Alkaliphilus halophilus sp. nov., a strictly anaerobic and halophilic bacterium isolated from a saline lake, and emended description of the genus *Alkaliphilus*

Xiao-Yue Wu,[1] Kong-Lan Shi,[1] Xue-Wei Xu,[2,3] Min Wu,[1] Aharon Oren[4] and Xu-Fen Zhu[1]

Correspondence
Xu-Fen Zhu
xufenzhu@zju.edu.cn

[1]College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China

[2]Laboratory of Marine Ecosystem and Biogeochemistry, State Oceanic Administration, Hangzhou 310012, PR China

[3]Second Institute of Oceanography, State Oceanic Administration, Hangzhou 310012, PR China

[4]Institute of Life Sciences, and Moshe Shilo Minerva Center for Marine Biogeochemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

A novel strictly anaerobic, halophilic and fermentative strain, designated E2R^T, was isolated from sediments of Xiaokule salt lake in Xinjiang Province, China. Cells were straight to slightly curved, Gram-stain-positive rods that were motile by means of flagella and formed endospores. Strain E2R^T was moderately halophilic and grew optimally in the presence of 7.5 % NaCl, at pH 8.0 and at 32 °C. Substrates used include yeast extract, Casamino acids, tryptone, fructose, sucrose, xylose, ribose, lactate and tartrate. Thiosulfate could be used as an accessory electron acceptor and stimulated growth. The main fermentation products from fructose were formate and acetate. The predominant fatty acids were iso-$C_{15:0}$, iso-$C_{15:1}$ F and iso-$C_{13:0}$. 16S rRNA gene sequence analyses revealed that strain E2R^T was related most closely to members of the genus *Alkaliphilus* (95.5–91.1 % similarity). The G+C content of strain E2R^T was 28.5 mol%. Strain E2R^T could be differentiated from its closest relatives based on its halophilic nature and its lower DNA G+C content. It could also be differentiated based on its substrate utilization pattern and relatively high levels of iso-$C_{15:0}$. On the basis of these data, strain E2R^T is considered to represent a novel species of the genus *Alkaliphilus*, for which the name *Alkaliphilus halophilus* sp. nov. is proposed. The type strain is E2R^T (=CGMCC 1.5124^T =JCM 16124^T). An emended description of the genus *Alkaliphilus* is also provided.

Inland saline and hypersaline lakes harbour diverse communities of halophilic micro-organisms, including many anaerobic species (Ollivier *et al.*, 1994). Most anaerobic, moderately halophilic bacteria described are classified in the order *Halanaerobiales*, and grow optimally at about 6.0 % NaCl (Oren, 2006). In this paper, we describe a novel, obligately anaerobic and halophilic bacterium with optimum growth at 7.5 % NaCl, isolated from sediments of Xiaokule salt lake (38° 35′ N 89° 29′ E) in Xinjiang Province, China.

Xiaokule salt lake is located on a plateau at an altitude of about 4480 m above sea level. There is no river supply to the lake, and atmospheric water and groundwater are the only water sources. The low temperature, low nutrient levels and high salt content make it a relatively isolated ecosystem. A mud sample was collected from the bottom of Xiaokule salt lake from a depth of about 0.3 m. About 1 g of this sample was incubated anaerobically in 40 ml enrichment medium (DSMZ medium 210) at 32 °C for 1 week and pure cultures were obtained by the Hungate roll-tube technique (Hungate, 1969; Bryant, 1972) under a gas phase of O_2-free N_2. Three strains were isolated and purified. Two of them stained Gram-negative and were identified as belonging to the genus *Halanaerobium*. The third, designated strain E2R^T, stained Gram-positive and is characterized in detail herein.

