Extremophiles (2011) 15:565–571
DOI 10.1007/s00792-011-0387-y

ORIGINAL PAPER

Characterization of *Halanaerocella petrolearia* gen. nov., sp. nov., a new anaerobic moderately halophilic fermentative bacterium isolated from a deep subsurface hypersaline oil reservoir

New taxa: *Firmicutes* (Class *Clostridia*, Order *Halanaerobiales*, *Halobacteroidaceae*, *Halobacteroides*)

G. Gales · N. Chehider · C. Joulian · F. Battaglia-Brunet · J.-L. Cayol · A. Postec · J. Borgomano · I. Neria-Gonzalez · B. P. Lomans · B. Ollivier · D. Alazard

Received: 25 March 2011 / Accepted: 27 June 2011 / Published online: 15 July 2011

Abstract An anaerobic, halophilic, and fermentative bacterium, strain S200^T, was isolated from a core sample of a deep hypersaline oil reservoir. Cells were rod-shaped, non-motile, and stained Gram-positive. It grew at NaCl concentrations ranging from 6 to 26% (w/v), with optimal growth at 15% (w/v) NaCl, and at temperatures between 25 and 47°C with an optimum at 40–45°C. The optimum pH was 7.3 (range 6.2–8.8; no growth at pH 5.8 and pH 9). The doubling time in optimized growth conditions was 3.5 h. Strain S200^T used exclusively carbohydrates as carbon and energy sources. The end products of glucose degradation were lactate, formate, ethanol, acetate, H_2, and CO_2. The predominant cellular fatty acids were non-branched fatty acids $C_{16:1}$, $C_{16:0}$, and $C_{14:0}$. The G + C mole% of the DNA was 32.7%. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain S200^T formed a distinct lineage within the family *Halobacteroidaceae*, order *Halanaerobiales*, and was most closely related to *Halanaerobaculum tunisiense* DSM 19997^T and *Halobacteroides halobius* DSM 5150^T, with sequence similarity of 92.3 and 91.9%, respectively. On the basis of its physiological and genotypic properties, strain S200^T is proposed to be assigned to a novel species of a novel genus, for which the name *Halanaerocella petrolearia* is proposed. The type strain of *Halanaerocella petrolearia* is strain S200^T (=DSM 22693^T = JCM 16358^T).

Keywords Anaerobic · Halophilic · Taxonomy · *Halanaerocella petrolearia* · Oil reservoir

Communicated by A. Oren.

The GenBank accession number for the 16S rRNA gene sequence of strain S200^T is FJ931099.

G. Gales · J. Borgomano
Université de Provence, Laboratoire de Géologie des Systèmes et des Réservoirs Carbonatés, EA 4234, case 67, 3 Place Victor Hugo, 13331 Marseille Cedex 03, France

N. Chehider · J.-L. Cayol · A. Postec · I. Neria-Gonzalez · B. Ollivier · D. Alazard (✉)
Laboratoire de Microbiologie IRD, UMR 180, Universités de Provence et de la Méditerranée, ESIL, case 925, 163 Avenue de Luminy, 13288 Marseille Cedex 9, France
e-mail: didier.alazard@univmed.fr

C. Joulian · F. Battaglia-Brunet
BRGM, Unité Ecotechnologies, 3 Avenue Claude Guillemin, BP 36009, 45060 Orléans Cedex 2, France

B. P. Lomans
Emerging Technologies—Subsurface, Projects & Technologies, Shell International Exploration and Production B.V, Kessler Park 1, 2288 GS Rijswijk, The Netherlands

Introduction

Mesophilic halophilic fermentative anaerobes have frequently been isolated from saline terrestrial and subterrestrial environments (Ollivier et al. 1994; Oren 2006). Despite the large microbial diversity of such microorganisms in terrestrial ecosystems (e.g., *Halanaerobium*, *Halobacteroides*, *Orenia*, *Halanaerobacter*), it appears quite restricted in the subterrestrial ones, especially oil field ecosystems, from where only members of the genus *Halanaerobium*, order *Halanaerobiales*, family *Halanaerobiaceae*, have been isolated so far.

