Halorubrum ejinorensense sp. nov., isolated from Lake Ejnor, Inner Mongolia, China

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A novel halophilic archaeon, strain EJ-32^T, was isolated from water from Lake Ejnor in Inner Mongolia, China. The taxonomy of strain EJ-32^T was studied by using a polyphasic approach. On the basis of 16S rRNA gene sequence similarities, strain EJ-32^T was shown to be phylogenetically related to *Halorubrum coriense* (97.9%), *Halorubrum trapanicum* (97.9%), *Halorubrum sodomense* (97.8%), *Halorubrum tebenquichense* (97.8%), *Halorubrum xinjiangense* (97.6%), *Halorubrum terrestre* (97.4%), *Halorubrum distributum* (97.1%) and *Halorubrum saccharovororum* (96.4%). Strain EJ-32^T was found to be neutrophilic, non-motile and Gram-negative. It grew in medium containing saturation concentrations of NaCl and did not require magnesium for optimal growth. The G+C content of the DNA is 64.0 mol%. Values for DNA–DNA hybridization with respect to phylogenetically related *Halorubrum* species were ≤49%, indicating that EJ-32^T constitutes a different genospecies. The data show that strain EJ-32^T represents a novel species of the genus *Halorubrum*, for which the name *Halorubrum ejinorensense* sp. nov. is proposed. The type strain is EJ-32^T (=CECT 7194^T=CGMCC 1.6782^T=JCM 14265^T).

Hypersaline ecosystems such as salt lakes, soda lakes, solar salterns and saline soils show great variability in total salt concentration, ionic composition and pH (Rodríguez-Valera, 1988; Oren, 2002). Members of the family *Halobacteriaceae* are ubiquitous in these hypersaline environments. They are aerobic or facultatively anaerobic, red-pigmented due to the presence of carotenoid pigments (except for a few species), chemo-organotrophic archaea requiring at least 1.5 M NaCl for growth (Grant *et al.*, 2001; Ventosa, 2006). The halophilic archaea have been classified into 23 genera on the basis of 16S rRNA gene sequence comparisons and chemotaxonomic criteria, particularly polar lipid composition, but there is no strict correlation between the two (Ventosa, 2006).

At the time of writing the genus *Halorubrum* (McGenity & Grant, 1995, 2001) contained 16 recognized species: *Halorubrum aidingense* (Cui *et al.*, 2006), *Halorubrum alkaliphilum* (Feng *et al.*, 2005), *Halorubrum coriense* (Kamekura & Dyall-Smith, 1995; Oren & Ventosa, 1996), *Halorubrum distributum* (Zvyagintseva & Tarasov, 1987; Oren & Ventosa, 1996), *Halorubrum ezzemoulense* (Kharroub *et al.*, 2006), *Halorubrum lacusprofundi* (Franzmann *et al.*, 1988), *Halorubrum lipolyticum* (Cui *et al.*, 2006), *Halorubrum orientale* (Castillo *et al.*, 2006), *Halorubrum sodomense* (Oren, 1983), *Halorubrum tebenquichense* (Lizama *et al.*, 2002), *Halorubrum terrestre* (Ventosa *et al.*, 2004), *Halorubrum tibetense* (Fan *et al.*, 2004), *Halorubrum trapanicum* (Petter, 1931; McGenity & Grant, 1995), *Halorubrum vacuolatum* (Mwatha & Grant, 1993; Kamekura *et al.*, 1997), *Halorubrum xinjiangense* (Feng *et al.*, 2004) and *Halorubrum saccharovororum* (type species) (Tomlinson & Hochstein, 1976). These species can be

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EJ-32^T is AM491830.

classified into two groups according to their growth at various pH values and their origins. Group 1 contains neutrophilic species, such as *Hrr. aidingense*, *Hrr. coriense*, *Hrr. distributum*, *Hrr. ezzemoulense*, *Hrr. lacusprofundi*, *Hrr. lipolyticum*, *Hrr. orientale*, *Hrr. saccharovororum*, *Hrr. sodomense*, *Hrr. tebenquichense*, *Hrr. terrestre*, *Hrr. trapanicum* and *Hrr. xinjiangense*. Group 2 contains three alkaliphilic species, *Hrr. alkaliphilum*, *Hrr. tibetense* and *Hrr. vacuolatum* (Grant *et al.*, 2001).

