Halanaerobacter jeridensis sp. nov., isolated from a hypersaline lake

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An obligatory anaerobic, moderately halophilic bacterium, designated strain CEJFG43^T, was isolated from a sample of sediment collected below the salt crust on the hypersaline El Jerid lake, in southern Tunisia. The cells of this novel strain were Gram-staining-negative, non-sporulating, motile, short rods. They grew in media with 6–30 % (w/v) NaCl (optimum 15 %), at 20–60 °C (optimum 45 °C) and at pH 5.5–9.5 (optimum pH 8.3). The micro-organism fermented glucose, fructose, ribose, raffinose, galactose, mannose, sucrose, maltose, xylose, mannitol, pyruvate and glycerol. The products of glucose fermentation were lactate, ethanol, acetate, H_2 and CO_2. The genomic G+C DNA content of strain CEJFG43^T was 33.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain CEJFG43^T belonged in the genus *Halanaerobacter* and was most closely related to *Halanaerobacter lacunarum* DSM 6640^T (95.3 % gene sequence similarity) and *Halanaerobacter chitinivorans* DSM 9569^T (95.3 %). The predominant cellular fatty acids were non-branched ($C_{16:0}$ and $C_{16:1}$). Based on the phylogenetic and phenotypic evidence, strain CEJFG43^T represents a novel species in the genus *Halanaerobacter* for which the name *Halanaerobacter jeridensis* sp. nov. is proposed. The type strain is CEJFG43^T (=DSM 23230^T=JCM 16696^T).

Intense evaporation from inland salt lakes can leave the water in such lakes extremely saline, with salt contents close to saturation. In Tunisia, these hypersaline lakes are called *sebkhat* or *chott*. Although usually appearing dry, with a thin crust of salt covering a layer of spongy, brine-soaked soil, heavy winter rains can result in surface water. The largest salt lake in Tunisia, El Jerid, covers about 5000 km^2 in the south of the country. Although the salts in El Jerid lake come from the geological formations of the surrounding mountains, the lake contains similar ions to seawater, with a salt concentration generally varying between 250 and 330 g l^{-1} (Kbir-Ariguib *et al.*, 2001). Salt lakes are inhabited by halophilic and halotolerant micro-organisms. The anaerobic halophilic fermentative bacteria represent a diverse but closely related group of micro-organisms. Such bacteria have been placed in a single order, the *Halanaerobiales*. This order is currently divided into two families: the *Halanaerobiaceae* (Oren *et al.*, 1984) and the *Halobacteroidaceae* (Rainey *et al.*, 1995). At the time of writing, the genus *Halanaerobacter* (in the family *Halobacteroidaceae*) comprises just three species: *Halanaerobacter chitinivorans* (Liaw & Mah, 1992), *Halanaerobacter lacunarum* (formerly *Halobacteroides lacunaris*; Zhilina *et al.*, 1991) and *Halanaerobacter salinarius* (Mouné *et al.*, 1999). The definition of this genus, like those of other genera in the order *Halanaerobiales*, has been largely based on the results of phylogenetic analyses of 16S rRNA gene sequences.

In this report, we describe a strain of anaerobic, halophilic, fermentative bacterium isolated from a sample of sediment from El Jerid lake. This strain, designated CEJFG43^T, was found to represent a novel species in the genus *Halanaerobacter*.

Strain CEJFG43^T was isolated from a sample of black sediment collected below the salt crust on the edge of El Jerid lake, in southern Tunisia. The sample was collected in a sterile glass bottle that was completely filled with water from the lake and kept at 4 °C until the isolation attempt. The water used to fill the bottle was at pH 7.6 and highly saline, with a total mineral content of 370 g l^{-1}. Standard anaerobic techniques were used throughout the study (Hungate, 1969; Balch *et al.*, 1979). A small subsample of sediment (1 g) was inoculated into an enrichment medium containing (l^{-1}): 1 g NH_4Cl, 0.5 g K_2HPO_4, 0.8 g KCl,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CEJFG43^T (=DSM 23230^T=JCM 16696^T) is GU265900.