Indeed, halanaerobes originating from oil field brines included *Halanaerobium acetethylicum*, (Rengpipat et al. 1988; Patel et al. 1995; Rainey et al. 1995), *Halanaerobium salsuginis* (Bhupathiraju et al. 1994; Trüper and De'Clari

1998), *H. congolense* (Ravot et al. 1997), and *H. kushneri* (Bhupathiraju et al. 1999). These four species are recognized as heterotrophic moderate fermentative halophiles growing optimally in the presence of around 10% NaCl.

We investigated the microbiology of core material from an onshore West African oil reservoir rock. This deep saline anaerobic reservoir belongs to a cretaceous formation, contemporary of the south Atlantic opening. Here we report on the first isolation from a deep hypersaline petroleum reservoir of a bacterium pertaining to the family *Halobacteroidaceae*, order *Halanaerobiales*.

Materials and methods

Origin of the sample

A subsurface core sample was obtained from an onshore oil reservoir located in Gabon at a depth of 1,153 m. Coring was performed in an unflooded oil field, which ensured the absence of contamination by any anthropogenic fluid brought to enhance oil recovery. Specifically designed techniques for coring in soft materials and adapted to integrity preservation were used (aluminium sleeves, adequate drilling fluid pressure). Formation waters exhibited salinities close to saturation. The sediment consisted in unconsolidated sandstone with quartz-felspar grains coated with green clays and salt crystals, underlying the transgressive Gamba Formation and thick salt deposits. It was stored immediately at −80°C until used. This core corresponded to evaporitic facies belonging to early Cretaceous (ca. 120 My). Local pressure and temperature were 120 bars and 43°C, respectively. Porosity and permeability of this formation were estimated at 24–32% and 1–3.5 Da. An alkaline pH value of 8.8 was measured under atmospheric condition. Only the central part of the core (excluding ca. 2 cm of the outer part of the 10 cm diameter core) was sampled in order to avoid a contamination possibly brought by the drilling fluids. Samples from the inside core were then homogenized in a sterilized mortar and dispatched into sub-samples for subsequent analyses. Core manipulation and sub-sample preparation were performed in an anaerobic chamber under an O_2-free nitrogen atmosphere.

Enrichment, isolation, and cultivation

Enrichment and isolation were performed in the following glucose-containing medium (per liter): NH_4Cl, 1.0 g; K_2HPO_4, 0.3 g; KH_2PO_4, 0.3 g; $MgCl_2$ $6H_2O$, 10 g; $MgSO_4$ $7H_2O$, 5 g; $CaCl_2$ $2H_2O$, 0.1 g; NaCl, 250 g; KCl, 0.1 g; yeast extract, 1 g; 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. It was boiled under a stream of O_2-free nitrogen gas and cooled to room temperature. Aliquots (5 or 20 ml) were then dispensed into Hungate tubes (Bellco Glass, Inc.) or serum bottles, respectively, under a stream of N_2–CO_2 (80:20) gas mixture, and the vessels were autoclaved for 30 min at 120°C. Before inoculation (5 ml culture medium), 0.1 ml 10% $NaHCO_3$, 0.1 ml 2% Na_2S, and 0.1 ml glucose (1 M) were injected from sterile stock solutions. This medium was anaerobically inoculated with 1 g of homogenized deep subsurface sandstone core sample and incubated under anaerobic conditions at 43°C for 7 days. From this enrichment culture, serial dilutions in roll-tubes with agar medium were positive after incubation at 43°C for 2 weeks. The culture was purified by repeated use of the roll-tube method (Hungate 1969). Other culture conditions were tested with the aim to isolate nitrate-reducing, sulfate-reducing, methanogenic, and autotrophic sulfur-oxidizing prokaryotes (Galès et al. in preparation).