Here, we report the isolation and description of a novel neutrophilic strain (EJ-32^T) isolated from Lake Ejnor in Inner Mongolia, China, and its assignment as a novel species of the genus *Halorubrum*.

Strain EJ-32^T was isolated from a water sample collected from Lake Ejnor (45° 14' N, 116° 32' E) in Inner Mongolia, China. At the time of sampling (September 2003) the water of the lake had a temperature of 32 °C, a pH of 8.5 and a salinity of 18 % (w/v). The isolation procedure was as described previously (Castillo *et al.*, 2006; Gutiérrez *et al.*, 2007). Characterization of strain EJ-32^T was performed according to the proposed minimal standards for the description of novel taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Cell motility and morphology were examined by phase-contrast microscopy of exponentially growing liquid cultures by using an Olympus BX41 microscope equipped with phase-contrast optics. The cells of strain EJ-32^T were rod-shaped and non-motile, with a tendency to form clumps. Colony morphology was observed under optimal growth conditions on agar medium with 25 % (w/v) salts, after incubation at 37 °C for 10 days. Anaerobic growth was tested in filled, stoppered tubes in the presence of 5 g nitrate l⁻¹ or 5 g arginine l⁻¹. Tests for the following features were carried out as described by Oren *et al.* (1997): catalase and oxidase activities; hydrolysis of starch, Tween 80, gelatin, casein, DNA and aesculin; reduction of nitrate and nitrite;

formation of H₂S and indole; utilization of sugars, alcohols, amino acids and organic acids. Antibiotic-sensitivity tests were performed by spreading bacterial suspensions on culture plates and applying discs impregnated with the following concentrations (µg unless indicated otherwise): ampicillin (10), bacitracin (10 U), cephalothin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (10), novobiocin (30), penicillin G (10 U), rifampicin (30), polymyxin (300 U), streptomycin (10), sulfamethoxazole (25), tetracycline (30) and vancomycin (30). The physiological and biochemical characteristics, as well as the antibiotic susceptibilities, of strain EJ-32^T are provided in the species description below.

Polar lipids were extracted with chloroform/methanol as described by Kamekura (1993). TLC was performed using Merck HPTLC silica gel 60 plates (Art. 5641) in a solvent system comprising chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.). The polar lipid composition of strain EJ-32^T comprised phosphatidylglycerol and phosphatidylglycerophosphate methyl ester, but no glycolipids were detected.

The 16S rRNA gene sequence was amplified by PCR using three universal primer sets as described by Lopez-Garcia *et al.* (2001) and Arahall *et al.* (1996), and the almost-complete nucleotide sequence was determined by NBT-Newbiotechnic (Sevilla, Spain) using an automated DNA sequencer (model 3100; Applied Biosystems). A subsequent sequence analysis was conducted using the ARB software package (Ludwig *et al.*, 2004). Alternative treeing methods (maximum parsimony, neighbour joining and maximum likelihood) were used according to the recommendations of Ludwig *et al.* (1998). A comparison of 16S rRNA gene sequences revealed that the sequence of strain EJ-32^T (1404 bp) displayed the highest level of similarity with those of *Halorubrum* species. Fig. 1 shows the phylogenetic

Fig. 1. Maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain EJ-32^T among the species of the genus *Halorubrum*. The sequence data used were obtained from the EMBL database (accession numbers are given in parentheses). Bar, 1 % sequence divergence.

tree constructed with the maximum-parsimony method (Fitch, 1971), in which strain EJ-32^T clustered with the type strains of species of the genus *Halorubrum*. Sequence similarity calculations indicated that strain EJ-32^T was related to *Hrr. coriense* (97.9% sequence similarity), *Hrr. trapanicum* (97.9%), *Hrr. sodomense* (97.8%), *Hrr. tebenquichense* (97.8%), *Hrr. xinjiangense* (97.6%), *Hrr. terrestre* (97.4%), *Hrr. distributum* (97.1%) and *Hrr. saccharovorum* (96.4%). Similar tree topologies were obtained when other tree-construction methods were used.

