

Novel anaerobic thermoalkaliphilic bacterium *Anaerobranca californiensis* sp. nov., with dissimilatory reduction of sulfur, Fe(III) and other inorganic electron acceptors

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The GenBank accession numbers for the 16SrRNA sequences of *Anaerobranca californiensis* sp.nov strain Paoha-1 (DSMZ 14826), type strain is AY064217 and strain Paoha-2 is AY064218.

Summary

A novel obligately anaerobic thermoalkaliphilic bacterium was isolated from the beds of alkaline hot springs located on the shore line of Paoha island of Mono Lake (California, USA). This rod shape bacterium is motile via peritrichous flagella. The thin cell wall is of a Gram positive type, but cells stain Gram negative. The GC content of the DNA is 30.3 mol%. Isolated strains grow optimally in 10–25 g/l NaCl, at pH 9.0 – 9.5 and at a temperature of 58 °C and are chemoorganotrophic and mainly proteolytic, utilizing peptone, casamino acids, and yeast extract. Some sugars and carboxylic acids are slowly utilized. The best growth was found when S⁰, polysulfide or thiosulfate was used as an electron acceptor with concomitant reduction to hydrogen sulfide. Thiosulfate disproportionates to hydrogen sulfide and sulfite. The new isolate can also reduce Fe(III), Mn(IV), and Se (IV) on media that contains tryptone peptone and yeast extract. Based on physiological properties, the data of 16S rRNA sequence, and the data of DNA-DNA hybridization, the strain PAOHA-1 (DSM 14826) belongs to the genus *Anaerobranca* and represents a new species *Anaerobranca californiensis* sp. nov. For the first time it was shown that type strains of two other species of the genus *Anaerobranca* are able to reduce S⁰, polysulfide, thiosulfate, Fe(III), and Se (IV) on media with tryptone peptone for *A. horikoshii* and on media with glucose for *A. gottschalkii*. These three strains of the *Anaerobranca* genus differ in salt tolerance, ability to ferment glucose, and ability to use fumarate as an electron acceptor.

INTRODUCTION

Alkaline environments are found in only a few locations throughout the world (Grant *et al.*, 1990; Zavarzin, 1993; Jones *et al.*, 1998). Usually, such niches are rather isolated areas (lakes or seeps) often separated by many hundreds or thousands of kilometers. Such isolation raises questions about biodiversity and innovation, as presumably similar physiological and ecological strategies will be needed. Even more rare are alkaline environments combined with high temperatures. Most known alkaliphilic isolates are mesophiles belonging to different phylogenetic taxa (Grant *et al.*, 1990; Grant & Horikoshi, 1992; Duckworth *et al.*, 1996; Jones *et al.*, 1998). The anaerobic thermoalkaliphiles are a new group of extremophiles (Wiegel, 1998), and only few true (pH opt > 8.5, T opt > 50) species are known. These include two *Clostridium* species (*C. paradoxum*, *C. thermoalkaliphilum*), a methanogen (*Methanohalophilus zhilinae*), organoheterotrophic bacteria (*Thermosyntropha lipolytica*, '*Thermopallium natronophilum*', *Thermobrachium celere*, *Caloramator indicus*), and two sulfur reducing Archaea (*Thermococcus alkaliphilus*, *T. fumicolans*) (Blotevogel *et al.*, 1985; Horikoshi, 1991; Keller *et al.*, 1995; Li *et al.*, 1993; Engle *et al.*, 1993, 1996; Svetlitsnyi *et al.*, 1996; Godfroy *et al.*, 1996; Mathrani *et al.*, 1988; Duckworth *et al.*, 1996; Chrisostomos *et al.*, 1996). Of particular relevance to this report, though, are two species of the genus *Anaerobranca* that have recently been described: *A. horikoshii* (Engle *et al.*, 1995) and *A. gottschalkii* (Prowe & Antranikian, 2001). Both of these organisms are thermoalkaliphilic fermentative anaerobes with temperature optima near 60°C, pH optima of 8.5-9.5 and phylogenetically distinct from other anaerobic alkalithermophiles.

