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ORIGINAL PAPER

Characterization of *Halanaerobaculum tunisiense* gen. nov., sp. nov., a new halophilic fermentative, strictly anaerobic bacterium isolated from a hypersaline lake in Tunisia

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Abstract A new halophilic anaerobe was isolated from the hypersaline surface sediments of El-Djerid Chott, Tunisia. The isolate, designated as strain 6SANG, grew at NaCl concentrations ranging from 14 to 30%, with an optimum at 20–22%. Strain 6SANG was a non-spore-forming, non-motile, rod-shaped bacterium, appearing singly, in pairs, or occasionally as long chains (0.7–1 × 4–13 μm) and showed a Gram-negative-like cell wall pattern. It grew optimally at pH values between 7.2 and 7.4, but had a very broad pH range for growth (5.9–8.4). Optimum temperature for growth was 42°C (range 30–50°C). Strain 6SANG required yeast extract for growth on sugars. Glucose, sucrose, galactose, mannose, maltose, cellobiose, pyruvate, and starch were fermented. The end products from glucose fermentation were acetate, butyrate, lactate, H_2, and CO_2. The G + C ratio of the DNA was 34.3 mol%. Strain 6SANG exhibited 16S rRNA gene sequence similarity values of 91–92% with members of the genus *Halobacteroides*, *H. halobius* being its closest phylogenetic relative. Based on phenotypic and phylogenetic characteristics, we propose that this bacterium be classified as a novel species of a novel genus, *Halanaerobaculum tunisiense* gen. nov., sp. nov. The type strain is 6SANG^T (=DSM 19997^T = JCM 15060^T).

Keywords *Halanaerobaculum tunisiense* · Halanaerobiales · Anaerobic · Extreme halophilic

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Introduction

Members of the order Halanaerobiales inhabit a wide variety of saline to hypersaline ecosystems, both terrestrial (e.g., thalassohaline and athalassohaline lakes) and subterrestrial (e.g., oil reservoirs) (Rainey et al. 1995; Magot et al. 2000; Oren 2000, 2006). They form a coherent phylogenetic group within the *Bacillus/Clostridium* subphylum of the Gram-positive bacteria and are separated into two families, the Halanaerobiaceae and the Halobacteroidaceae, consisting of only anaerobic microorganisms. All these anaerobes, with the exception of *Halanaerobium lacusroseus* (Cayol et al. 1995), family Halanaerobiaceae, grow optimally at NaCl concentrations lower than 20%. By contrast, *H. lacusroseus* isolated from a hypersaline lake in Senegal (Africa) is the most halophilic species in the family Halanaerobiaceae, growing in the presence of NaCl concentrations ranging from 7.5 to 34%, with an optimum around 20% (Cayol et al. 1995). However, no halophilic representative of family Halobacteroidaceae has been reported to grow at NaCl concentration equal to or higher than 20% (w/v). Here, we describe the first strictly anaerobic, halophilic, fermentative bacterium isolated from sediments of a hypersaline lake in Tunisia (El-Djerid Chott) belonging to this family which grows optimally at 20% NaCl. As the phenotypic and phylogenetic characteristics of this bacterium are different from those of all previously described halophilic anaerobes, we propose that

it be classified as a novel species of a novel genus in this family, *Halanaerobaculum tunisiense* gen. nov., sp. nov. The type strain is 6SANG (DSM 19997 = JCM 15060).

Materials and methods

Origin of the strain

Strain 6SANG was isolated from the sediment of the hypersaline Lake El-Djerid located in southern Tunisia. Sediment samples containing 20% NaCl (w/w) were collected from the surface (20 cm). Temperature and pH were 21°C and 8.2, respectively. Samples were dispatched on the day of collection in sterile bottles and stored in ice boxes in our laboratory.

