is considered the type strain of a novel species of the genus *Halorubrum*, for which the name *Halorubrum yunnanense* sp. nov. is proposed. As differential characteristics, *ef-2* gene sequence dissimilarity, and optimum pH and Mg2+ concentration for growth between strains Q85T and Q86 were observed, strain Q86 may represent a different subspecies in *Halorubrum yunnanense*.

**Description of *Halorubrum yunnanense* sp. nov.**

*Halorubrum yunnanense* [yun.nan.en’se. N.L. neut. adj. yunnanense from (a salt mine) Yunnan, China, where the type strain was isolated].

Cells are motile, short rod- or oval-shaped (0.7–1.2 × 0.8–1.4 μm) and Gram-stain-negative. Colonies on solid medium after incubation at 37 °C for 2 weeks are red, smooth, convex, round and 1.0–2.0 mm in diameter. Cell lysis occurs in distilled water. Growth occurs in the presence of 1.7–5.1 M NaCl (optimum, 3.4 M), at 25–50 °C (optimum, 42 °C) and at pH 6.0–8.5 (optimum, pH 7.5 for the type strain Q85T and pH 6.6 for strain Q86). Mg2+ is required for growth. The optimum concentration of Mg2+ for growth is 0.7 M for Q85T and 0.5 M for Q86. Oxidase and catalase activities are positive. Anaerobic growth occurs with nitrate, but not with L-arginine or DMSO.
H$_2$S is produced from cysteine; indole is not produced from tryptophan. Nitrate is reduced to nitrite. Starch, gelatin, casein, and Tweens 80, 60, 40 and 20 are not hydrolysed. For the type strain, the following substrates are utilized for growth as sole source of carbon and energy: D-glucose, D-galactose, maltose, sucrose, lactose, mannitol, sorbitol, malate and L-lysine. The following compounds are not utilized: D-mannose, D-fructose, L-sorbose, D-ribose, D-xylose, starch, glycerol, acetate, pyruvate, lactate, succinate, fumarate, citrate, glycine, L-alanine, L-arginine, L-aspartate, L-glutamate and L-ornithine. Glucose, galactose, fructose, sorbose, maltose, sucrose and mannitol are utilized and

Halarobrum litoreum Fa-1$^T$ (EF028067)
Halarobrum distributum JCM 9100$^T$ (D63572)
Halarobrum coriense Ch2$^T$ (L00922)
Halarobrum xinjiangense BD-1$^T$ (AY510707)
Halarobrum trapanicum NRC 34021$^T$ (X82168)
Halarobrum chaoviator Halo-G$^T$ (AM048786)
Halarobrum ezzemoulense 5.1$^T$ (DQ118426)
Halarobrum sodense ATCC 33756$^T$ (D13379)
Halarobrum californiens SF3-213$^T$ (EF139654)
Halarobrum tebenquichense JCM 12290$^T$ (EF468473)
Halarobrum ejinorense EJ-32$^T$ (AM491830)
Halarobrum terrestre VKM B-1739$^T$ (AB090169)
Halarobrum arcis AJ201$^T$ (DQ355793)
Halarobrum salinum GX71$^T$ (HM063951)
Halarobrum laminariae R60$^T$ (KF680551)
Halarobrum saccharovorum JCM 8865$^T$ (U17364)
Halarobrum halophilum B8$^T$ (EF077637)
Halarobrum lipolyticum 9-3$^T$ (DQ355814)
Halarobrum lacusprofundi ATCC 49239$^T$ (NR_028244)
Halarobrum kocurii BG-1$^T$ (AM900832)
Halarobrum aidingense 31-hong$^T$ (DQ355813)
Halarobrum yunnanense Q86 (KJ644186)
Halarobrum yunnanense Q85$^T$ (KJ644187)
Halarobrum orientale EJ-52$^T$ (AM235786)
Halarobrum alkaliphilum DZ-1$^T$ (AY510708)
Halarobrum tibetense BW8$^T$ (AY149598)
Halarobrum luteum CGSA15$^T$ (DQ987877)
Halarobrum vacuolatum JCM 9060$^T$ (D87972)
Halarobrum aquaticum EN-2$^T$ (AM268115)
Halarobrum rubrum YC87$^T$ (JQ237124)
Halarobrum cibi B31$^T$ (EF077639)
Halarobrum aquaticum DC30$^T$ (JF979130)
Halarobrum rubrum JCM 9276$^T$ (AB081732)
Halarobrum cibi B31$^T$ (EF077639)
acids are produced. The polar lipid pattern consists of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol, phosphatidylglycerol sulfate and sulfated diglycosyl dietherglycolipids as major lipids.

The type strain, Q85\textsuperscript{T} (=CGMCC 1.15057\textsuperscript{T} = JCM 30665\textsuperscript{T}), was isolated from a subterranean salt mine in Yunnan province, China. The DNA G+C content of the type strain is 66.3 mol\% (\(T_m\)). Q86 (=CGMCC 1.15058 = JCM 30666)
may represent a different subspecies belonging to the same species.

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References


