

Halorubrum kocurii sp. nov., an archaeon isolated from a saline lake

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A Gram-negative, non-motile, neutrophilic, rod-shaped, extremely halophilic archaeon, designated strain BG-1^T, was isolated from a salt lake, Lake Bagaejinnor, in Inner Mongolia, China. Strain BG-1^T was able to grow at 25–55 °C, required at least 2.5 M NaCl for growth (with an optimum at 3.4 M NaCl) and grew at pH 6.0–9.0 (with an optimum at pH 7.5). Hypotonic treatment with less than 2.0 M NaCl caused cell lysis. Phylogenetic analysis of the almost-complete 16S rRNA gene sequence positioned the isolate within the genus *Halorubrum* in the family *Halobacteriaceae*. Strain BG-1^T was most closely related to *Halorubrum aidingense* 31-hong^T (98.8% sequence similarity), *Halorubrum saccharovororum* NCIMB 2081^T (98.6%), *Halorubrum lacusprofundi* ACAM 34^T (98.6%) and *Halorubrum lipolyticum* 9-3^T (98.4%). However, values for DNA–DNA hybridization between strain BG-1^T and the most closely related members of the genus *Halorubrum* were below 40%. Analysis of the polar lipids of strain BG-1^T revealed the presence of mannosyl-2-sulfate-(1-4)-glycosyl-archaeol, the main glycolipid found in neutrophilic species of the genus *Halorubrum*. The G + C content of the genomic DNA was 69.4 mol% (T_m). Comparison of the phenotypic characteristics of the strain with those of *Halorubrum* species supported the conclusion that BG-1^T represents a novel species within this genus, for which the name *Halorubrum kocurii* sp. nov. is proposed. The type strain is BG-1^T (=CECT 7322^T =CGMCC 1.7018^T =JCM 14978^T).

Aerobic, extremely halophilic archaea typically comprise red-pigmented micro-organisms that belong to the family *Halobacteriaceae* (Grant *et al.*, 2001). They are the most halophilic organisms known and are predominant in hypersaline environments in which the salt concentration exceeds 250 g l⁻¹ (Rodríguez-Valera *et al.*, 1981; Ventosa, 2006). Cell densities may be sufficiently high in hypersaline brines to impart a red colour. Recent studies based on phylogenetic analyses of 16S rRNA genes have revealed a

very high taxonomic diversity at both the genus and species level within the family *Halobacteriaceae* (Grant *et al.*, 2001; Ventosa, 2006). At the time of writing, the family *Halobacteriaceae* includes 26 genera and 85 species (Oren *et al.*, 2007).

The genus *Halorubrum* was originally proposed by McGenity & Grant (1995) to accommodate several species previously included in the genus *Halobacterium*. Currently, the genus *Halorubrum* comprises 19 species with validly published names: *Halorubrum saccharovororum* (Tomlinson & Hochstein, 1976), *Hrr. trapanicum* (Petter, 1931), *Hrr. sodomense* (Oren, 1983), *Hrr. lacusprofundi* (Franzmann *et al.*, 1988), *Hrr. coriense* (Oren & Ventosa, 1996), *Hrr. distributum* (Oren & Ventosa, 1996), *Hrr. vacuolatum*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BG-1^T is AM900832.

A thin-layer chromatogram of polar lipids extracted from strain BG-1^T and some other haloarchaea is available as supplementary material with the online version of this paper.

(Kamekura *et al.*, 1997), *Hrr. tebenquichense* (Lizama *et al.*, 2002), *Hrr. tibetense* (Fan *et al.*, 2004), *Hrr. terrestre* (Ventosa *et al.*, 2004), *Hrr. xinjiangense* (Feng *et al.*, 2004), *Hrr. alkaliphilum* (Feng *et al.*, 2005), *Hrr. ezzemoulense* (Kharroub *et al.*, 2006), *Hrr. orientale* (Castillo *et al.*, 2006), *Hrr. lipolyticum* (Cui *et al.*, 2006), *Hrr. aidingense* (Cui *et al.*, 2006), *Hrr. arcis* (Xu *et al.*, 2007), *Hrr. litoreum* (Cui *et al.*, 2007) and *Hrr. ejinorensis* (Castillo *et al.*, 2007). The aim of this study was to describe a novel non-alkaliphilic halophilic archaeon isolated from Lake Bagaejinnor in Inner Mongolia, China. We propose that it represents a novel species of the genus *Halorubrum*. This archaeon is the host for a halovirus, designated BJ1, the complete genomic sequence of which has been reported recently (Pagaling *et al.*, 2007).