Anaerobic enrichments were also set up to mimic the oil reservoir conditions, i.e., high pressure using a liquid-generated pressure system and energy and carbon sources only provided by the core material. The system consisted in a 2 l high-pressure reactor (Autoclave Engineer Inc.) pressurized with water using a simple pressure-generating pump. The pressure was transmitted into Hungate tubes through the rubber septa (Kallmeyer et al. 2003). The enrichment medium consisted of an anaerobic saline solution containing 250 g/l sea salts (Sigma) and 30% (v/v) homogenized sandstone without any carbon or energy source amendment. Hungate tubes filled up to capacity were incubated at in situ pressure (120 bars) and temperature (43°C) for 6 months.

Phenotypic characterization

Light-microscope examination of morphology was performed using a Nikon Eclipse 600 phase-contrast microscope. Gram reaction was performed by the Hucker staining method (Murray et al. 1994). Tests for sporulation and heat resistance of cells were determined as follows: cultures were heated at 80, 90, and 100°C for 10 and 20 min and then subcultured in fresh growth medium (20% inoculum). Growth experiments were performed in duplicate, using Hungate tubes. Cultures were subcultured at least twice under the same experimental conditions before determination of growth rates. The temperature, pH, and NaCl ranges for growth were determined in the optimized growth medium containing glucose as energy source. The temperature range for growth was tested from 20 to 55°C. For pH experiments, the medium was adjusted to different pH values (between 4.8 and 9) by injecting adequate amounts of $NaHCO_3$ or Na_2CO_3 from 10 and 8% (w/v) sterile anaerobic solutions, respectively. After autoclaving,

the pH of the medium was measured and re-adjusted with sterile KOH if necessary. NaCl requirement was determined by directly weighing NaCl in Hungate tubes before dispensing 5 ml growth medium. The volume taken up by salt had been taken into account. Substrate utilization was studied at a final substrate concentration of 20 mM in the presence of 0.2 g/l yeast extract. To test for electron acceptors, sodium thiosulfate, sodium sulfate, sodium sulfite, and elemental sulfur were added to the medium at final concentrations of 20 mM, 20 mM, 2 mM, and 2% (w/v), respectively. Sulfide contents were determined photometrically as collodial CuS using the method of Cord-Ruwisch (1985). Nitrate and nitrite were tested at concentration of 10 and 2 mM, respectively. The end products from glucose fermentation were determined by HPLC using an Aminex HPX-87H (Biorad) column with 5 mM H_2SO_4 as the mobile phase. Bacterial growth was measured by optical density with a Varian model Cary 50 Scan spectrophotometer at 580 nm by inserting tubes directly into the cuvette holder. Unless otherwise indicated, duplicate culture tubes were used throughout.

Chemotaxonomic characterization

The determination of fatty acid composition of strain S200^T was performed at the Identification Service of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Fatty acid methyl esters (FAMEs) were extracted from fresh biomass and identified following the procedure recommended by Microbial Identification System (MIDI, Sherlock Microbial Identification System Version 4.0, MIS Operating Manual March 2001, Newark, Del.). The MIS system was used to compare the fatty acid methyl esters of strain S200^T with fatty acid patterns stored in MIDI fatty acid database. The G + C content of the DNA was determined at the DSMZ. DNA was isolated and purified by chromatography on hydroxyapatite (Cashion et al. 1977) and its G + C content was determined by using HPLC as described by Mesbah et al. (1989). Non-methylated lambda DNA (Sigma) with a G + C content of 49.8 mol% was used as a standard.

