Anaerobranca californiensis sp. nov., an anaerobic, alkalithermophilic, fermentative bacterium isolated from a hot spring on Mono Lake

Vladimir Gorlenko,[1] Alexandre Tsapin,[2,3] Zorigto Namsaraev,[1] Tracy Teal,[4] Tatyana Tourova,[1] Diane Engler,[2] Randy Mielke[2] and Kenneth Nealson[2,3]

[1]Institute of Microbiology, Russian Academy of Science, Moscow 117811, Russia

[2]Jet Propulsion Laboratory/California Institute of Technology, Pasadena, CA 91109-8099, USA

[3]University of Southern California, Los Angeles, CA 90089, USA

[4]California Institute of Technology, Pasadena, CA 91125, USA

Correspondence
Vladimir Gorlenko
vgorlenko@mail.ru

A novel, obligately anaerobic, alkalithermophilic, chemo-organotrophic bacterium was isolated from the sediment of an alkaline hot spring located on Paoha Island in Mono Lake, California, USA. This rod-shaped bacterium was motile via peritrichous flagella. Isolated strains grew optimally in 5–25 g NaCl l^{-1}, at pH 9·0–9·5 and at a temperature of 58 °C and were fermentative and mainly proteolytic, utilizing peptone, Casamino acids and yeast extract. Optimal growth was seen in the presence of elemental sulfur, polysulfide or thiosulfate with concomitant reduction to hydrogen sulfide. Sulfite was also formed in an equal ratio to sulfide during reduction of thiosulfate. The novel isolate could also reduce Fe(III) and Se(IV) in the presence of organic matter. On the basis of physiological properties, 16S rRNA gene sequence and DNA–DNA hybridization data, strain PAOHA-1^T (=DSM 14826^T=UNIQEM 227^T) belongs to the genus *Anaerobranca* and represents a novel species, *Anaerobranca californiensis* sp. nov.

INTRODUCTION

Only a few species among the anaerobic bacteria are capable of growth at alkaline pH (pH optima > 8·5) and elevated temperatures (optima > 50 °C) (Wiegel, 1998). Of particular relevance to this report are two species of the genus *Anaerobranca* that have recently been described: *Anaerobranca horikoshii* (Engle *et al.*, 1995) and *Anaerobranca gottschalkii* (Prowe & Antranikian, 2001). Both are alkalithermophilic, fermentative anaerobes with temperature optima near 60 °C and pH optima of 8·5–9·5.

We studied the microbial community of alkaline hot springs on Paoha Island located in alkaline, hypersaline Mono Lake (California, USA): the temperatures at the outflow were in the range 72–94 °C, the pH was 9·5 and the total salt concentration was 25 g l^{-1} (Mono Basin Ecosystem Study Committee, 1987; Oremland *et al.*, 2000). From samples of biofilms collected from the beds of the springs, we isolated several strains belonging to the genus *Anaerobranca*. In this paper, we present the description of a novel species, the thermoalkaliphilic and halotolerant *Anaerobranca californiensis* sp. nov. (type strain PAOHA-1^T). Cultures of *A. horikoshii* DSM 9786^T and *A. gottschalkii* DSM 13577^T were used for comparative studies.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Anaerobranca californiensis* sp. nov. strains PAOHA-1^T and PAOHA-2 are AY064217 and AY064218.

METHODS

For isolation and enrichment of bacteria, the following basal medium (BM) was used (l^{-1}): KH_2PO_4, 0·5 g; NH_4Cl, 0·5 g; KCl, 0·5 g; NaCl, 25 g; Na_2SO_4, 0·5 g; $MgSO_4$, 0·2 g; $NaHCO_3$, 5 g; Na_2CO_3, 5 g; $Na_2S.9H_2O$, 0·3 g; trace-element solution (Pfennig & Lippert, 1966), 1 ml; vitamin B_{12}, 10 μg; yeast extract, 1 g; peptone, 2 g; cysteine hydrochloride, 0·25 g; thiosulfate, 20 mM. The pH of the medium was adjusted to 9·2–9·6 at 25 °C; the medium was dispensed into 60 ml screw-cap tubes or 500 ml bottles. The containers were filled completely so that no gas space remained and then incubated at 58 °C. Cultures were purified from single colonies by serial dilution on plates with Gel-Gro (ICN Biochemicals) as a solidifying agent (1·2 %, w/v). Incubation of the plates was done at 58 °C in anaerobic jars filled with pure nitrogen. For cultivation of two previously described representatives of the genus *Anaerobranca*, BM with the following modifications was used: for *A. gottschalkii* (l^{-1}), NaCl, 10 g; glucose, 2 g; no polysulfide; final medium pH, 9·3–9·7 at 25 °C; for *A. horikoshii* (l^{-1}), NaCl, 0·5 g; disodium fumarate, 1·5 g; Na_2CO_3, 1·8 g; $NaHCO_3$, 1·8 g; final medium pH, 9·3–9·7 at 25 °C. The pH was adjusted by adding 1 M HCl after the addition of $NaHCO_3$ (pH between 6·0 and 7·5) and pH values between 8·0 and 10·5 were obtained by varying the amount of sodium carbonate. In a medium containing yeast extract (0·1 g l^{-1}) as growth factor