The medium for the cultivation of strain E2R^T had the following composition (per litre distilled water): 2.0 g yeast extract (BD), 2.0 g tryptone (BD), 1.0 g KCl, 0.3 g KH_2PO_4, 0.5 g $MgSO_4 . 7H_2O$, 75 g NaCl, 10 ml $Na_2S . 9H_2O$ (5 %, w/v), 0.4 g L-cysteine, 0.001 g resazurin and 10 ml trace element solution M144 (see DSMZ medium 144). The medium was adjusted to pH 8.0 with NaOH. To test the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain E2R^T is EU627628.

A 16S rRNA gene sequence-based maximum-parsimony tree is available as supplementary material with the online version of this paper.

effect of salt concentration on growth, NaCl was added at 0, 0.5, 1, 2, 3, 5, 7.5, 10, 12, 15 or 20 % (w/v). To examine the pH range for growth, MES (pH 5.5–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), CAPSO (pH 9.0–9.5) or CAPS (10.0–11.5) was added at a concentration of 25 mM. The temperature range for growth was determined by incubating at 4, 10, 15, 20, 25, 28, 32, 37, 42, 45, 50, 55 and 60 °C. To test the use of substrates and potential electron acceptors, a basal medium was prepared based on JCM medium 378 without tryptone, crotonic acid or trace vitamins and with 0.1 % yeast extract (BD). For substrate utilization tests, Casamino acids (BBI), yeast extract (BD), tryptone (BD), starch (each at 10 g l^{-1}), alcohols (0.1 %, v/v) and organic acids (20 mM) were added directly to the basal medium; sugars (20 mM) were added from filter-sterilized concentrated solutions. To test for the use of accessory electron acceptors, S^0, sodium thiosulfate, fumarate or crotonate was added at a concentration of 10 mM. Strain E2R^T and the reference strains listed below were incubated under a gas phase of O_2-free N_2 and utilization was confirmed by growing the strain in the same substrate for two subcultures. All experiments were conducted in duplicate. For comparison of phenotypic and other properties, *Alkaliphilus oremlandii* DSM 21761^T and *Alkaliphilus crotonatoxidans* JCM 11672^T were grown under identical conditions. These physiological properties are detailed in Table 1 and in the species description.

Cell morphology and motility were examined by using optical (BX 40; Olympus) and transmission electron (H-600;

Table 1. Differential phenotypic and genotypic characteristics between strain E2R^T and type strains of recognized *Alkaliphilus* species

Strains: 1, E2R^T; 2, *A. oremlandii* DSM 21761^T; 3, *A. crotonatoxidans* JCM 11672^T; 4, *A. transvaalensis* JCM 10712^T (data from Takai *et al.*, 2001); 5, *A. peptidifermentans* DSM 18978^T (Zhilina *et al.*, 2009). Data were obtained in this study unless indicated. All utilized yeast extract and Casamino acids. None utilized glucose, formate, acetate, oxalate, methanol, ethanol, 2-propanol, 1-butanol, glycerol, dimethyl formamide, DMSO or soluble starch. NR, Not reported.

Characteristic	1	2	3	4	5
Optimum temperature (°C)	32	37	37	40	35
Optimum pH	8.0	8.5	8.0	10.0	9.1
NaCl concentration for growth (%, w/v)					
Range	0.5–15	0–2	0–7	0.1–3.3*	0–5
Optimum	7.5	0	1.0	0.5	2
Use of electron acceptors					
S^0	−	+	−	+	−
Crotonate	−	−	+	NR	+
Fumarate	−	−	+†	+	+
Utilization of:					
Tryptone	+	−	−	+	+
Cellobiose	−	−	+	−	−
Fructose	+	−‡	+	−	−
Galactose	−	−	+	−	−
Lactose	−	−	+	−	−
Maltose	−	−	+	−	−
Ribose	+	−	+	−	−
Sucrose	+	−	−	−	−
Trehalose	−	−	+	−	−
Xylose	+	−	+	−	−
Citrate	−	−	+	−	−
Lactate	+	+	−	−	−
Malate	−	−	+	−	NR
Tartrate	+	−	+	−	NR
Main fermentation products§	Fo, Ac	Ac, Pr	Ac, Pr	NR	Fo, Ac
DNA G+C content (mol%)	28.5	36.5‖	33.3¶	36.4	33.8

*Based on addition of sea salts rather than NaCl.