Strain BG-1^T was isolated from a sediment and water sample from a saline lake, Lake Bagaejinnor (45° 09' N 116° 36' E). At the time of sampling (September 2003), the water of this lake had a temperature of 20.5 °C, a pH of 8.5 and a conductivity of 146.7 mS cm⁻¹. Approximately 0.5 g of the sample was dissolved in the medium and serially diluted; 100 µl of each dilution was plated on plates with solid isolation medium containing the following (l⁻¹): yeast extract, 10 g; Casamino acids, 7.5 g; NaCl, 130 g; MgCl₂·7H₂O, 71.16 g; Na₂SO₄, 45.6 g; trisodium citrate, 3 g; KCl, 2.48 g; Na₂B₄O₇, 1.62 g; NaBr, 0.84 g; NaHCO₃, 0.62 g; Na₂CO₃, 0.36 g; the pH was adjusted to 8.0. Strain BG-1^T was isolated and a pure culture was obtained after several transfers on the same medium.

Strain BG-1^T grew at temperatures in the range 25–55 °C (optimally at 37 °C) and at pH 6.0–9.0 (optimally at pH 7.5). Routine cultivation was conducted at 37 °C and pH 7.5. NaCl and magnesium requirements for growth were tested in media with 1.0–5.2 M NaCl or 0–0.5 M MgCl₂. Strain BG-1^T was capable of growth over a wide range of NaCl concentrations, ranging from 2.5 M (approx. 15%) to 5.0 M (approx. 30%). It grew optimally in the presence of 3.4 M (20%) NaCl, as has been shown for most extremely halophilic archaea (Grant *et al.*, 2001). MgCl₂ was not required for growth.

Phenotypic characterization was carried out in accordance with the recommended minimal standards for the description of novel taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Anaerobic growth was tested in the presence of 5% nitrate and 3% L-arginine in filled, stoppered tubes (Oren *et al.*, 1997). The formation of acid from different sugars was tested in medium with 0.05% (w/v) yeast extract and supplemented with 1% (w/v) of the sugar tested (sterilized separately). Strain BG-1^T was oxidase- and catalase-positive. The following tests produced negative results: methyl red, Voges–Proskauer, indole production from tryptone and Simmons' citrate. The strain was positive for nitrate reduction. Casein, aesculin, DNA and starch were not hydrolysed. The urease test was positive. Other phenotypic characteristics of strain BG-1^T are summarized in Table 1 and the species description.

Table 1. Differentiation of strain BG-1^T from closely related species of the genus *Halorubrum*

Taxa: 1, strain BG-1^T; 2, *Hrr. saccharovorum* NCIMB 2081^T; 3, *Hrr. aidingense* JCM 13560^T; 4, *Hrr. lacusprofundi* ACAM 34^T; 5, *Hrr. lipolyticum* JCM 13559^T. Data are from this study, McGenity & Grant (2001) and Cui *et al.* (2006). +, Positive; –, negative; w, weak; ND, no data available.

Characteristic	1	2	3	4	5
Motility	–	+	+	+	+
Mg ²⁺ required	–	+	–	+	+
H ₂ S formation	–	–	+	–	+
Nitrate reduction	+	–	+	w	+
Indole formation	–	–	+	–	–
Starch hydrolysis	–	–	–	–	+
Urease	+	–	ND	–	ND
Gelatin liquefaction	–	–	–	–	+
Utilization of:					
D-Glucose	–	+	+	+	+
D-Galactose	–	–	+	+	+
D-Mannose	–	+	+	+	+
D-Ribose	–	–	–	+	–
D-Xylose	+	+	–	–	–
Acetate	–	+	+	+	–
DNA G + C content (mol%)	69.4	71.2	64.2	65.3	65.9

To determine the utilization of different organic substrates as carbon and energy sources or as carbon, nitrogen and energy sources, a medium containing yeast extract at 0.05% (w/v), supplemented with 1% (w/v) of the tested substrate, was used (Torreblanca *et al.*, 1986). Susceptibility to antibiotics was determined on agar plates by using antibiotic discs with the following amounts: ampicillin (10 µg), bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg); gentamicin (10 µg), nalidixic acid (30 µg), neomycin (10 µg), novobiocin (30 µg), penicillin G (10 U), rifampicin (30 µg), streptomycin (10 µg) and tetracycline (30 µg). The results for the utilization of different substrates and for antibiotic susceptibility are included in the species description.