and thiosulfate (20 mM), organic substrates were tested at a concentration of 2 g l^{-1}. Growth was estimated by measuring the turbidity of the medium at 650 nm with an HP-5342 spectrophotometer (Hewlett Packard). Enumeration of cells was performed by means of direct cell counting using a Reichert model 334 012 microscope.

Reduction of various inorganic compounds was tested on BM with peptone (2 g l^{-1}) and 0·1 g yeast extract l^{-1} for strain PAOHA-1^T and for *A. horikoshii*, and with glucose for *A. gottschalkii*. Inorganic substrates were added at the following concentrations: sulfate, 10 mM; thiosulfate, 10 mM; sulfite, 4 mM; polysulfide, 20 mM; nitrate, 5 mM; fumarate, 5 mM; Se(IV) as sodium selenite, 0·5 mM; Fe(III) in the form of ferric citrate, 10 mM; and Fe(III) in the form of insoluble ferric hydroxide, $Fe(OH)_3$. Elemental sulfur was also tested at a concentration of 10 g l^{-1}. Data were collected after 3 days incubation.

Iron reduction was determined by measuring Fe(II) using the ferrozine method (Stookey, 1970). Sterile samples were used as a control. Sulfide was measured colorimetrically using the methylene blue method (Trüper & Schlegel, 1964). Thiosulfate and sulfite concentrations were determined by iodometric titration with formaldehyde as a blocking agent for sulfite (Reznikov *et al.*, 1970). Selenite reduction was determined visually.

DNA extraction, DNA G+C content determination and DNA–DNA hybridization were performed according to standard protocols (Marmur, 1961; De Ley *et al.*, 1970). A PCR was performed on whole cells obtained from pure cultures. 16S rRNA genes were selectively amplified using primers 5′-GTTTGATCCTGGCTCAG-3′ (forward) and 5′-ACGGYTACCTTGTTACGACTT-3′ (reverse). PCR products were cloned using a TA cloning kit (Invitrogen). Sequencing was performed on a LI-COR sequencer by MWG Biotech (High Point, NC, USA).

Sequences were aligned manually with sequences obtained from the database of small-subunit rRNAs collected from the international nucleotide sequence library EMBL. The sequences were compared with the members of the *Bacillus–Clostridium* subphylum of the Gram-positive bacteria. Regions that were not sequenced in one or more reference organisms were omitted from the analyses. Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed by using the method of Jukes & Cantor (1969). The resulting phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with bootstrap analysis of 100 trees using programs of the TREECON package (Van de Peer & De Wachter, 1994). Bootstrap analysis (100 replications) was used to validate the reproducibility of the branching pattern of the trees.

RESULTS AND DISCUSSION

The inoculated bottles were incubated at 60 °C. After 12 h, thin, long, sometimes curved, rod-shaped bacteria (Fig. 1) were found. After purification by serial dilution on agar plates cultivated in an anaerobic jar and regrowth, the isolated strains were used for future investigation. The physiological properties of strain PAOHA-1^T were investigated in greater detail.

Cells of strain PAOHA-1^T were rod-shaped, 0·26–0·32 μm wide and 2·4–5·0 μm long (Fig. 1). The cells exhibited a low frequency of branch formation, and dividing cells were often visible (Fig. 1). The cells were peritrichously flagellated. Only single cells (Fig. 1) exhibited mobility in young cultures. PAOHA-1^T also formed slime clusters and lost motility during growth with peptone as the carbon source and polysulfide as the electron acceptor. Spores were not observed under any growth conditions. The cells stained Gram-negative in both the exponential and stationary growth phases, but an ultrathin-section electron micrograph revealed a Gram-positive-type cell wall (Fig. 1c).

Strain PAOHA-1^T was an obligate anaerobe that grew without the addition of reductants such as sulfide or dithionite. Growth did not occur under microaerophilic (resazurin test) conditions in liquid and solid media. However, the cells are not sensitive to oxygen and could be stored under aerobic conditions for several months at room temperature without losing viability.

