Halorubrum aquaticum sp. nov., an archaeon isolated from hypersaline lakes

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Members of the family Halobacteriaceae, the single recognized family within the order Halobacteriales, have long been known as the most abundant microorganisms in hypersaline environments (Oren, 1994). At the time of writing, the family Halobacteriaceae comprises 27 genera (Oren et al., 2009). The genus Halorubrum was established by McGinity & Grant (1995) and currently contains 23 species: four haloalkaliphilic species, Halorubrum vacuolatum (Kamekura et al., 1997), Hrr. tibetense (Fan et al., 2004), Hrr. alkaliphilum (Feng et al., 2005) and Hrr. luteum (Hu et al., 2008), and 19 neutrophilic species, Hrr. trapanicum (Petter, 1931), Hrr. saccharovorum (Tomlinson & Hochstein, 1976), Hrr. sodomense (Oren, 1983), Hrr. distribution (Oren & Ventosa, 1996), Hrr. lacusprofundi (Franzmann et al., 1988), Hrr. coriense (Kamekura & Dyall-Smith, 1995; McGinity & Grant, 1995), Hrr. tebenquichense (Lizama et al., 2002), Hrr. terrestre (Ventosa et al., 2004), Hrr. xinjiangense (Feng et al., 2004), Hrr. ezeemoulense (Kharroub et al., 2006), Hrr. lipolyticum and Hrr. aegingense (Cui et al., 2006), Hrr. orientale (Castillo et al., 2006), Hrr. arcis (Xu et al., 2007), Hrr. litoreum (Cui et al., 2007), Hrr. ejinorense (Castillo et al., 2007), Hrr. kocurii (Gutiérrez et al., 2008), Hrr. californiense (Pesenti et al., 2008) and Hrr. cibi (Roh & Bae, 2009).

In the present study, we characterized two strains, EN-2¹ and SH-4, which were isolated from water samples of the saline lakes Erliannor (43° 44′ N 112° 02′ E) and Shangmatala (43° 12′ N 114° 01′ E), respectively, located in the Inner Mongolia Autonomous Region, China. At the time of sampling, the temperatures and pH of the lakes were 18.4 °C and 19.8 °C and pH 7.5 and 8.0, respectively. Water samples were plated on agar plates of halophilic medium (MH), containing (per litre distilled water) 195 g NaCl, 32.5 g MgCl₂·6H₂O, 50.8 g MgSO₄·7H₂O, 0.8 g CaCl₂, 5 g KCl, 0.16 g NaHCO₃, 0.6 g NaBr, 5 g yeast extract and 20 g agar (pH 7.5), and incubated at 37 °C for 1–2 weeks. Pure culture was obtained by repeated subcultivation on the same medium.

For comparative purposes, the following strains were obtained from the Japan Collection of Microorganisms (JCM) and the Spanish Type Culture Collection (CECT): Hrr. cibi JCM 15757T, Hrr. alkaliphilum JCM 12358T, Hrr. lipolyticum 9-3T, Hrr. luteum CECT 7303T, Hrr. kocurii JCM 14978T and Hrr. tibetense JCM 11889T. The Hrr. cibi, Hrr. luteum...
Hrr. kocurii and Hrr. lipolyticum strains were routinely cultivated on JCM medium no. 168, Hrr. tibetense JCM 11889T and Hrr. alkaliphilum JCM 12358T were cultivated on JCM medium no. 167 and Hrr. luteum CECT 7303T was cultivated on CECT medium no. 248.

Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa of the order Halobacteriales (Oren et al., 1997). Motility and morphology of cells from exponentially growing liquid cultures were examined using an Olympus BX41 microscope equipped with phase-contrast optics. Cells of strains EN-2T and SH-4 were motile rods (Supplementary Fig. S1, available in IJSEM Online). Colony morphology, colour and size were observed by growth on MH agar after incubation at 37 °C for 10 days. Growth with 0, 0.5, 1, 3, 5, 7, 10, 15, 20, 25 and 30% (w/v) NaCl was determined on MH agar. Growth at pH 5.0–11.0 (at intervals of 0.5 pH unit) was determined in liquid MH with the pH readjusted after sterilization and growth was assessed by measuring the optical density at 600 nm. The temperature range for growth was assessed on MH agar at 15–55 °C (at intervals of 5 °C). Tests for catalase and oxidase, hydrolysis of starch, Tween 80, gelatin, casein, DNA and aesculin, reduction of nitrate and nitrite, formation of H2S and the optical density at 600 nm. The temperature range for growth with 0, 0.5, 1, 3, 5, 7, 10, 15, 20, 25% (w/v) NaCl was determined on MH agar. Growth at pH 5.0–11.0 (at intervals of 0.5 pH unit) was determined in liquid MH with the pH readjusted after sterilization and growth was assessed by measuring the optical density at 600 nm. The temperature range for growth with 0, 0.5, 1, 3, 5, 7, 10, 15, 20, 25% (w/v) NaCl was determined on MH agar. Growth at pH 5.0–11.0 (at intervals of 0.5 pH unit) was determined in liquid MH with the pH readjusted after sterilization and growth was assessed by measuring the optical density at 600 nm. The temperature range for growth was assessed on MH agar at 15–55 °C (at intervals of 5 °C). Tests for catalase and oxidase, hydrolysis of starch, Tween 80, gelatin, casein, DNA and aesculin, reduction of nitrate and nitrite, formation of H2S and indole and utilization of sugars, alcohols, amino acids and organic acids were carried out as described by Oren et al. (1997). Susceptibility to antibiotics was determined on MH agar using antibiotic discs containing (μg per disc unless otherwise stated): ampicillin (10) bacitracin (10 U), chloramphenicol (30), erythromycin (15), nalidixic acid (30), neomycin (30), novobiocin (30), rifampicin (5) and streptomycin (10). The physiological and biochemical characteristics as well as the antibiotic susceptibilities of strains EN-2T and SH-4 are provided in the species description and Table 1.

Polar lipids of both isolates were extracted with chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.). Glycolipids were detected as purple spots by spraying with 0.5 % a-naphthol solution and 0.5 % phosphomolybdic acid. Phosphatidylglycerol sulfate was detected as a dark green black spot after spraying with 0.5% phosphomolybdic acid. Phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, sulfated diglycosyl diethers and several unidentified glycolipids. Phosphatidylglycerol sulfate was not detected. This polar lipid profile is typical of neutrophilic species of the genus Halorubrum (McGenity & Grant, 2001).

Chromosomal DNA of strains EN-2T and SH-4 was isolated and purified according to the method described by Marmur (1961). The G+C content of genomic DNA was determined from the mid-point (Tm) of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979) as described previously (Ventosa et al., 2004). The DNA G+C content of strains EN-2T and SH-4 was 62.1 mol%. This value is within the range described for the genus Halorubrum (60.0–71.2 mol%; Grant et al., 2001). The 16S rRNA genes of strains EN-2T and SH-4 were sequenced by PCR using universal primers as described elsewhere (López-García et al., 2001; Arahal et al., 1996). The almost-complete 16S rRNA gene sequences of strains EN-2T (1420 bp) and SH-4 (1381 bp) were determined. ARB software (Ludwig et al., 2004) was used for sequence analysis. Following the

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**Table 1. Characters that differentiate Halorubrum aquaticum sp. nov. from other closely related species of the genus Halorubrum**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<td>Motility</td>
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<td>Colony colour†</td>
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<td>Mg2⁺ requirement</td>
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<td>Nitrate reduction</td>
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<td>+†</td>
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<td>Indole production</td>
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<td>Oxidase</td>
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<td>–</td>
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<td>Succinate</td>
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<td>Bacitracin (10 U)</td>
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<td>+</td>
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<td>Erythromycin (15 μg)</td>
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<td>ND</td>
<td>+</td>
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<td>–</td>
<td>+</td>
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<td>Rifampicin (5 μg)</td>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>62.1</td>
<td>61.7</td>
<td>63.3</td>
<td>62.1</td>
<td>60.2</td>
<td>65.9</td>
<td>60.9</td>
<td>61.2</td>
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</tbody>
</table>

*pl, Pleomorphic; RO, rods; SR, short rods.
†Data obtained in this study.
‡O, Orange; PK, pink; RE, red.
recommendations of Ludwig et al. (1998), phylogenetic
trees were generated using alternative treeing methods
(maximum parsimony, neighbour joining and maximum
likelihood) (Saitou & Nei, 1987). Base-frequency filters
were applied in the sequence comparison analysis and the
effects on the results were evaluated. The topologies of
the neighbour-joining and maximum-likelihood trees were
similar to that of the maximum-parsimony tree (not
shown). The identification of phylogenetic neighbours and
calculation of pairwise sequence similarity were achieved
using the EzTaxon server (http://www.eztaxon.org/; Chun
et al., 2007). The maximum-parsimony phylogenetic tree
(Fig. 1) indicated that strains EN-2T and SH-4 were closely
related to Hrr. cibi B31T (97.9 and 98.0 % 16S rRNA gene
sequence similarity, respectively), Hrr. tibetense 8W8T
(97.3 and 97.7 %), Hrr. alkaliphilum DZ-1T (96.8 and 97.1 %),
Hrr. luteum CGSA15T (96.8 and 97.0 %) and Hrr.
lipolyticum JCM 13559T (96.8 and 97.0 %).