The DNA G+C content 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 EJ-32^T was 64.0 mol%, which is within the G+C range reported for the species of the genus *Halorubrum* (62.7–71.2 mol%) (Grant *et al.*, 2001).

DNA–DNA hybridizations between strain EJ-32^T and the type strains of the phylogenetically most closely related *Halorubrum* species were performed using the competition procedure of Johnson (1994), as described in detail by

Gutierrez *et al.* (2002). The levels of DNA–DNA relatedness between strain EJ-32^T and *Hrr. coriense* JCM 9275^T, *Hrr. trapanicum* NRC 34021^T, *Hrr. sodomense* ATCC 33755^T, *Hrr. tebenquichense* CECT 5317^T, *Hrr. xinjiangense* JCM 12388^T, *Hrr. terrestre* VKM B-1739^T, *Hrr. distributum* JCM 9100^T and *Hrr. saccharovorum* NCIMB 2081^T were 40, 46, 31, 44, 49, 17, 36 and 3%, respectively. These levels of DNA–DNA hybridization are low enough to justify the classification of strain EJ-32^T as representing a genotypically distinct species within the genus *Halorubrum* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

The differentiating characteristics of strain EJ-32^T compared with those of the other *Halorubrum* species are listed in Table 1. Overall, our data show that strain EJ-52^T represents a novel species of the genus *Halorubrum*, for which the name *Halorubrum ejinorensense* sp. nov. is proposed.

Description of *Halorubrum ejinorensense* sp. nov.

Halorubrum ejinorensense (e.ji.no.ren'se. N.L. neut. adj. *ejinorensense* of Ejinor, referring to the isolation of the organism from the saline Lake Ejinor, in Inner Mongolia, China).

Table 1. Characteristics that distinguish strain EJ-32^T from non-alkaliphilic *Halorubrum* species

Strains: 1, EJ-32^T; 2, *Hrr. saccharovorum* ATCC 29252^T; 3, *Hrr. aidingense* JCM 13560^T; 4, *Hrr. coriense* JCM 9275^T; 5, *Hrr. distributum* JCM 9100^T; 6, *Hrr. ezzemoulense* CECT 7099^T; 7, *Hrr. lacusprofundi* JCM 8891^T; 8, *Hrr. lipolyticum* JCM 13559^T; 9, *Hrr. orientale* CECT 7145^T; 10, *Hrr. sodomense* ATCC 33755^T; 11, *Hrr. tebenquichense* DSM 14210^T; 12, *Hrr. terrestre* VKM B-1739^T; 13, *Hrr. trapanicum* JCM 10477^T; 14, *Hrr. xinjiangense* AS 1.3527^T. Data are from Cui *et al.* (2006), Kharroub *et al.* (2006), Feng *et al.* (2004), Ventosa *et al.* (2004), Lizama *et al.* (2002), McGenity & Grant (2001), Feng *et al.* (2005) and this study. +, Positive; –, negative; +/-, doubtful; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Morphology	Rods	Rods	Rods	Pleo-morphic short rods and cup shapes	Pleo-morphic rods	Pleo-morphic rods	Pleo-morphic rods	Rods	Pleo-morphic rods	Rods	Irregular discs	Pleo-morphic	Pleo-morphic rods	Short rods
Cell size (µm)	1.0–1.5 × 5.0–8.0	0.6–1.2 × 2.5	0.9–1.0 × 3.6–6.4	5.0–0.5	0.8–1.0 × 2.7–7.0	0.6–1.5 × 3.0	>12	0.9–1.2 × 1.8–5.5	1.5–2.0 × 1.0–2.0	0.5 × 2.5–5	0.8–1.2 × 1.5–1.0	1.0–1.5 × 1.5–2.5	0.7–1.0 × 1.5–3.0	0.8–1.2 × 1.8–2.6
Motility	–	+	+	+	+	+	+	+	+	+	ND	+	–	+
Colony pigmentation	Red	Orange–red	Red	Orange–red	Orange–red	Red	Red	Red	Red	Orange–red	Red–orange	Orange–red	Pale orange	Red
NaCl range for growth (M)	2.5–5.0	1.5–5.2	1.7–4.3	2.0–5.2	1.7–5.2	2.5–4.3	1.5–5.2	1.7–4.8	2.5–5.0	0.5–4.3	2.5–5.2	2.5–5.2	2.5–5.2	2.0–5.2
Temperature optimum (°C)	37	50	40–42	50	37–45	37–40	31–37	45–48	37	40	40	37–45	37	40
Nitrate reduction	+	–	+	ND	+	+	+/-	+	+	+/-	+	–	+	–
H ₂ S production	–	+	+	ND	+	ND	–	+	–	ND	+	–	ND	+
Utilization of:														
D-Fructose	–	+	+	ND	ND	–	–	+	+	+	+	–	+	+
Lactose	–	+	–	+	ND	–	+	–	–	+	–	ND	–	–
D-Galactose	–	+	+	+	–	–	+	+	–	–	+	ND	+	–
Maltose	–	+	+	ND	ND	ND	–	+	+	+	–	ND	+	+
D-Glucose	–	+	+	+	–	+	+	+	+	+	+	–	+	+
Sucrose	–	+	+	ND	–	+	ND	+	ND	+	–	ND	+	+
DNA G+C content (mol%)	64.0	71.2	64.2	ND	63.6	61.9	65.3	65.9	64.2	67.4	63.2	64.4	64.3	68.0