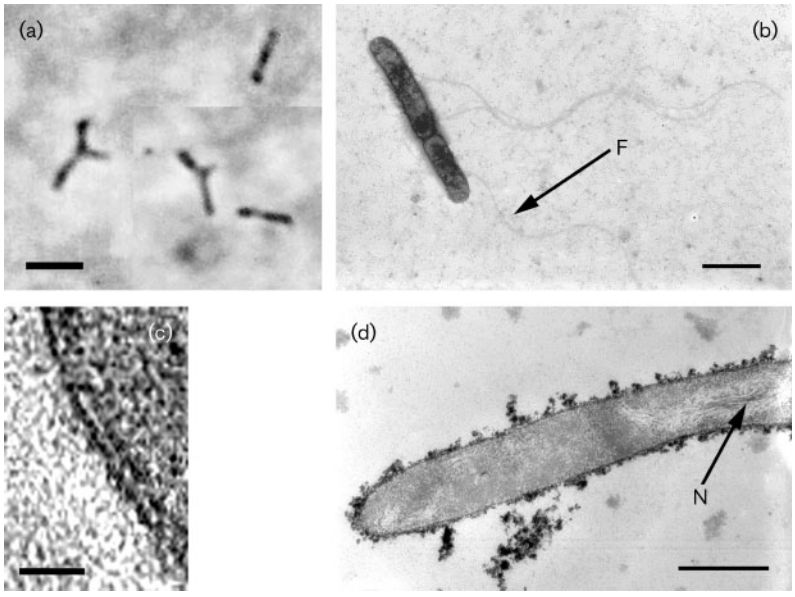

Fig. 1. Cell morphology of *A. californiensis* sp. nov. strain PAOHA-1^T growing on BM medium under optimal conditions. (a) Phase-contrast micrograph (bar, 2 μm); (b) total preparation stained with phosphotungstic acid (bar, 1 μm); (c) thin section showing cell-wall construction (bar, 50 nm); (d) thin section (bar, 0·5 μm). F, Flagellum; N, nuclear material.

Strain PAOHA-1^T was a moderate thermophile with a temperature range for growth of 45–70 °C, with an optimum of 58 °C. The pH range for growth at 58 °C was 8·6–10·4, with an optimum at 9·0–9·5 (measured at 25 °C). At temperatures between 47 and 58 °C, the NaCl concentration for growth (at pH 9·5) had a broad optimum from 5 g l^{-1} (85 mM) to 60 g l^{-1} (1 M), while at 70 °C a sharp maximum was observed at 25 g NaCl l^{-1} (430 mM). Under optimal conditions, the doubling time was 40 min.

In the presence and absence of thiosulfate, strain PAOHA-1^T grew on a variety of substrates, with a preference for peptides: the best carbon sources were peptone, tryptone, soytone peptone, yeast extract, malt extract and Casamino acids. Final concentrations greater than 10^8 cells ml^{-1} were reached with thiosulfate. Slow growth (and sulfide production from thiosulfate) was seen on fructose, sucrose, maltose, starch, glycogen, cellobiose and glycerol. Strain PAOHA-1^T was unable to utilize cellulose, glucose, lactate or acetate, but could utilize pyruvate as a carbon source, making the metabolism of strain PAOHA-1^T similar to that of *A. horikoshii* (Engle *et al.*, 1995). As with the other described anaerobic alkalithermophiles, strain PAOHA-1^T had a requirement for yeast extract that could not be satisfied by vitamins (Wiegel, 1998).

Growth without thiosulfate was 18–28 % of that achieved with thiosulfate (three observations). Sulfide was detected during the growth of strain PAOHA-1^T in the presence of sulfur compounds such as polysulfide, sulfur and thiosulfate. The maximal final concentration of sulfide was 40 mM when strain PAOHA-1^T was grown on media containing peptone (2 g l^{-1}), yeast extract (0·5 g l^{-1}) and sulfur. Sulfite was also formed in an equal ratio to sulfide during reduction of thiosulfate (Fig. 2). Sulfite is stable in alkaline media, and was not oxidized to sulfate. No dissimilatory fumarate, sulfate, sulfite or nitrate reduction was detected. Strain PAOHA-1^T was also able to reduce Se(IV), ferric citrate and hydrous ferric oxide. In control experiments (without micro-organisms), 5–7 % reduced iron was found, confirming the biological nature of the process. Extracellular magnetic material (possibly magnetite) was one of the end-products of Fe(III) reduction. The reduction of selenite (Na_2SeO_3) led to the formation of intermediate elemental selenium or polyselenites (red amorphous precipitate) and finally Se(II) as colourless sodium selenide (Na_2Se) (data not shown). We also showed that *A. horikoshii* and *A. gottschalkii* are able to reduce ferric citrate, selenite and elemental sulfur (Table 1).