†Cao *et al.* (2003) reported a negative reaction.

‡We could not reproduce growth on fructose, as reported by Fisher *et al.* (2008).

§Ac, Acetate; Fo, formate; Pr, propionate. Data for strain E2R^T and *A. crotonatoxidans* JCM 11672^T are products from fructose; data for *A. oremlandii* DSM 21761^T are products from lactate; data for *A. peptidifermentans* DSM 18978^T are products from yeast extract.

‖Based on our determination; Fisher *et al.* (2008) reported 36.1 mol%.

¶Based on our determination; Cao *et al.* (2003) reported 30.6 mol%.

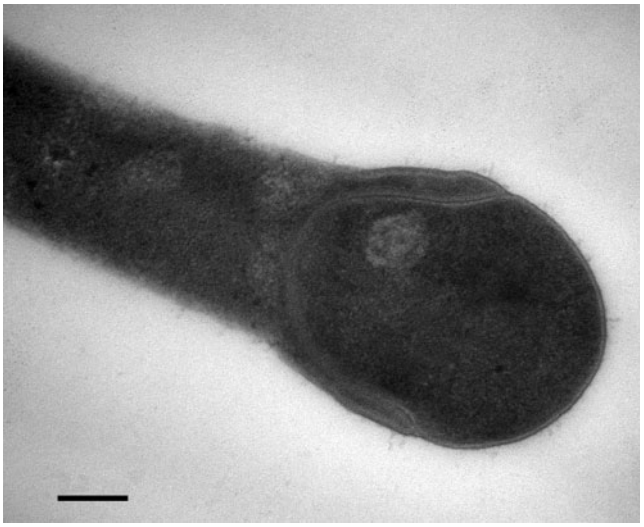

Fig. 1. Phase-contrast micrograph of a cell of strain E2R^T in the stationary phase of growth, showing formation of a terminal spore. Bar, 0.2 μm.

Hitachi) microscopy. Cells of strain E2R^T were straight to slightly curved rods (0.5–0.9 × 1.6–6 μm). In the late-exponential and stationary phases of growth, the rods formed terminal endospores (Fig. 1). Strain E2R^T was obligately anaerobic; no growth was observed in the presence of air. The generation time was about 7 h under optimal conditions. No growth occurred in the absence of NaCl; growth was obtained at NaCl concentrations between 0.5 and 15 %, with an optimum at 7.5 %. Growth occurred at 15–42 °C, with an optimum at 32 °C. The pH range for growth was 5.5–9.0 (optimum, pH 8.0).

Fatty acid methyl esters were extracted from cells grown in DSMZ medium 104b PYX without fructose until an OD_{600} of 0.1 was reached, and were analysed by using GC/MS (Kuykendall *et al.*, 1988). The fatty acid profiles of strains E2R^T, *A. oremlandii* DSM 21761^T and *A. crotonatoxidans* JCM 11672^T are compared in Table 2. The predominant component of all three strains was iso-$C_{15:0}$, but a much higher level was found for strain E2R^T. In addition, iso-$C_{15:1}$ F (double bond position unknown) represented 13.6 % of the total fatty acids in strain E2R^T and 6.3 % in *A. oremlandii* DSM 21761^T, but only 1.4 % in *A. crotonatoxidans* JCM 11672^T.

For determination of the G+C content of the genomic DNA and for 16S rRNA gene sequence determination and phylogenetic analysis, DNA was prepared and purified as described by Marmur (1961). The DNA G+C content of strain E2R^T was determined by thermal denaturation (T_m) (Marmur & Doty, 1962) with *Escherichia coli* K-12 DNA as the calibration standard; the DNA G+C content of strain E2R^T was 28.5 mol%. The 16S rRNA gene was amplified with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGYTACCTTGTTACGACTT-3′). The

Table 2. Fatty acid compositions of strain E2R^T and type strains of related *Alkaliphilus* species

Strains: 1, E2R^T; 2, *A. oremlandii* DSM 21761^T; 3, *A. crotonatoxidans* JCM 11672^T. Values are percentages of total fatty acids and were determined in this study. Major components (>10 %) in each strain are highlighted in bold. −, Not detected.