Phylogenetic analysis

Genomic DNA of strain S200^T was extracted using the Wizard Genomic DNA Purification kit (Promega Corp. Madison, WI, USA), according to the instructions of the manufacturer. The 16S rRNA gene was amplified by PCR using the universal primers Rd1 (5′-AAGCGGTTGCCGC CGACCGACC-3′) and Fd1 (5′-AGAGTTTGATCCTGG CTCAG-3′) and its sequence was determined and analyzed (Maidak et al. 2001; Weisburg et al. 1991). For the high-pressure enrichment, genomic DNA was extracted (Fast DNA Spin Kit for Soil, Bio101) from the supernatant of the culture, the 16S rRNA gene was amplified with primers Fd1 and 1406R (5′-GACGGGCGGTGTGTRCA-3′), and cloned (TOPO-TA Cloning Kit, Invitrogen). 16S rRNA gene insert sequences sharing 1,360/1,364 identity with strain S200^T were found and the retrieved sequence was included in the phylogenetic analysis (MIGSAL5SN 16S rRNA gene sequence GenBank accession number: HQ676590). The nearly complete sequence (1,487 nt) of the 16S rRNA gene of S200^T was aligned with closely related sequences from GenBank database and the high-pressure enrichment using programs provided by the Ribosomal data Project II (Maidak et al. 2001). All the sequences were imported into the sequence editor BioEdit v 5.0.9 (Hall 1999). Positions of sequence with alignment uncertainties were omitted, and in total 1,228 positions of alignment were used in the analysis. A phylogenetic tree was inferred using algorithms implemented in the TREECON (Van de Peer and De Watcher 1994) and PHYLIP software packages (Felsenstein 1993).

Results and discussion

Phenotypic characteristics

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 phylogeny (99–100% similarity), in morphology and with the same profile with regard to glucose metabolism were obtained. Strain S200^T was retained for further characterization. Cells were long and thin rods (0.8–1.2 μm wide by 8–15 μm long) that stained Gram-positive. They showed a high degree of flexibility, and bent or curved cells (Fig. 1). Cells were non

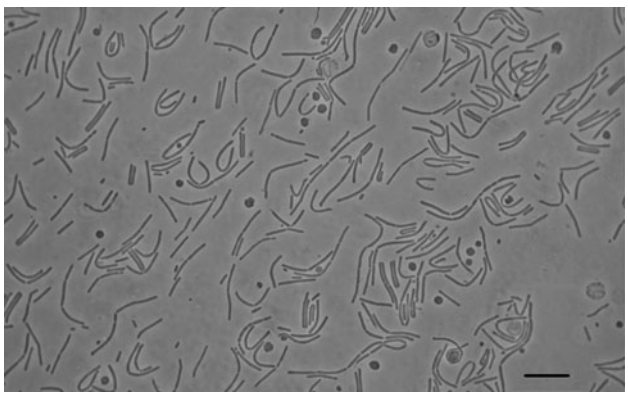

Fig. 1 Morphology of strain S200^T: vegetative cells of a culture under phase-contrast microscope, *bar* 10 μm

motile, appearing singly, in pairs or occasionally forming aggregates. Formation of spherical degenerating forms (spheroplasts) was observed at the end of the exponential growth phase, spherical shapes being predominant in old cultures. Interestingly, spheroplasts were also observed as well as rod-shaped forms in the high-pressure enrichment on the sandstone saline suspension.

Endospores were not detected either by phase-contrast microscopy or after pasteurization. Strain S200^T required anaerobic conditions for growth. The organism was an obligate halophile that grew in medium containing 6–26% NaCl. Optimal growth occurred in medium containing 15% NaCl. Strain S200^T was mesophilic and grew at temperatures ranging from 25 to 47°C, with an optimum at 40–45°C. The pH range was 6.2–8.8 (no growth at pH 5.8 and pH 9). The optimum pH was 7.3. The doubling time in optimized conditions was 3.5 h. Yeast extract (0.1%) was required for growth, but could not be used as sole carbon and energy source. Strain S200^T fermented exclusively carbohydrates, i.e., cellobiose, fructose, glucose, galactose, lactose, maltose, mannose, sucrose, xylose, pyruvate, and mannitol. Substrates that did not supported growth included lactose, ribose, raffinose, sorbose, succinate, fumarate, glycerol, lactate, acetate, casamino acids, gelatin, or peptone. The end products of glucose fermentation were lactate, formate, ethanol, acetate, CO_2, and H_2. Thiosulfate, sulfate, sulfite, sulfur, nitrate, and nitrite were not used as electron acceptors and did not stimulate the growth of isolate S200^T.