The sequences of 16S rRNA genes for two strains, PAOHA-1^T and PAOHA-2, were obtained. The complete (1527 nucleotides) sequences of the 16S rRNA genes of these strains were determined. In the initial analysis, the 16S rRNA sequences of these strains were compared with the corresponding sequences from the EMBL RNA database. This analysis revealed that the novel isolates, PAOHA-1^T and PAOHA-2, were members of the *Clostridium–Bacillus* subphylum of the Gram-positive bacteria. Additional sequence alignment and phylogenetic analyses were performed with a set of related species of this subphylum. Positions of sequence and alignment uncertainty were omitted and a total of 1214 nucleotides were used in the analysis. According to this analysis, strains PAOHA-1^T and PAOHA-2 belong to the *Anaerobranca*-genus cluster with a maximum-level bootstrap value (100; Fig. 3). The 16S rRNA gene sequences of strains PAOHA-1^T and PAOHA-2 were almost identical (99·3 %) and showed some differences with respect to *A. horikoshii* (98·4–98·8 %) and *A. gottschalkii* (97·0–97·2 %) (Fig. 3).

The DNA G+C content of strain PAOHA-1^T was 30 mol%, which is similar to that of *A. gottschalkii* LBS3^T (30 mol%), but differs from the G+C content of *A. horikoshii* (33–34 mol%) (Table 2). DNA–DNA hybridization of strain PAOHA-1^T with the type strains of

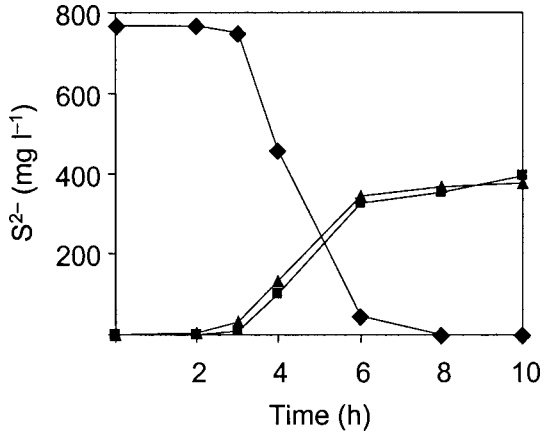

Fig. 2. Reduction of thiosulfate by strain PAOHA-1^T grown on BM medium. ◆, $S/S_2O_3^{2-}$; ▲, S/S^{2-}; ■, S/SO_3^{2-}.

Table 1. Sulfide production and ferric citrate reduction by different *Anaerobranca* species

Cells were grown on BM medium with modifications for *A. gottschalkii* and *A. horikoshii* (see Methods). Values are concentrations in mg l^{-1}.

Product	*A. californiensis* sp. nov.	*A. gottschalkii*	*A. horikoshii*
S^{2-} from:			
Polysulfide	480	10–20	130–185
S^0	288	140–185	165
Thiosulfate	110	76	66
Fe^{2+} from ferric citrate	210–360	380–420	310–350

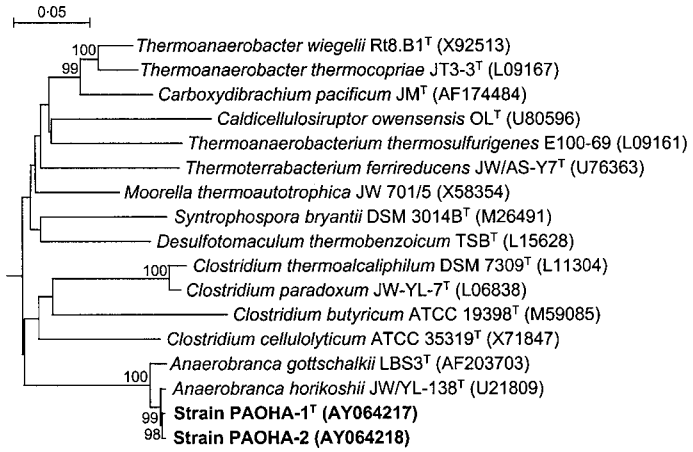

Fig. 3. Phylogenetic tree for *A. californiensis* strains PAOHA-1^T and PAOHA-2 and related organisms, based on 16S rRNA gene sequences and generated by the neighbour-joining method. Numbers at nodes indicate levels of bootstrap support (of 100 trees). Bar, 5 inferred changes per 100 nucleotides.