Fatty acid	1	2	3
Unbranched			
$C_{14:0}$	3.2	4.9	**11.1**
$C_{15:0}$	0.2	0.8	2.6
$C_{16:0}$	5.0	8.5	8.9
$C_{18:0}$	1.3	4.4	0.9
$C_{14:0}$ 2-OH	−	0.8	0.3
$C_{15:1}\omega 8c$	0.2	1.1	1.1
$C_{18:1}\omega 7c$	0.6	−	0.4
$C_{18:1}\omega 9c$	4.0	4.2	0.7
iso-Branched			
iso-$C_{11:0}$	0.3	0.8	−
iso-$C_{13:0}$	9.7	**10.5**	4.7
iso-$C_{14:0}$	0.2	0.6	1.4
iso-$C_{14:0}$ 3-OH	1.0	3.1	4.7
iso-$C_{15:0}$	**41.4**	**27.9**	**26.5**
iso-$C_{15:1}$ F*	**13.6**	6.3	1.4
iso-$C_{17:0}$	1.8	1.3	1.0
iso-$C_{17:1}\omega 9c$	3.2	1.2	0.7
anteiso-Branched			
anteiso-$C_{13:0}$	0.3	1.0	0.2
anteiso-$C_{15:0}$	1.4	3.3	6.0
Summed features†			
1	0.8	0.8	3.3
2	−	−	3.1
3	8.7	**10.1**	5.5
4	1.1	3.1	9.7

*Letters indicate unknown double-bond positions.

†Summed features represent groups of two or three fatty acids that cannot be separated with the MIDI system. Summed feature 1, iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH; summed feature 2, unknown fatty acid; summed feature 3, $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH; summed feature 4, anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I.

sequence between positions 28 and 1491 (*E. coli* numbering) was compared with all closely related sequences by using the EzTaxon service (Chun *et al.*, 2007). Preliminary 16S rRNA gene sequence comparisons showed that strain E2R^T was related most closely to the type strains of recognized *Alkaliphilus* species, namely *A. oremlandii* DSM 21761^T (95.5 % similarity), *Alkaliphilus transvaalensis* JCM 10712^T (93.2 %), *Alkaliphilus peptidifermentans* DSM 18978^T (92.9 %) and *A. crotonatoxidans* JCM 11672^T (91.2 %). The almost-complete 16S rRNA gene sequence (1449 nt) of strain E2R^T was aligned by using CLUSTAL X software (version 1.8). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with the MEGA4 program package (Tamura *et al.*, 2007) and by