Phylogenetic characteristics

The nearly complete 16S rRNA gene sequence from strain S200^T was aligned with species of the order *Halanaerobiales* to verify its taxonomic position. Strain S200^T belongs to the family *Halobacteroidaceae*. Neighbor-joining analysis indicated that strain S200^T formed a distinct phylogenetic line within this family related to members of the genus *Halanaerobaculum* and *Halobacteroides* with a high bootstrap value, meaning that the phylogenetic topology is stable (Fig. 2). Analysis with the maximum-likelihood and maximum-parsimony algorithms also showed that strain S200^T formed a distinct phyletic clade (data not shown).

Comparative analysis of 16S rRNA gene sequences indicated that the isolate was most closely related to *Halanaerobaculum tunisiense* DSM 19997^T and *Halobacteroides halobius* DSM 5150^T with 92.3 and 91.9% similarity, respectively. Comparative analyses also indicated that isolate S200^T shared almost identical 16S rRNA gene sequence (1,360/1,364 bp) with the bacteria enriched in the high-pressure test on sandstone saline suspension (clone MIGSAL1-B5 and MIGSAL5SN on Fig. 2).

Strain S200^T exhibited similar phenotypic characteristics to members of the family *Halobacteroidaceae*

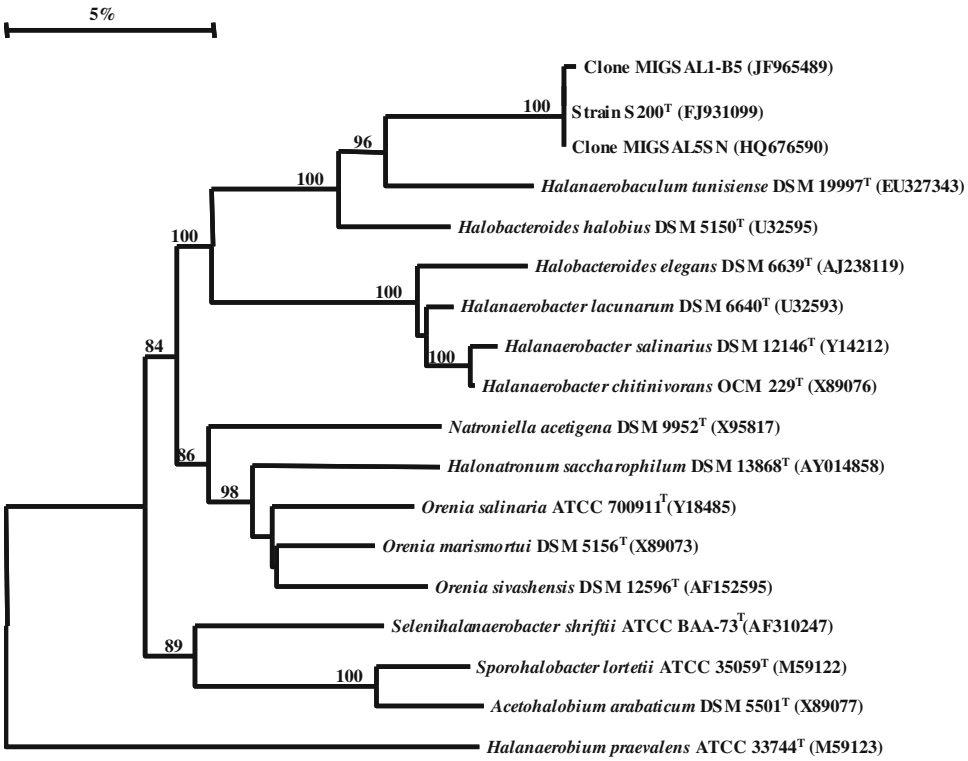

Fig. 2 Phylogenetic position of strain S200T among the family *Halobacteroidaceae* based on 16S rRNA gene sequence analysis. The tree was constructed using neighbor-joining algorithm. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. Bootstrap values, expressed as a percentage of 1000 replications, are shown at branching points. Only values above 80% are shown. The sequence of *Halanaerobium praevalens* was taken as an outgroup

Table 1 Main characteristics discriminating strain S200^T from its closest phylogenetic relatives *Halanaerobaculum tunisiense* and *Halobacteroides halobius*