A. horikoshii and *A. gottschalkii* showed only 38 and 29 % relatedness, respectively. Relatedness between the type strains of *A. horikoshii* and *A. gottschalkii* according to our data was 51 %.

These data demonstrate clearly that the novel isolate represents a novel species within the genus *Anaerobranca*. This is also supported by the physiological and morphological data presented in Table 2. The main differences between these three species of the genus *Anaerobranca* are as follows: cell-wall properties, salt tolerance, the ability to ferment glucose and the ability to use fumarate as an electron acceptor.

We suggest placement of strains PAOHA-1^T and PAOHA-2 within a novel species, *Anaerobranca californiensis* sp. nov.

Description of *Anaerobranca californiensis* sp. nov.

Anaerobranca californiensis (ca.li.for.ni.en′sis. N.L. fem. adj. *californiensis* pertaining to California, the location of the hot spring from which the micro-organism was isolated).

Cells are rod-shaped and sometimes branching. Cells are 0·26–0·31 μm wide and 2·4–5·0 μm or more long. Division

Table 2. General characteristics of the three known species of the genus *Anaerobranca*

Data were obtained from the present study (*A. californiensis* sp. nov.), Prowe *et al.* (2001) (*A. gottschalkii*) and Engle *et al.* (1995) (*A. horikoshii*). All of these bacteria have rod-shaped, motile, peritrichously flagellated cells, sometimes showing branching. All are obligate anaerobes.

Characteristic	*A. californiensis* sp. nov.	*A. gottschalkii*	*A. horikoshii*
Habitat	Hot springs of Paoha Island in Mono Lake (CA, USA)	Hot inlet of Lake Bogoria (Kenya)	Hot spring at Old Faithful Hotel (Yellowstone National Park, USA)
Cell length (μm)	2·4–5	3–5	8–22
Cell diameter (μm)	0·26–0·31	0·3–0·5	0·5–0·65
Gram staining	Negative	Negative	Positive
Cell wall	Gram-positive, thin	Gram-positive, thin	Gram-positive, thick
pH for growth:			
Range	8·6–10·4[a]*	6·0–10·5[a]	6·5–10·3[b]
Optimum	9·0–9·5[a]	9·5[a]	8·5[b]
Temperature for growth (°C):			
Range	45–67	30–65	30–66
Optimum	58	50–55	57
NaCl concentration for growth (%, w/v):			
Range	0–6	0–4	ND
Optimum	1–2·5	1	ND
Type of metabolism	Fermenting proteins	Fermenting sugars	Fermenting proteins
Ammonification	+	ND	ND
DNA G+C content (mol%)	30	30	32–34

*pH measured at 20 (*a*) or 60 (*b*) °C.

occurs by binary fission. Spores are not observed. Colonies are 3–5 mm in diameter, pale-whitish and lens-shaped. Cell walls are of the Gram-positive type, but cells stain Gram-negative. Growth temperature ranges from 45 to 70 °C, with an optimum of 58 °C. The pH range for growth is 8·6–10·4, with an optimum of pH 9·0–9·5. Growth occurs at NaCl concentrations in the range 0–60 g l^{-1}, with an optimum of 5–25 g l^{-1}. Obligately anaerobic chemo-organotroph with fermentative metabolism. Able to grow on a variety of substrates, but grows preferentially on proteins and peptides. The best carbon sources are peptone, tryptone peptone, soytone peptone, Casamino acids, yeast extract and malt extract. Able to grow slowly on fructose, sucrose, maltose, starch, glycogen, cellobiose and glycerol in the presence of yeast extract as a growth factor. Cannot utilize glycogen, glucose or cellulose. Pyruvate can be used, but acetate and lactate do not support growth. No dissimilatory nitrate, fumarate, sulfate or sulfite reduction is detected. Optimal fermentative growth is seen in the presence of elemental sulfur, polysulfide or thiosulfate, with concomitant reduction to hydrogen sulfide. Sulfite and sulfide are formed in an equal ratio during reduction of thiosulfate. The organism has a high tolerance to sulfide (up to 40 mM). Capable of reducing, in addition to sulfur compounds, ferric citrate, insoluble ferric hydroxide and Se(IV) (as sodium selenite).

The type strain, PAOHA-1^T (=DSM 14826^T=UNIQEM 227^T), and strain PAOHA-2 were isolated from alkaline hot springs (pH 9·7, salinity 25 g l^{-1}, temperature 90 °C) located on Paoha Island in Mono Lake (California, USA). The DNA G+C content of the type strain is 30 mol%.

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