0.1 g $CaCl_2$, 10 g $MgSO_4 . 7H_2O$, 15 g $MgCl_2 . 7H_2O$, 1 mg NaBr, 250 g NaCl, 0.5 g yeast extract, 0.5 g tryptone, 0.5 g cysteine-HCl, 0.001 g resazurin and 10 ml trace element solution (Balch *et al.*, 1979). The pH of the medium was adjusted to 7.6 with 1 M KOH before the medium was boiled under a stream of O_2-free N_2 gas and then cooled to room temperature. The cooled medium was dispensed into Hungate tubes (at 5 ml per tube) and serum bottles (at 20 ml per bottle), under a stream of N_2/CO_2 (80 : 20 %, v/v) gas, and then sterilized by autoclaving at 110 °C for 45 min. Prior to inoculation of the medium in each bottle, 0.4 ml 10 % (w/v) $NaHCO_3$, 0.4 ml 2 % (w/v) Na_2S and 0.4 ml 1 M glucose were injected into the bottle, from sterile stock solutions. The medium in each Hungate tube was similarly supplemented, using 0.1 ml of each stock solution.

Each enrichment culture was incubated at 35 °C for 2 weeks. A novel strain, designated CEJFG43^T, was obtained and this was purified by two rounds of culture using the roll-tube method (Hungate, 1969) and enrichment medium that had been solidified by the addition of 2 % (w/v) agar. After 6 days in roll tubes, on the solid medium, the novel strain formed cream-coloured colonies with regular edges and diameters of 0.8–1.0 mm. The strain grew as motile, short rods that measured approximately 1.2 × 2.5–6.0 μm and occurred singly or in pairs (Fig. 1). These rods were Gram-staining-negative. Spores were never observed. Examination of ultrathin sections of the rods, by transmission electron microscopy, revealed that each had a tri-layered cell wall and an envelope that was typical of Gram-staining-negative bacteria (Fig. 1). The purity of the isolate was checked by microscopic observations.

Strain CEJFG43^T was strictly anaerobic and moderately halophilic, growing in media containing 6–30 % (w/v) NaCl (optimum 15 %). No growth was observed in the absence of NaCl. It grew at pH 5.5–9.5 (optimum pH 8.3) and at 20–60 °C (optimum 45 °C). Substrates were tested at a final concentration of 20 mM in the basal medium (with 0.2 g yeast extract and 0.2 g tryptone l^{-1}). Experiments were conducted in duplicate and, in each case, two successive subcultures were performed under the same conditions. To test for electron acceptors, sodium thiosulfate, sodium sulfate, sodium sulfite, elemental sulfur and nitrate were added to the medium at final concentrations of 20 mM, 20 mM, 2 mM, 2 % (w/v) and 10 mM, respectively. Strain CEJFG43^T used glucose, fructose, sucrose, maltose, mannose, raffinose, ribose, xylose, trehalose, starch, mannitol, pyruvate and glycerol. No growth was observed on cellobiose, sorbose, arabinose, lactose, rhamnose, acetate, lactate, succinate, fumarate, Casamino acids, betaine, trimethylamine or peptone. The end products from the fermentation of glucose (20 mM) were determined by HPLC, using an Aminex HPX-87H column (Bio-Rad) with 5 mM H_2SO_4 as the mobile phase. Strain CEJFG43^T formed almost equal amounts of lactate and ethanol (13 and 12 mM, respectively) as well as smaller amounts of acetate (1.5 mM), CO_2 (1.6 mM) and H_2.

The cellular fatty acid composition of strain CEJFG43^T was determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. After cells had been grown in basal liquid medium at 45 °C for 5 days, fatty acid methyl esters were produced using the method of Miller (1982), with the modifications of Kuykendall *et al.* (1988), before being separated and identified using version 4.0 of the

