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

M. C. Gutiérrez,1 A. M. Castillo,1 P. Corral,1 M. Kamekura2 and A. Ventosa1

1Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain
2Halophiles Research Institute, 677-1 Shimizu, Noda-shi, Chiba-ken 278-0043, Japan

Two halophilic archaea, strains EN-2T and SH-4, were isolated from the saline lakes Erliannor and Shangmatala, respectively, in Inner Mongolia, China. Cells were strictly aerobic, motile rods. Colonies were red. Strains EN-2T and SH-4 were able to grow at 25–50 °C (optimum 35–40 °C), with 2.5–5.0 M NaCl (optimum 3.4 M NaCl) and at pH 6.0–9.0 (optimum pH 7.5). MgCl2 was not required for growth. Cells lysed in distilled water and the lowest NaCl concentration that prevented cell lysis was 12 % (w/v). On the basis of the 16S rRNA gene sequence analysis, strains EN-2T and SH-4 were closely related to Halorubrum cibi B31T (97.9 and 98.0 % similarity, respectively), Hrr. tibetense BW8T (97.3 and 97.7 %), Hrr. alkaliphilum DZ-1T (96.8 and 97.1 %), Hrr. lutum CGSA15T (96.8 and 97.0 %) and Hrr. lipolyticum 9-3T (96.8 and 97.0 %). DNA–DNA hybridization showed that strains EN-2T and SH-4 did not belong to the same species as any of these strains (<45 % DNA–DNA relatedness) but that they are members of the same species (>70 % DNA–DNA relatedness). Polar lipid analysis revealed that strains EN-2T and SH-4 contained phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, sulfated diglycosyl diethers and several unidentified glycolipids. The DNA G+C content of both isolates was 62.1 mol%. It was concluded that strains EN-2T and SH-4 represent a novel species of the genus Halorubrum, for which the name Halorubrum aquaticum sp. nov. is proposed. The type strain is EN-2T (=CECT 7174T =CGMCC 1.6377T =JCM 14031T).

Hrr. cibi (Pesenti et al., 2006), Hrr. cibi (Castillo 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-2T 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. lutum CECT 7303T, Hrr. kocurii JCM 14978T and Hrr. tibetense JCM 11889T. The Hrr. cibi, 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).

Members of the family Halobacteriaceae, the single recognized family within the order Halobacterales, have long been known as the most abundant micro-organisms 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 McGenity & Grant (1995) and currently contains 23 species: four haloalkaliphilic species, Halorubrum vaculatum (Kamekura et al., 1997), Hrr. tibetense (Fan et al., 2004), Hrr. alkaliphilum (Feng et al., 2005) and Hrr. lutum (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 (Frimannsdóttir et al., 1988), Hrr. corense (Kamekura & Dyall-Smith, 1995; McGenity & Grant, 1995), Hrr. tebenquichense (Lizama et al., 2002), Hrr. terrestre (Ventosa et al., 2004), Hrr. xinjiangense (Feng et al., 2004), Hrr. ezeemouense (Kharroub et al., 2006), Hrr. lipolyticum and Hrr. aidingense (Cui et al., 2006), Hrr. orientale (Castillo et al., 2006), Hrr. arcis (Xu et al., 2007), Hrr. alkaliphilum (Feng et al., 2005) and Hrr. lutum (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 (Frimannsdóttir et al., 1988), Hrr. corense (Kamekura & Dyall-Smith, 1995; McGenity & Grant, 1995), Hrr. tebenquichense (Lizama et al., 2002), Hrr. terrestre (Ventosa et al., 2004), Hrr. xinjiangense (Feng et al., 2004), Hrr. ezeemouense (Kharroub et al., 2006), Hrr. lipolyticum and Hrr. aidingense (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).

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain EN-2T is AM268115.

Two supplementary figures are available with the online version of this paper.
**Hrr. kocurii** and **Hrr. lipolyticum** strains were routinely cultivated on JCM medium no. 168, **Hrr. tibetense** JCM 11889^T^ and **Hrr. alkaliphilum** JCM 12358^T^ were cultivated on JCM medium no. 167 and **Hrr. luteum** CECT 7303^T^ 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 **Halobacteria** (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-2^T^ 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 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-2^T^ and SH-4 are provided in the species description and Table 1.

Polar lipids of both isolates were extracted with chloroform/methanol as described previously (Kamekura, 1993) and separated by TLC using Merck HPTLC silica gel 60 plates (no. 5641) and chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.). Glycolipids were detected as purple spots by spraying with 0.5 % naphthol in methanol/water (1:1) and then with sulfuric acid/ethanol (1:1), followed by heating at 160 °C. The polar lipid profiles of the isolates and the reference strains are shown in Supplementary Fig. S2. Strains EN-2^T^ and SH-4 contained phosphatidylglycerol, phosphatidylglycerol phosphate, unsaturated 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-2^T^ 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 (T_m) 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-2^T^ 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-2^T^ 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-2^T^ (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>
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<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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*PL, Pleomorphic; RO, rods; SR, short rods.
†Data obtained in this study.
‡O, Orange; PK, pink; RE, red.

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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-2^T and SH-4 were closely related to \textit{Hr. cibi} B31^T (97.9 and 98.0\% 16S rRNA gene sequence similarity, respectively), \textit{Hr. tibetense} 8W8^T (97.3 and 97.7\%), \textit{Hr. alkaliphilum} DZ-1^T (96.8 and 97.1\%), \textit{Hr. luteum} CGSA15^T (96.8 and 97.0\%) and \textit{Hr. lipolyticum} JCM 13559^T (96.8 and 97.0\%).

DNA–DNA hybridization between strains EN-2^T and SH-4 and the phylogenetically most closely related type strains of species of the genus \textit{Halorubrum} was performed by the competition procedure of Johnson (1994), as described in detail by Gutierrez et al. (2002). DNA–DNA relatedness between strains EN-2^T 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-2^T and SH-4 and \textit{Hr. cibi} JCM 15757^T, \textit{Hr. tibetense} JCM 11889^T, \textit{Hr. alkaliphilum} JCM 12358^T, \textit{Hr. luteum} CECT 7303^T and \textit{Hr. lipolyticum} JCM 13559^T 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 \textit{Halorubrum}.

Differences in phenotypic characteristics (such as motility, 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 hybridization data, justify the creation of a novel species within the genus \textit{Halorubrum} to accommodate strains EN-2^T and SH-4 (Wayne et al., 1987; Stackebrandt & Goebel, 1994). The name \textit{Halorubrum aquaticum} sp. nov. is proposed.

**Description of \textit{Halorubrum aquaticum} sp. nov.**

\textit{Halorubrum aquaticum} (a.qua’ti.cum. L. neut. adj. aquaticum living, growing or found in or by water, aquatic).

Cells are rods (1.0–1.2 × 2.0–6.0 μm). Non-motile. Colonies are circular, entire, smooth, red and 0.5–1.5 mm in diameter on MH agar after 7 days at 37 \degree 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 \degree C (optimum 35–40 \degree 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 produced. 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-xylose, 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 diglycosyl diethers and several unidentified glycolipids. The DNA G+C content of the type strain is 62.1 mol% (Tm).

The type strain, EN-2^T (=CECT 7174^T =CGMCC 1.6377^T =JCM 14031^T), was isolated from a saline lake, Lake Erliannor, in Inner Mongolia, China. Strain SH-4, from a similar source, is a second strain of the species.

Acknowledgements

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References