Cell morphology and motility were examined using an Olympus BX41 microscope equipped with phase-contrast optics. Cells were non-motile and rod-shaped (Fig. 1). Colony morphology was observed on solid medium under optimal growth conditions after incubation at 37 °C for 5 days. Colonies of strain BG-1^T that formed on agar plates were circular, smooth, entire, opaque, red-pigmented and 0.5–1.5 mm in diameter.

Polar lipids were extracted with chloroform/methanol as described previously (Kamekura, 1993). TLC was done using Merck HPTLC silica gel 60 plates (art. 5641) in the solvent system, chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.). Glycolipids were detected as purple spots by spraying with 0.5% α-naphthol in

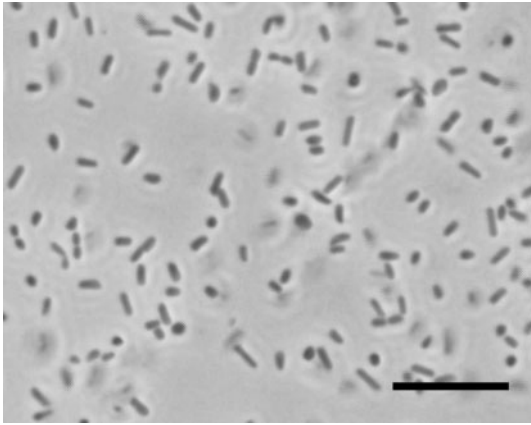


Fig. 1. Phase-contrast micrograph of cells of strain BG-1^T grown in liquid medium under optimal conditions. Bar, 15 μm.

methanol/water (1:1) and then with sulfuric acid/ethanol (1:1), followed by heating at 160 °C. The polar lipids of strain BG-1^T are C₂₀C₂₀ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and a sulfated diglycosyl archaeol [mannosyl-2-sulfate-(1-4)-glycosyl-archaeol] (see Supplementary Fig. S1, available in IJSEM Online). This polar lipid profile has been shown to be a distinctive feature of non-alkaliphilic species of the genus *Halorubrum* (McGenity & Grant, 2001).

Chromosomal DNA of strain BG-1^T 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). The DNA G+C content of strain BG-1^T was 69.4 mol%. The 16S rRNA gene of strain BG-1^T was amplified using a PCR with three universal primer sets, as described previously (Lopez-Garcia *et al.*, 2001; Arahall *et al.*, 1996) and the

almost-complete nucleotide sequence (1369 bp) was determined. The ARB software package (Ludwig *et al.*, 2004) was used for 16S rRNA gene sequence analysis. Base-frequency filters were applied in the sequence-comparison analysis and the effects on the results were evaluated. In the phylogenetic tree constructed with the maximum-parsimony method (Saitou & Nei, 1987), strain BG-1^T clustered with the type strains of species of the genus *Halorubrum* (Fig. 2). The 16S rRNA gene sequence similarities between strain BG-1^T and the most closely related *Halorubrum* type strains, *Hrr. aidingense* 31-hong^T, *Hrr. saccharovororum* NCIMB 2081^T, *Hrr. lacusprofundi* ACAM 34^T and *Hrr. lipolyticum* 9-3^T, were 98.8, 98.6, 98.6 and 98.4 %, respectively. Similar tree topologies were obtained when other treeing methods (neighbour joining and maximum likelihood) were used.

To verify the species status of strain BG-1^T, DNA–DNA hybridization studies were carried out with the type strains of the most closely related species of *Halorubrum*. These studies were performed using the competition procedure of the membrane method (Johnson, 1994) as described in detail by Ventosa *et al.* (2004). The levels of DNA–DNA relatedness for strain BG-1^T with respect to *Hrr. aidingense* JCM 13560^T, *Hrr. saccharovororum* NCIMB 2081^T, *Hrr. lipolyticum* JCM 13559^T and *Hrr. lacusprofundi* ACAM 34^T were 27, 28, 36 and 39 %, respectively. These data indicate that strain BG-1^T represents a novel species of the genus *Halorubrum*, having DNA–DNA hybridization values <70 % with respect to members of recognized species (Stackebrandt & Goebel, 1994). In addition, several phenotypic differences were observed between strain BG-1^T and the most closely related *Halorubrum* species, including those concerning motility, urease production and the utilization of glucose and other sugars (Table 1).