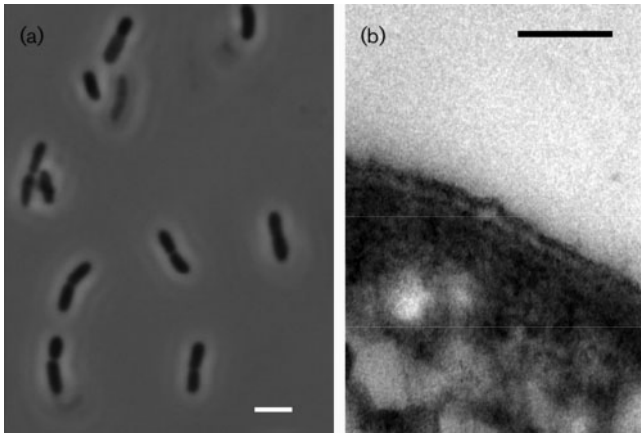

Fig. 1. Phase-contrast light micrograph of cells of strain CEJFG43^T (a) and a transmission electron micrograph of an ultrathin section of part of a cell of the same strain (b). Bars, 5 μm (a) and 100 nm (b).

Table 1. Cellular fatty acid contents of strain CEJFG43^T

Fatty acid	Content (%)
$C_{9:0}$	0.2
$C_{10:0}$	6.1
$C_{10:0}$ 3-OH	0.4
$C_{14:0}$	7.9
$C_{15:1}\omega 9c$	0.5
$C_{15:0}$	1.1
$C_{16:1}\omega 7c$	4.5
$C_{16:1}\omega 9c$	42.5
$C_{16:1}\omega 11c$	2.3
$C_{16:0}$	21.7
$C_{16:1}\omega 9c$ DMA	0.6
$C_{16:0}$ DMA	0.3
$C_{18:1}\omega 11c$	1.1
$C_{18:0}$	1.1
Summed feature*	
2	8.2
4	0.9
8	0.6

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 comprises $C_{12:0}$ 3-OH and/or $C_{13:0}$ DMA. Summed feature 4 comprised $C_{15:2}$ and/or $C_{15:1}\omega 7c$. Summed feature 8 comprised $C_{17:1}\omega 9c$ and/or $C_{17:2}$.

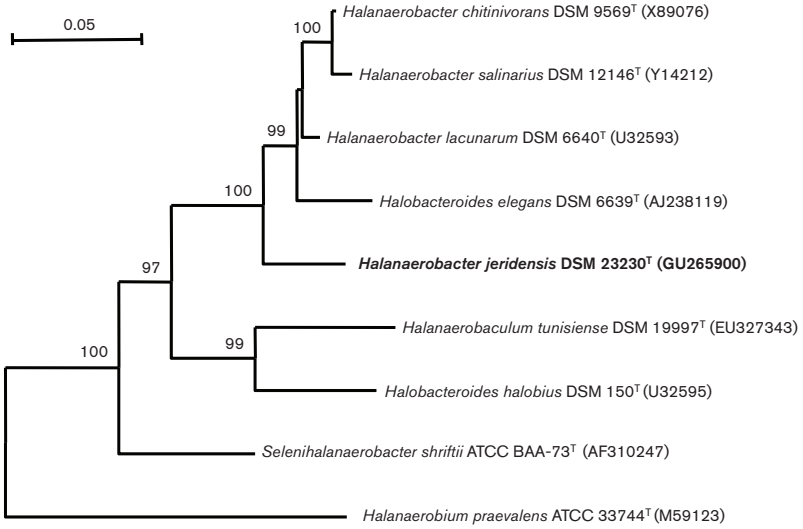

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between strain CEJFG43^T and closely related species in the family *Halobacteroidaceae*. *Halanaerobium praevalens* ATCC 33744^T was used as an outgroup. Bootstrap values >80 % (based on 1000 resampled datasets) are shown at nodes. Bar, 0.05 substitutions per nucleotide position.

Microbial Identification System (MIDI; Sasser, 1990). The predominant fatty acids of strain CEJFG43^T, like those of the recognized members of the genus *Halanaerobacter* (Oren, 2006), were found to be $C_{16:0}$ and $C_{16:1}$ (Table 1).

The genomic DNA G+C content of the novel strain (33.3 mol%) was also determined by the Identification Service of the DSMZ, by using HPLC to investigate DNA that had been extracted using the Wizard Genomic DNA Purification kit (Promega).