Enrichment, isolation and growth conditions

Strain 6SANG was isolated in medium containing (per liter) 1.0 g of NH_4Cl, 0.3 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.1 g of $CaCl_2{\cdot}2H_2O$, 250 g of NaCl, 0.1 g of KCl, 2.0 g of KNO_3, 1.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 20-mM glucose, 10 ml of the trace mineral element solution of Balch et al. (1979), and 1,000 ml of distilled water. The pH was adjusted to 8.3 with 10-M KOH. The medium was boiled under a stream of O_2–free N_2 gas and cooled to room temperature; 5- or 20-ml aliquots were then dispensed into Hungate tubes or serum bottles, respectively, under a stream of N_2–CO_2 (80:20) gas mixture, and the vessels were autoclaved for 45 min at 110°C. Before inoculation (5 ml culture medium), 0.1 ml 10% $NaHCO_3$ and 0.1 ml 10% $MgCl_2{\cdot}6H_2O$ were injected from sterile stock solutions.

Enrichments were performed in Hungate tubes or serum bottles inoculated with 10% of sample and incubated at 37°C. The culture was purified by repeated use of the Hungate roll tube method (Hungate 1969).

Optimum growth conditions

The pH, temperature and NaCl ranges for growth were determined in growth medium containing glucose as energy source. The medium was adjusted to different pH values (between 4.8 and 8.4) by injecting adequate amounts of $NaHCO_3$ or Na_2CO_3 from 10% sterile anaerobic stock solutions. Growth of strain 6SANG was tested at temperatures ranging from 30 to 50°C. NaCl requirement was determined by directly weighing NaCl in Hungate tubes before dispensing medium and volume taken up by salt and has been taken in account. Cultures were subcultured at least twice under the same experimental conditions before determination of growth rates.

The Gram reaction was determined with heat-fixed liquid cultures stained with Difco kit reagents.

Substrate utilization tests

Substrates were tested at a final concentration of 20 mM in growth medium that lacked glucose. To test for electron acceptors, sodium thiosulfate, sodium sulfate and elemental sulfur were added to the medium at final concentrations of 20 mM, 20 mM, and 2% (w/v), respectively.

Sporulation test

Heat resistance of cells was determined as follows: after incubation at 42°C, duplicated cultures were heated at 80, 90 or 100°C for 10 min and subcultured with 20% inoculum in fresh growth medium; thereafter, the resulting cultures were incubated at 42°C.

Light and electron microscopy

Light microscopy was performed as previously described (Cayol et al. 1994a). Exponentially grown cells were negatively stained with 1% sodium phosphotungstic acid (pH 7.2) or with 1% uranyl acetate. For preparation of thin sections, exponentially grown cells were centrifuged and fixed overnight in 1% w/v osmium tetroxide prepared in 0.75-M sucrose, or in other cases, the cells were fixed for 1 h in 3% w/v glutaraldehyde prepared in HCl-cacodylate buffer (pH 6.0) containing 0.7-M sucrose and fixed in osmium tetroxide as described above. After fixation, the cells were washed, embedded in 2% agarose and stained with 4% uranyl acetate. The agar was cut into small cubes, dehydrated in acetone and embedded in Araldite. Thin sections were stained with 5% uranyl acetate for 20 min and with 2% lead citrate for 10 min. Micrographs were taken with a Hitachi model H600 electron microscope at an accelerating voltage of 75 kV.

Analytical techniques

Unless otherwise indicated, duplicate culture tubes were used throughout. Growth was measured by inserting tubes directly into a model UV-160 A spectrophotometer (Shimadzu Corp., Kyoto, Japan) and measuring the optical density at 600 nm. Sulfide contents were determined photometrically as collodial CuS using the method of Cord-Ruwisch (1985). H_2, CO_2 and fermentation products (alcohols and volatile and non-volatile fatty acids) were measured as described previously (Fardeau et al. 1993).

Cellular fatty acid analysis

The whole-cell fatty acid composition was performed at DSMZ using cell material grown under identical conditions (culture medium contained 25% NaCl and glucose was used as energy source).

Determination of G + C content

The G + C content of DNA was determined at DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The DNA was isolated and purified by chromatography on hydroxyapatite, and the G + C content was determined by high-performance liquid chromatography (HPLC) as described by Mesbah et al. (1989). Non-methylated lambda DNA (Sigma) was used as standard.