Characteristics	Strain S200^T DSM22693^T	*Halanaerobaculum tunisiense*[a] 6SANG	*Halobacteroides halobius*[b] MD-1
Morphology	Flexible rods	Rods	Flexible rods
Cell size (μm)	15–8 × 0.8–1.2	13–4 × 0.7–1	10–20 × 0.3–0.5
Gram stain reaction	Positive	Positive	Negative
Motility	–	–	+
Temperature range (°C)	25–47	30–50	30–47
Temperature optimum (°C)	40–45	42	37–42
pH range	6.2–8.8	5.9–8.4	ND
pH optimum	7.3	7.2–7.4	6.5
NaCl range (%)	6–26	17.5–35	5.8–17.5
NaCl optimum (%)	15	25	8.8–14.6
Doubling time (h)	3.5	2.1	1
Use of substrates			
Cellobiose	+	+	–
Fructose	+	–	+
Raffinose	–	–	+
D-Xylose	+	–	–
Mannitol	+	–	–
Pyruvate	+	+	+
Fermentation products from glucose	Acetate, ethanol, formate, lactate, H_2, CO_2	Acetate, butyrate, lactate, H_2, CO_2	Acetate, ethanol, H_2, CO_2
DNA G + C mol%	32.7	34.3	30.7

[a] Data from Hedi et al. (2009)

[b] Data from Oren et al. (1984)

ND not determined, + supported growth, − did not support growth

including a rod shape with elongated cells and the formation of spheroplasts in late exponential growth phase (Oren et al. 1984). However, it differed from its closest relatives based on the range of substrates used (Table 1).

Chemotaxonomic characteristics

The whole-cell fatty acid composition of strain S200^T was similar to that of the type strains of *Halanaerobaculum tunisiense* and *Halobacteroides halobius* (Table 2). Major fatty acids were unsaturated $C_{16:1}$, saturated $C_{16:0}$, and $C_{14:0}$. No branched or cyclopropane chains were detected. The presence of large amounts of unsaturated fatty acids, mainly $C_{16:1}$ was in relation with the plasticity of their membranes. The DNA of strain S200^T showed a G + C content (32.7 mol%) which is in the range of other members of the family *Halobacteroidaceae*.

Table 2 Cellular fatty acids composition (%) of strain S200^T and members of phylogenetically related species

Compounds	% of total fatty acids		
	Strain S200^T DSM22693^T	*Halanaerobaculum tunisiense*[a]	*Halobacteroides halobius*[b]
C10:0	2.5	2.7	–
C10:0 3OH	0.6	–	–
C12:0	1.7	–	–
$C_{12:0}$ 3OH	5.3	7.5	–
C14:0	14.8	8.1	11.9
C14:1 ω9c	0.3	–	4.3
C16:0	16.5	19.3	25.6
C16:1 ω7c	7.0	6.5	–
C16:1 ω9c	47.0	52.2	54
C16:1 ω11c	2.9	3.6	–
C18:0	0.5	–	0.4
C18:1 ω9c	0.9	–	2.5

[a] Data from Hedi et al. (2009)

[b] Data from Oren et al. (1984)

Ecological aspects

The presence of microorganisms in oilfield production waters has been long thought to be the consequence of the drilling or oil recovery processes (Magot 2005). However, the concept of a deep biosphere constituted of adapted indigenous bacterial communities has been widely accepted by the scientific community (Teske 2005). Several criteria can be used to evaluate if an isolate can be considered as native to the formation of interest or not. This mainly includes the sampling technique and the

physiological properties of the microorganism to the in situ physico-chemical conditions of the oil reservoir (Magot 2005). Strain S200^T was isolated from a core sampled before oil exploitation and any flooding operation. Mineralogical and visual observation showed no penetration of the drilling fluid or mud into the inner core. Redox state of the clays showed no oxidation during core processing, indicating that the inner core was not chemically and therefore even less likely microbiologically contaminated, enhancing the probability to recover indigenous prokaryotes. Strain S200^T also grows anaerobically at temperature and pH existing in situ and under high saline conditions thus suggesting that it should be considered as indigenous to the saline oil reservoir that it inhabits.