The 16S rRNA gene of strain CEJFG43^T was amplified by PCR, using the universal primers Rd1 and Fd1 (Weisburg *et al.*, 1991). The nearly complete sequence of the 16S rRNA gene (1423 nt) was aligned with the corresponding sequences of closely related taxa from the GenBank database by using programs provided by the Ribosomal Database Project II (Maidak *et al.*, 2001). All the sequences were imported and verified manually using version 5.0.9 of the BioEdit sequence editor (Hall, 1999). Overall, after positions of sequence and alignment uncertainty were omitted, 1228 positions of alignment were computed using the method of Jukes & Cantor (1969). Phylogenetic trees were then produced in the TREECON (Van de Peer & De Wachter, 1994) and PHYLIP software packages (Felsenstein, 1993) by using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms. In the neighbour-joining tree (Fig. 2), as in the maximum-likelihood and maximum-parsimony trees (data not shown), strain CEJFG43^T was clustered with members of the genus *Halanaerobacter*. In pairwise comparisons, the novel strain appeared most closely related to *H. lacunarum* DSM 6640^T (95.3 % 16S rRNA gene sequence similarity) and *H. chitinivorans* DSM 9569^T (also 95.3 %). Unlike *H. lacunarum* and *H. chitinivorans*, however, strain CEJFG43^T was able to ferment xylose, raffinose, pyruvate and glycerol (Table 2). Glycerol is considered to be one of the main carbon and energy sources for heterotrophic bacterial communities in hypersaline environments such as salterns because the microalgae (*Dunaliella* species) in such environments produce large amounts of this compound in response to the extracellular osmotic pressure (Ben-Amotz & Avron, 1973).

Based on the phylogenetic and phenotypic evidence, strain CEJFG43^T represents a novel species in the genus *Halanaerobacter* for which the name *Halanaerobacter jeridensis* sp. nov. is proposed.

Table 2. Differentiating physiological and biochemical characteristics of strain CEJFG43^T and its closest relatives in the genus *Halanaerobacter*

Strains: 1, CEJFG43^T (data from this study); 2, *H. lacunarum* DSM 6640^T (Zhilina *et al.*, 1991); 3, *H. chitinivorans* DSM 9569^T (Liaw & Mah, 1992). +, Positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3
Temperature range for growth (°C)	20–60	25–52	23–50
Optimum temperature (°C)	45	35–40	30–45
pH range for growth	5.5–9.5	6–8	6–8
Optimum pH	8.3	6.5–7	7
NaCl range for growth (%, w/v)	6–30	10–30	3–30
Optimum NaCl (%, w/v)	15	15–18	12–18
Assimilation of:			
Ribose	+	−	ND
Cellobiose	−	+	+
Raffinose	+	−	−
Galactose	+	−	−
Sorbose	−	+	−
Xylose	+	−	−
Pyruvate	+	−	−
Glycerol	+	−	−
Peptone	−	−	W
Elemental sulphur as electron acceptor	−	+	ND
DNA G+C content (mol%)	33.3	32.4	34.8

Description of *Halanaerobacter jeridensis* sp. nov.

Halanaerobacter jeridensis (je.ri.den′sis. N.L. masc. adj. *jeridensis* of El Jerid, the lake from which the type strain was recovered).

Cells are strictly anaerobic, motile rods (1.2×2.5–6 μm) that occur singly or in pairs. Mesophilic, with a maximum temperature for growth of 60 °C (optimum 45 °C). Grows with 6–30 % (w/v) NaCl (optimum 15 %) and at pH 5.5–9.5 (optimum pH 8.3). Ferments glucose, fructose, ribose, raffinose, galactose, mannose, sucrose, maltose, mannitol, xylose, trehalose, starch, pyruvate and glycerol but not arabinose, cellobiose, rhamnose, sorbose, lactose, succinate, fumarate, acetate, choline, glycine, leucine, serine, Casamino acids or peptone. Glucose is converted into lactate, ethanol, acetate, H_2 and CO_2. Does not use elemental sulfur, sulfate, thiosulfate, sulfite, fumarate, nitrate or nitrite as electron acceptors. The predominant cellular fatty acids are $C_{16:1}$ and $C_{16:0}$.

The type strain, CEJFG43^T (=DSM 23230^T=JCM 16696^T), was isolated from sediment collected from El Jerid lake in southern Tunisia. The genomic DNA G+C content of the type strain is 33.3 mol%.

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