16S rRNA sequence studies

The methods for the purification and extraction of DNA and the amplification and sequencing of the 16S rRNA gene have been described previously by Miranda-Tello et al. (2003) except for the use of primer Rd1 (5′-AAGGAGGTGATCCAGCC-3′) instead of primer R6. The 16S rRNA gene sequence we determined was imported into the sequence editor BioEdit version 5.0.9. (Hall 1999). Reference sequences were obtained from the Ribosomal Database Project II (Maidak et al. 2001) and GenBank databases (Benson et al. 1999). Positions of sequence and alignment uncertainty were omitted from the analysis. The sequence position and pairwise evolutionary distances based on 1,239 unambiguous nucleotides were computed using the method of Jukes and Cantor (1969). Dendrograms were constructed with Treecon program using the neighbor-joining method (Saitou and Nei 1987). Its topology was also supported by using the maximum-parsimony and maximum-likelihood algorithms.

Nucleotide sequence accession number

The 16S rRNA sequence of strain 6SANG has been deposited in the GenBank database under accession number EU327343.

Results

Enrichment and isolation

Enrichment cultures were positive after incubation at 37°C for 2 weeks. Colonies in roll tubes were round with entire edges, smooth, flat, opaque, and yellowish-cream. They were 0.5–1 mm in diameter after 3 weeks of incubation. Single colonies were picked, and serial dilution in roll tubes were repeated at least twice before the culture was considered pure. Several pure cultures similar in morphology and with the same profile for glucose metabolism were obtained. Strain 6SANG was retained for further characterization.

Cell properties

Strain 6SANG was a strictly anaerobic rod-shaped bacterium occurring singly and in pairs, or occasionally as long chains in young cultures with size ranging from 0.7–1 to 4–13 μm (Fig. 1). Electron microscopy of ultrathin sections of strain 6SANG revealed a Gram-negative-like thin, and wavy cell wall, with an irregular electron-density and a clear periplasm but stained Gram-positive. Spores were not observed, and no growth was obtained after pasteurization at 80°C for 10 min.

Optimum growth conditions

The optimal growth temperature of strain 6SANG was 42°C (range 30–50°C). The isolate was a halophilic bacterium growing optimally at 20–22% NaCl with growth occurring in the presence of NaCl concentrations between 14 and 30% NaCl (Fig. 2). The optimum pH for growth was 7.2–7.4 (range 5.9–8.4) (Table 1).

Substrates used for growth

Yeast extract or bio-trypticase was required for growth and could also be used as sole energy source. Strain 6SANG fermented carbohydrates, i.e., galactose, D-glucose,

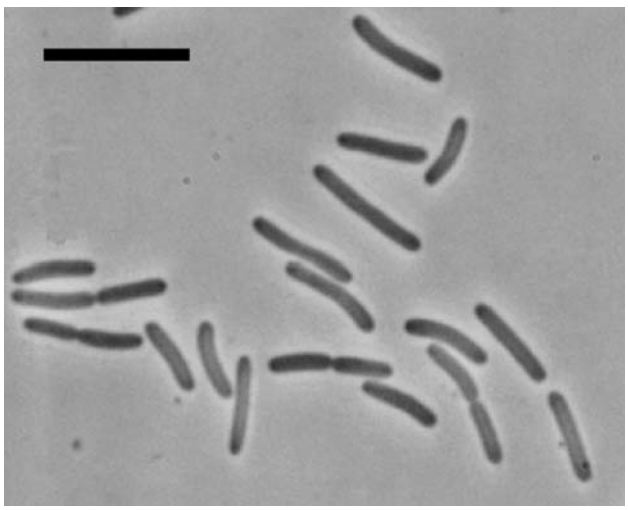

Fig. 1 Phase contrast photomicrograph of strain 6SANG (*Bar* 10 μm)