Furthermore, the experiments that have been undertaken to enrich indigenous anaerobes at the temperature and high pressure of the oil reservoir and on a saline sandstone suspension (25% sea salts) without any other organic carbon or energy sources amendment allowed us to enrich bacteria sharing identical 16S rRNA gene sequence with that of strain S200^T. This result thus strengthens the indigenous feature of this isolate and strongly suggests that it is able to develop, although slowly, at high pressure and temperature conditions of the oil reservoir, consuming the energy and carbon sources available in situ. Compared with its closest relatives, strain S200^T is unique in its existence in subsurface. It has been isolated from oil-bearing sandstone core at depth of 1,153 m. The close relationship of strain 200^T and isolates from hypersaline environments like shallow sediments (*Halanaerobacter salinarius*, *Selenihalanaerobacter shriftii*), soda lake (*Natroniella acetigena*, *Halonatronum saccharophilum*) or chotts (*Halanaerobaculum tunisiense*), similar to the Cretaceous paleoenvironment, suggests that this strain could be a remnant of the bacterial population contemporary of the sediment deposition which has survived and evolved as long as the sediment was buried (Galès et al. in preparation).

Because of significant phenotypic, chemotaxonomic, and phylogenetic differences between strain S200^T and all the previously described members of the family *Halobacteroidaceae*, we propose it to be classified as the type strain of a novel species of a new genus: *Halanaerocella petrolearia* gen. nov., sp. nov.

Description of *Halanaerocella* gen. nov

Hal.an.ae.ro.cel'la. Gr.n. *hals*, salt; Gr. pref. *an,* not; Gr.n. *aer,* air; L.n.fem. cella, cell; N.L. fem. n. *Halanaerocella*, salt cell not living in air.

Cells stain Gram-positive, non-motile, non-sporulating rods occurring singly, in pairs, or occasionally as long chains. Halophilic. Obligate anaerobe metabolizing only carbohydrates. The end products from glucose fermentation are lactate, ethanol, acetate, formate, H_2, and CO_2.

The type species is *Halanaerocella petrolearia*

Description of *Halanaerocella petrolearia* sp. nov.

Halanaerocella petrolearia (pe.tro.le.a'ria L. fem. n. petra rock; L. fem. adj. olearia related to vegetal oil; petrolearia N. L. fem. adj. related to mineral oil). Description as for the genus.

Rods are 0.8–1 μm by 8–15 μm. Growth at NaCl concentrations ranging from 6 to 26%, with an optimum at 15%. Optimal growth at pH value of 7.3 (range 6.2–8.8: no growth at pH 5.8 and 9). Optimum temperature for growth is 40–45°C (range 25–47°C). Glucose, galactose, mannose, fructose, xylose, cellobiose, lactose, maltose, sucrose, and mannitol are fermented. Yeast extract is required for growth on sugars. The G + C ratio of the DNA is 32.7 mol%. 16S rRNA gene sequence similarity values with members of the genus *Halobacteroides* are around 91–92%, *Halanaerobaculum tunisiense* being its closest phylogenetic relative (92.3%). The type strain is S200^T (=DSM 22693^T = JCM 16358^T).

Isolated from deep subsurface oil-bearing sandstone core.

Acknowledgments We thank the SHELL petroleum company for its financial support, Prof. Jean Euzéby for his suggestion on Latin nomenclature for the novel microorganism, and Dr. Marie-Laure Fardeau for HPLC analysis.

References

Balch WE, Fox GE, Magrum RJ, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43:260–296

Bhupathiraju VK, Oren A, Sharma PK, Tanner RS, Woese CR, McInerney MJ (1994) *Haloanaerobium salsugo* sp. nov., a moderately halophilic, anaerobic bacterium from a subterranean brine. Int J Syst Evol Microbiol 44:565–572

Bhupathiraju VK, McInerney MJ, Woese CR, Tanner RS (1999) *Haloanaerobium kushneri* sp. nov., an obligately halophilic, anaerobic bacterium from an oil brine. Int J Syst Evol Microbiol 49:953–960