Cells are Gram-negative rods $1.0\text{--}1.5 \times 5.0\text{--}8.0\text{ }\mu\text{m}$ in size. Colonies on agar plates containing 25 % (w/v) total salt are red, elevated and round. Growth occurs with 2.5–5.0 M NaCl at pH 6–10 and 25–50 °C. The optimal NaCl concentration, pH and temperature for growth are 3.4 M, pH 7.5 and 37 °C, respectively. Magnesium is not required for growth. Chemo-organotrophic and aerobic. Catalase- and oxidase-positive. Anaerobic growth with nitrate or L-arginine does not occur and nitrate reduction to nitrite is observed. H_2S is not produced from cysteine. Indole is not produced from tryptophan. Methyl red, Voges–Proskauer and Simmons citrate test results are negative. Tween 80 and DNA are hydrolysed. Casein, gelatin and starch are not hydrolysed. Acid is not produced from D-arabinose, D-fructose, D-galactose, D-glucose, glycerol, lactose, maltose, D-mannitol, sucrose, trehalose or D-xylose. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not produced. Growth occurs on trehalose as a single carbon and energy source. The following compounds are not used as sole carbon and energy sources: acetate, D-arabinose, fumarate, D-fructose, D-glucose, D-glutamate, glycerol, lactose, malate, maltose, D-mannitol, propionate, D-raffinose, D-ribose, D-sorbitol, succinate and D-xylose. The following compounds are used as sole carbon, nitrogen or energy sources: L-asparagine, isoleucine, L-lysine and L-threonine. Serine and glycine are not used as sole carbon, nitrogen or energy sources. Susceptible to bacitracin (10 U) and novobiocin (30 μg). Resistant to the following antibiotics (μg unless indicated otherwise): ampicillin (10), cephalothin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (10), penicillin G (10 U), rifampicin (30), polymyxin (300 U), streptomycin (10), sulfamethoxazole (25), tetracycline (30) and vancomycin (30). The polar lipids are phosphatidylglycerol and phosphatidylglycerophosphate methyl ester. Glycolipids not detected. The DNA G+C content is 64.0 mol%.

The type strain, EJ-32^T (=CECT 7194^T=CGMCC 1.6782^T=JCM 14265^T), was isolated from Lake Ejnor, a salt lake in Inner Mongolia, China.

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