DNA–DNA hybridization between strains EN-2T and SH-4
and the phylogenetically most closely related type strains of
species of the genus Halorubrum was performed by the
competition procedure of Johnson (1994), as described in
detail by Gutiérrez et al. (2002). DNA–DNA relatedness
between strains EN-2T and SH-4 was 98 and 95 %
(reciprocal hybridizations). These values showed that the
two isolates can be considered to represent the same species
(Stackebrandt et al., 2002). DNA–DNA relatedness
between strains EN-2T and SH-4 and Hrr. cibi JCM
15757T, Hrr. tibetense JCM 11889T, Hrr. alkaliphilum JCM
12358T, Hrr. luteum CECT 7303T and Hrr. lipolyticum
JCM 13559T was 38 and 27 %, 45 and 39 %, 37 and 43 %,
23 and 30 % and 25 and 18 %, respectively. These levels of
DNA–DNA relatedness are low enough to classify the two
isolates in a genotypically distinct species within the genus
Halorubrum.

Differences in phenotypic characteristics (such as mot-
ility, oxidase, reduction of nitrate and nitrite, hydrolysis
of different compounds and utilization of several
substrates) (Table 1), polar lipid profiles and 16S rRNA
gene sequences, together with the DNA–DNA hybridiza-
tion data, justify the creation of a novel species within
the genus Halorubrum to accommodate strains EN-2T
and SH-4 (Wayne et al., 1987; Stackebrandt & Goebel,
1994). The name Halorubrum aquaticum sp. nov. is proposed.

**Description of Halorubrum aquaticum sp. nov.**

*Halorubrum aquaticum* (aqua’ticum. L. neut. adj. aqua-
ticum living, growing or found in or by water, aquatic).

Cells are rods (1.0–1.2 × 2.0–6.0 μm). Non-motile. Colo-
nies are circular, entire, smooth, red and 0.5–1.5 mm in
diameter on MH agar after 7 days at 37 °C. Growth occurs
with 2.5–5.0 M NaCl (optimum 3.4 M NaCl). MgCl₂
is not required. Strict aerobe. Growth occurs at 25–50 °C
(optimum 35–40 °C) and pH 6.0–9.0 (optimum pH 7.5).
Chemo-organotrophic. Oxidase-negative and catalase-
positive. Nitrate and nitrite are reduced. Tween 80, urea
and aesculin are hydrolysed, but casein, DNA, starch
and gelatin are not. H₂S is not produced. Indole is not
produced from tryptophan. Methyl red and Voges–
Proskauer tests are negative. Arginine dihydrolase, lysine
decarboxylase and ornithine decarboxylase are not pro-
duced. Anaerobic growth with nitrate or arginine does
not occur. Acid is not produced from lactose, glycerol,
D-glucose, D-fructose, D-arabinose, maltose, D-xylene,
D-galactose, trehalose or D-mannose. Growth occurs with glycerol, D-mannitol, D-fructose, lactose, maltose, sucrose, trehalose, D-glucose, D-arabinose, starch, L-glutamate and fumarate as single carbon and energy sources, but not with D-sorbitol, mannose, raffinose, D-ribose, D-xylene, succinate, propionate, malate or acetate. Growth occurs with glycine as a sole carbon, nitrogen and energy source, but not with L-asparagine, L-lysine, L-serine or L-threonine. Susceptible to (μg per disc unless otherwise stated) novobiocin (30), bacitracin (10 U), erythromycin (15) and streptomycin (10) and resistant to rifampicin (5), ampicillin (10), neomycin (30), chloramphenicol (30) and nalidixic acid (30). The polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, sulfated diglycerol diethers and several unidentified glycolipids. The DNA G+C content of the type strain is 62.1 mol% (Tm).

The type strain, EN-2T (=CECT 7174T =CGMCC 1.6377T =JCM 14031T), was isolated from a saline lake, Lake Erlian nor, Inner Mongolia, China. Strain SH-4, from a similar source, is a second strain of the species.

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References