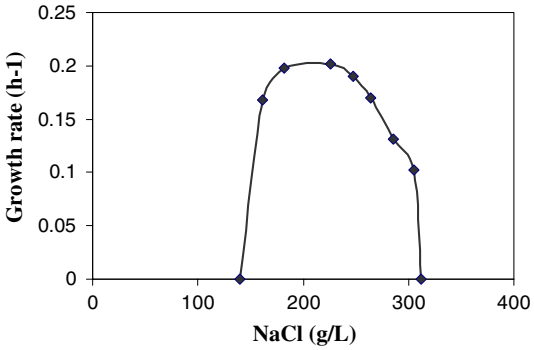

Fig. 2 Effect of NaCl concentration on growth rate of strain 6SANG cultivated in the basal medium containing glucose. Cultures were incubated at 37°C

maltose, D-mannose, sucrose, starch, and cellobiose. Pyruvate was also fermented. It could not utilize DL-fructose, mannitol, D-ribose, raffinose, succinate, D-xylose, fumarate, casamino acids, acetate or lactate. The end products of glucose fermentation were butyrate, lactate, acetate, CO_2, and H_2.

Its doubling time in the presence of glucose and yeast extract under optimal conditions was 2.1 h (Table 1). Thiosulfate, sulfate, sulfite, sulfur, nitrate, and nitrite were not used as electron acceptors.

Cellular fatty acid composition

The cell membrane fatty acid composition of strain 6SANG is shown in Table 2. Cellular fatty acids of *H. lacunarum* (DSM 6640^T), *Halobacteroides elegans* (Z-7287) and *Halobacteroides halobius* (MD-1) are also reported for comparison. Similarly to *H. halobius*, its closest phylogenetic relative, the major membrane fatty acids present in strain 6SANG were $C_{16:1}$ and $C_{16:0}$ acids; minor fatty acids were $C_{14:0}$ and $C_{10:0}$ acids.

G + C content

The G + C content of the DNA of strain 6SANG as determined by HPLC was 34.3 mol%.

16S rRNA sequence analysis

To analyze the phylogenetic position, the 16S rRNA gene sequence of strain 6SANG (comprising 1,498-bp) was determined. The sequence of strain 6SANG was aligned with the sequences of representatives of the family Halobacteroidaceae, and a phylogenetic tree was constructed (Fig. 3). The sequence was deposited in the GenBank database under accession number EU327343. The analysis indicated that strain 6SANG was a member of the low G + C (34.3%) containing Gram-positive phylum. The phylogenetic analysis indicated that strain 6SANG belonged to a subdivision of the family Halobacteroidaceae, forming a separate lineage in a cluster that also includes other genera of halophilic anaerobes, such as species of genus *Halanaerobacter* and *Halobacteroides*. The degree of similarity of the 16S rRNA of strain 6SANG and *H. halobius*, its closest relative, is 92% (Fig. 3).

Discussion

Members of the order Halanaerobiales have frequently been isolated from terrestrial or subterrestrial hypersaline habitats (Ollivier et al. 1994; Oren et al. 1984a). All of them are mesophilic chemoorganotrophic anaerobes growing fermentatively on sugars, *Halothermothrix orenii* being the only thermophilic representative of this order (Cayol et al. 1994b). Within the order Halanaerobiales, *H. lacusroseus*, isolated from a hypersaline African lake, represents the most halophilic microorganism, which grows optimally in the presence of 18–20% NaCl. Here, we report on a new halophilic anaerobe (strain 6SANG) of the order Halanaerobiales (optimum growth in the presence of 20–22% NaCl) isolated from a hypersaline Tunisian lake (El-Djerid Chott) that extends the optimum salinity for growth known for members of the family Halobacteroidaceae so far. Because of its atypical halophilism within this family, physiological studies of this isolate merit further attention to learn more about the biological mechanisms (e.g., osmoprotective processes) it uses to face extreme saline conditions when growing anaerobically. Phylogenetic analysis based on 16S rRNA sequences indicated that strain 6SANG had *H. halobius* (similarity of 92%) as its closest phylogenetic relative. Beside *H. halobius*, the genus *Halobacteroides* comprises another species, *H. elegans*, isolated from a cyanobacterial mat in the Crimea (Zhilina et al. 1997), which has very low phylogenetic similarity (87%) with our isolate. These two *Halobacteroides* species have similar metabolic patterns for glucose fermentation, acetate and ethanol being the major end products of metabolism. However, butyrate, and lactate, but not ethanol, are produced from glucose fermentation by the new isolate, which cannot therefore belong to genus *Halobacteroides*. Further differences between strain 6SANG and the type species of *Halobacteroides* (*H. halobius*) include not only the optimum NaCl concentration for growth, but also (1) the range of substrates used, and (2) the G + C content of the DNA (see Table 1).