Cashion P, Holder-Franklin MA, McCully J, Franklin M (1977) A rapid method for the base ratio determination of bacterial DNA. Anal Biochem 81:461–466

Cord-Ruwisch R (1985) A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. J Microbiol Methods 4:33–36

Felsenstein J (1993) PHYLIP (Phylogeny interference package), version 3.5c. Department of Genetics, Manual version 3.3. University of Washington, Seattle

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Hedi A, Fardeau M-L, Safdi N, Boudabous A, Ollivier B, Cayol J-L (2009) Characterization of *Halanaerobaculum tunisiense* gen. nov., a new halophilic fermentative, strictly anaerobic bacterium isolated from a hypersaline lake in Tunisia. Extremophiles 13:313–319

Hungate RE (1969) A roll-tube method for the cultivation of strict anaerobes. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 3B. Academic Press, London, pp 117–132

Kallmeyer J, Ferdelman T, Jansen KH, Jorgensen BB (2003) A high-pressure thermal gradient block for investigating microbial activity in multiple deep-sea samples. J Microbiol Meth 55:165–172

Magot M (2005) Indigenous microbial communities in oil fields. In: Ollivier B, Magot M (eds) Petroleum microbiology. American Society for Microbiology Press, Washington, pp 21–34

Maidak BL, Cole JR, Lilburn TG, Parker CT Jr, Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM, Tiedje JM (2001) The RDP-II (Ribosomal Database Project). Nucleic Acids Res 29:173–174

Mesbah M, Premchandran U, Whitman WB (1989) Precise measurement of the G + C content of deoxyribonucleic acid by high performance liquid chromatography. Int J Syst Bacteriol 39:159–167

Murray RGE, Doetsch RN, Robinow CF (1994) Determinative and cytological light microscopy. In: Gerhardt P, Murray RGE, Wood WA and Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, pp 22–41

Ollivier B, Caumette P, Garcia JL, Mah RA (1994) Anaerobic bacteria from hypersaline environments. Microbiol Rev 58:27–38

Oren A (2006) The order *Haloanaerobiales*. In: Dworkin M et al (eds) The Prokaryotes, Vol 3, 3rd edn. Springer, New York, pp 809–822

Oren A, Weisburg WG, Kessel M, Woese CR (1984) *Halobacteroides halobius* gen. nov., sp. nov., a moderately halophilic anaerobic bacterium from the bottom sediments of the Dead Sea. Syst Appl Microbiol 5:58–70

Patel BKC, Andrews KT, Ollivier B, Mah RA, Garcia JL (1995) Reevaluating the classification of *Halobacteroides* and *Haloanaerobacter* species based on sequence comparisons of the 16S ribosomal RNA gene. FEMS Microb Lett 134:115–119

Rainey FA, Zhilina TN, Boulygina ES, Stackebrandt E, Tourova TP, Zavarzin GA (1995) The taxonomic status of the fermentative halophilic anaerobic bacteria: description of *Haloanaerobiales* ord. nov., *Halobacteroidaceae* fam. nov., *Orenia* gen. nov. and further taxonomic rearrangements at the genus and species level. Anaerobe 1:185–199

Ravot G, Magot M, Ollivier B, Patel BKC, Ageron E, Grimont PAD, Thomas P, Garcia J-L (1997) *Haloanaerobium congolense* sp. nov., an anaerobic, moderately halophilic, thiosulfate- and sulfur-reducing bacterium from an African oil. FEMS Microbiol Lett 147:81–88

Rengpipat S, Langworthy A, Zeikus JG (1988) *Halobacteroides acetoethylicus* sp. nov., a new obligately anaerobic halophile isolated from deep subsurface hypersaline environments. System Appl Microbiol 11:28–35

Teske A (2005) The deep subsurface biosphere as alive and well. Trends Microbiol 13:402–404

Trüper HG, De'Clari L (1998) Taxonomic note: erratum and correction of further specific epithets formed as substantives (nouns) "in apposition". Int J Syst Bacteriol 48:615

Van de Peer Y, De Watcher R (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Appl Biosci 10:569–570

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703