Therefore, on the basis of the data from this polyphasic study, strain BG-1^T represents a novel species of the genus *Halorubrum*, for which the name *Halorubrum kocurii* sp. nov. is proposed.

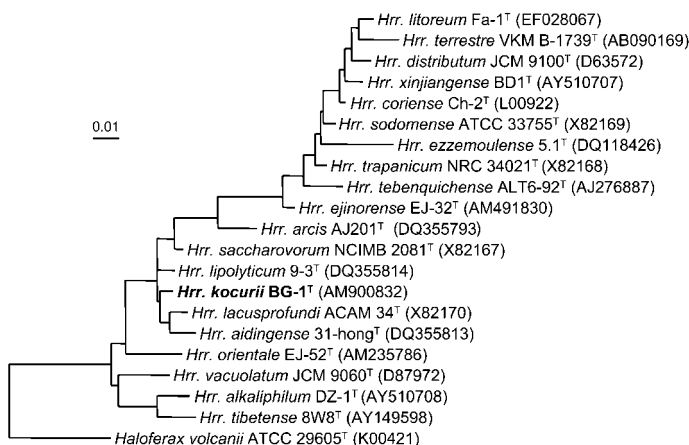


Fig. 2. Maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequence comparison, showing the position of strain BG-1^T among the species of the genus *Halorubrum*. EMBL accession numbers are given in parentheses. Bar, 1 % sequence divergence.

Description of *Halorubrum kocurii* sp. nov.

Halorubrum kocurii (ko.cu'ri.i. N.L. gen. masc. n. *kocurii* of Kocur, named for the Czech microbiologist M. Kocur, a pioneer in the study of halophilic archaea and bacteria).

Cells are non-motile, rod-shaped ($2\text{--}5 \times 0.9\text{--}1.1 \mu\text{m}$) (Fig. 1) and Gram-negative. Colonies are circular, smooth, entire, opaque, red-pigmented and 0.5–1.5 mm in diameter after 5 days at 37 °C on plates containing 20% (w/v) total salts. Cells are extremely halophilic and lyse in water. At least 2.5 M NaCl is required for growth, the optimum concentration being 3.4 M NaCl. MgCl_2 is not required for growth. The pH and temperature ranges for growth are pH 6.0–9.0 (optimum, pH 7.5) and 25–55 °C (optimum, 37 °C). Chemo-organotrophic, aerobic and oxidase- and catalase-positive. Indole is not produced from tryptophan. Methyl red, Voges–Proskauer and Simmons' citrate tests produce negative results. Acid is not produced from lactose, glycerol, D-glucose, sucrose, D-fructose, D-arabinose, maltose, D-galactose, trehalose or D-mannose. Does not grow anaerobically in the presence of nitrate or L-arginine. Starch, gelatin, DNA, aesculin and casein are not hydrolysed. Urea is hydrolysed. Does not produce arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Nitrate is reduced to nitrite; gas is not produced from nitrite. Utilizes D-xylose. No growth is observed on D-glucose, D-fructose, glycerol, maltose, trehalose, starch, propionate, fumarate, acetate, L-lysine, D-mannitol, D-sorbitol, lactose, D-arabinose, D-galactose, D-mannose, raffinose, D-ribose, malate, succinate, glutamate, isoleucine, L-serine or glycine. Sensitive to bacitracin, novobiocin and rifampicin. Resistant to ampicillin, chloramphenicol, erythromycin, gentamicin, nalidixic acid, neomycin, penicillin G, streptomycin and tetracycline. The polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and the glycolipid mannosyl-2-sulfate-(1-4)-glycosyl-archaeol. The DNA G + C content is 69.4 mol% (T_m).

The type strain, BG-1^T (=CECT 7322^T =CGMCC 1.7018^T =JCM 14978^T), was isolated from saline Lake Bagaejinnor in Inner Mongolia, China.

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