Because of significant phenotypic, phylogenetic, and genetic differences between this strain and all the validly published members of the family Halanaerobiaceae, we propose that it be classified as the type strain of a novel species in a novel genus *H. tunisiense* gen. nov., sp. nov.

Table 1 Differentiation of strain 6SANG from other halophilic anaerobes

Characteristics	Strain 6SANG	*Halobacteroides halobius*[a] MD-1	*Halanaerobacter lacunarum*[b] DSM 6640^T	*Halobacteroides elegans*[c] Z-7287	*Halanaerobium lacusroseus*[d] (H200^T)
Morphology	Rods	Flexible rods	Flexible rods	Flexible rods	Rods
Cell size (μm)	0.7–1 × 4–13	0.5 × 10–20	0.7–1 × 0.5–6	0.3–0.5 × 2–10	0.5 × 2–3
Gram stain reaction	Positive	Negative	Negative	Negative	Negative
Motility	−	+	+	+	+
NaCl range (%)	17.5–35	5.8–17.5	10–30	10–30	7.5–34
Optimum NaCl (%)	25	8.8–14.6	15–18	10–15	18–20
Temp range (°C)	30–50	30–47	25–52	28–47	20–50
Optimum temp (°C)	42	37–42	35–40	40	40
pH range	5.9–8.4	ND[e]	6.0–8.0	6.5–8.0	ND
Optimum pH	7.2–7.4	6.5	6.5–7.0	7.0	7.0
Doubling time (h)	2.1	1	4.3	2	2.4
G + C content (mol%)	34.3	30.7	32.4	30.5	32
Use of substrates[f]					
Casamino acids	−	ND	ND	ND	+
Cellobiose	+	−	±	±	+
Fructose	−	+	+	+	+
Galactose	+	+	±	±	+
Lactate	−	−	−	±	ND
Mannitol	−	−	+	±	+
Maltose	+	+	+	±	+
Pyruvate	+	+	±	±	ND
Raffinose	−	+	−	−	ND
D-Ribose	−	−	±	−	+
D-Xylose	−	ND	ND	ND	+
Fermentation products from glucose	Butyrate, lactate, acetate, H_2, CO_2	Acetate, ethanol, H_2, CO_2	Acetate, ethanol, H_2, CO_2	Acetate, ethanol, H_2, CO_2	Ethanol, acetate, H_2, CO_2
Reduction of S°	−	ND	+	+	ND

[a] Data from Oren et al. 1984b

[b] Data from Zhilina et al. 1991

[c] Data from Zhilina et al. 1997

[d] Data from Cayol et al. 1995

[e] ND, not determined

[f] +, supported growth; −, did not support growth

[g] *p*-aminobenzoic acid

Description of *Halanaerobaculum* gen. nov.

Halanaerobaculum (Hal.an.ae.ro.ba'cu.lum. Gr. n. *hals,* salt; Gr. pref. *an,* not; Gr. n. *aer,* air; L. neut. n. *baculum*, stick; N.L. neut. n. *Halanaerobaculum*, salt stick not living in air).

Cells are Gram-negative, non-motile, non-sporulating rods appearing singly, in pairs, or occasionally as long chains, halophilic, obligate anaerobes, metabolize only carbohydrates. It grows at NaCl concentrations ranging from 14 to 30%, with an optimum at 20–22%.

The end products from glucose fermentation are butyrate, lactate, acetate, H_2 and CO_2.

The type species is *H. tunisiense* sp. nov.

Description of *Halanaerobaculum tunisiense* sp. nov.