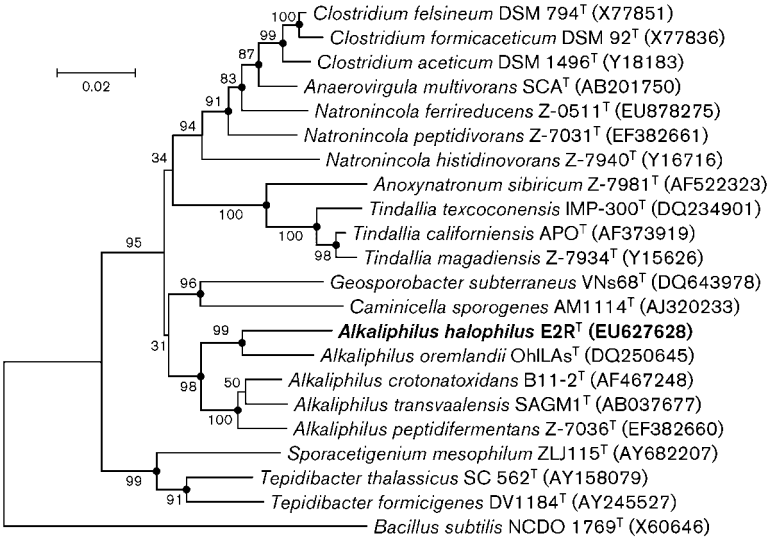

Fig. 2. Phylogenetic tree based on comparison of 16S rRNA gene sequences of strain E2R^T and the type strains of related species of the *Clostridiaceae*. The tree was constructed by the neighbour-joining method and was rooted with *Bacillus subtilis* NCDO 1769^T as an outgroup. Bootstrap values based on 1000 replicates are shown. Filled circles indicate branches that were also recovered in the tree generated with the maximum-likelihood method. Bar, 2 % sequence divergence.

using the maximum-likelihood method (Felsenstein, 1981) with the PHYLIP 3.6 program (Felsenstein, 1993). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method and indicated that the new isolate clustered within the genus *Alkaliphilus* (Fig. 2). Trees constructed with the other two methods showed similar topologies (Fig. 2 and Supplementary Fig. S1, available in IJSEM Online).

Strain E2R^T could be distinguished from the related alkaliphiles *A. transvaalensis* JCM 10712^T and *A. peptidifermentans* DSM 18978^T, both of which grow optimally above pH 9.0 and do not grow below pH 7.5 (Takai *et al.*, 2001; Zhilina *et al.*, 2009). In addition, under a nitrogen gas phase, strain E2R^T utilized not only proteinaceous substrates and some organic acids but also oligosaccharides, which were not metabolized by *A. oremlandii* DSM 21761^T. Strain E2R^T could be differentiated from *A. crotonatoxidans* JCM 11672^T based on its ability to utilize tryptone, sucrose and lactate, together with tolerance to NaCl. Based on the phenotypic characteristics described above, phylogenetic analysis, elevated amounts of iso-$C_{15:0}$ and relatively low DNA G+C content, strain E2R^T is considered to represent a novel species of the genus *Alkaliphilus*, for which the name *Alkaliphilus halophilus* sp. nov. is proposed.

Description of *Alkaliphilus halophilus* sp. nov.

Alkaliphilus halophilus [ha.lo′phi.lus. Gr. n. *hals*, *halos* salt; L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*) friendly to, loving; N.L. masc. adj. *halophilus* salt-loving].

Cells are Gram-stain-positive, straight to slightly curved rods (0.5–0.9 × 1.6–6.1 μm) that occur singly or in pairs. Cells are motile by means of flagella and form endospores. Strictly anaerobic. The temperature range for growth is 15–42 °C (optimum, 32 °C). The pH range for growth is 5.5–9.0 (optimum, pH 8.0). The NaCl concentration range for growth is 0.5–15 % (optimum, 7.5 %). Able to ferment fructose, sucrose, xylose, ribose, lactate, tartrate, yeast extract, Casamino acids and tryptone, but not cellobiose, galactose, glucose, lactose, maltose, trehalose, acetate, citrate, formate, malate, oxalate, acetone, 1-butanol, ethanol, methanol, 2-propanol, dimethyl formamide, DMSO, glycerol or soluble starch. The main fermentation products from fructose are formate and acetate. Thiosulfate can serve as an accessory electron acceptor and stimulates growth, but growth stimulation is not observed with sulfur, fumarate or crotonate. The predominant cellular fatty acids are iso-$C_{15:0}$, iso-$C_{15:1}$ F and iso-$C_{13:0}$. The DNA G+C content of the type strain is 28.5 mol% (T_m).

The type strain, E2R^T (=CGMCC 1.5124^T =JCM 16124^T), was isolated from sediment of a salt lake in Xinjiang Province, China.

Emended description of the genus *Alkaliphilus* Takai *et al.* 2001

The G+C content of the DNA is in the range 28–36 mol%. Other properties are as given by Cao *et al.* (2003).

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