Halanaerobaculum tunisiense (tu.ni.si.en'se. N.L. neutr. n., referring to Tunisia, the country where the bacterium was first recovered). Characteristics are the same as those described above for the genus. Rods are 0.7–1 by 4–13 μm. Growth at NaCl concentrations ranging from

Table 2 Cellular fatty acid compositions (%) of strain 6SANG^T, and members of phylogenetically related species

Fatty acid	Strain 6SANG	*Halobacteroides halobius*[a] MD-1	*Halanaerobacter lacunarum*[b] DSM 6640^T	*Halobacteroides elegans*[c] Z-7287
Major				
$C_{16:1}$	62.3	54	73.2	69.0
$C_{16:0}$	19.3	25.6	18.7	25.0
Minor				
$C_{14:0}$	8.1	11.9	4.6	5.2
$C_{14:1}$	ND	4.3	0.8	0.3
$C_{18:0}$	ND	0.4	1.0	0.4
$C_{18:1}$	ND	2.5	0.6	0.2
$C_{12:0}$ 3-OH	7.5	ND	ND	ND
$C_{10:0}$	2.6	ND	ND	ND

ND not detected

[a] Data from Oren et al. 1984b

[b] Data from Zhilina et al. 1991

[c] Data from Zhilina et al. 1997

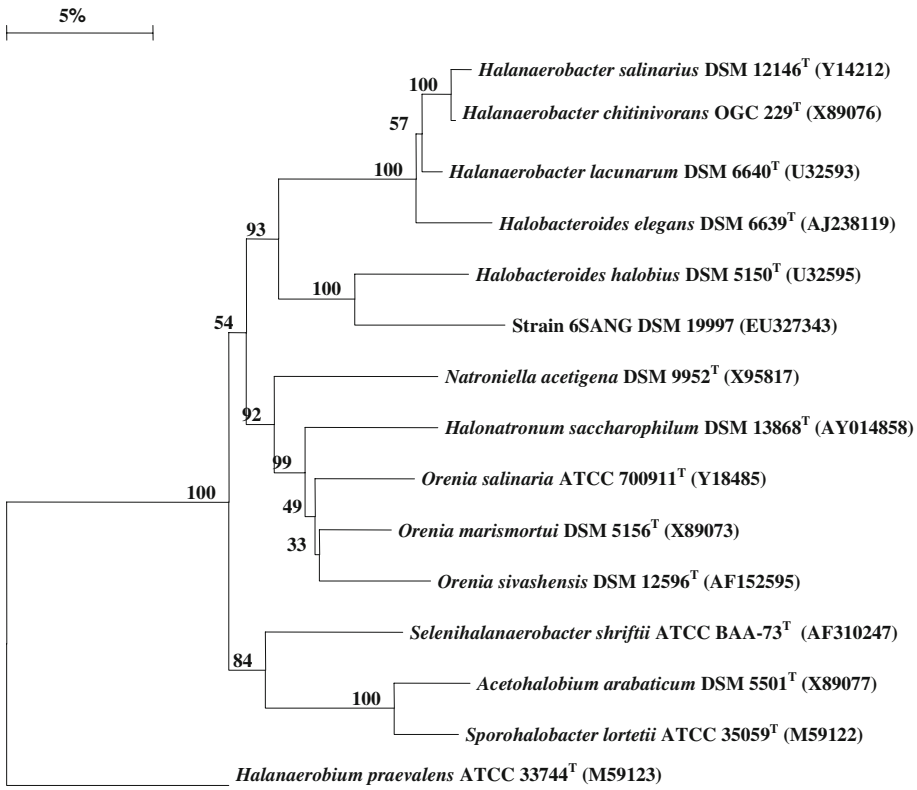

Fig. 3 *Dendrogram* showing the position of strain 6SANG, among members of the family Halobacteroidaceae. *Bar* indicates evolutionary distance

14 to 30%, with an optimum at 20–22%. Optimal growth at pH values between 7.2 and 7.4. Optimum temperature for growth is 42°C (range 30–50°C). Yeast extract is required for growth on sugars. Glucose, sucrose, galactose, mannose, maltose, cellobiose, pyruvate, and starch are fermented. The G + C ratio of the DNA is 34.3 mol%. 16S rRNA gene sequence similarity values with members of the genus *Halobacteroides* are 91–92%, *H. halobius* being its closest phylogenetic relative. The type strain is 6SANG, (=DSM 19997, =JCM 15060). Isolated from the hypersaline surface sediments of El-Djerid Chott, Tunisia.

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