It was shown that thiosulfate and elemental sulfur are reduced to hydrogen sulfide during growth of *A. gottschalkii* on glucose, but other electron acceptors have not been tested. There was no information available about ability of *A. horikoshii* to use inorganic electron acceptors.

We studied the microbial community of an alkaline hot spring with temperatures of up to 94°C and a total salt concentration of over 25 g/l, which is located on Paoha island (In: The Mono Basin Ecosystem, 1987) of Mono Lake (California, USA). We isolated several strains belonging to the genus *Anaerobranca*. In this paper we present the description of a new species, the thermoalkaliphilic and halotolerant (PAOHA-1) *A. californiensis* sp. nov. This organism can reduce elemental sulfur, polysulfide, thiosulfate, Se(IV), Fe(III) and Mn(IV) during its metabolism. We also tested type strains of *A. horikoshii* and *A. gottschalkii* for their ability to reduce the same inorganic electron acceptors.

METHODS

Sample collection.

During the summers of 2000 and 2001 water and biofilms were collected from rocks in the outlets of hot springs on Paoha Island. The temperatures of the sample sites ranged from 45 to 94°C and the pH was between 9.0 and 9.8. Usually the samples were kept at ambient temperature until inoculation in the laboratory. Some samples were inoculated directly after sampling.

A. horikoshii (DSM 9786) and *A. gottschalkii* (DSM 13577) cultures were obtained from the German Collection of Microorganisms (DSMZ), Braunschweig, Germany.

Media and culture conditions.

The basal mineral enrichment medium (BEM) contained (per L): 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; $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$, 0.3 g; trace element solution (Pfennig & Lippert, 1966), 1 ml; vitamin B12, 10 μg ; yeast extract, 2g; soytone peptone, 1 g; polysulfide, 0.02% (w/v). Pure cultures were grown in the BEM in early experiments, but most of the working cultures were grown on modified BEM so that it contained 2 g/L of peptone instead of soytone peptone and 0.25 g/l cysteine hydrochloride was added as source of reduced sulfur. After mixing of all sterilized components, the pH of the media was adjusted to 9.2-9.6 at 25°C and then was dispensed into 60-ml screw-cap tubes or 500-ml bottles. The containers were completely filled so that no gas space remained, and incubated at 58°C. The cultures were purified from single colonies by serial dilution on plates with Gel-Gro[™] (ICN Biochemicals, Ohio, USA) as a solidifying agent (1.2%). 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* the medium with following composition were used:

For *A. gottschalkii* (per L)

KH_2PO_4 , 0.5 g; NH_4Cl , 0.5 g; KCl , 0.5 g; NaCl , 10 g; Na_2SO_4 , 0.5 g; MgSO_4 , 0.2 g; Na_2CO_3 , 5 g; NaHCO_3 , 5 g; glucose, 2 g; cysteine hydrochloride, 0.25 g; yeast extract, 1 g; peptone, 1 g; trace element solution (Pfennig & Lippert, 1966), 1 ml; vitamin B12, 10 μg . Final medium pH was 9.3-9.7 at 25°C.

For *A. horikoshii*: (per L)

KH_2PO_4 , 0.5 g; NH_4Cl , 0.5 g; KCl , 0.5 g; NaCl , 0.5 g; Na_2SO_4 , 0.5 g; MgSO_4 , 0.2 g; Na_2 -fumarate, 1.5 g; Na_2CO_3 , 1.8 g; NaHCO_3 , 1.8 g; $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$, 0.125 g; cysteine hydrochloride, 0.25 g; yeast extract, 1 g; peptone, 1 g; trace element solution (Pfennig & Lippert, 1966), 1 ml; vitamin B12, 10 μg . Final medium pH was 9.3-9.7 at 25°C.

Cellular characteristics

Light microscopy was done with a Nikon Eclipse E600 phase contrast microscope. Gram staining was done using the Hucker method according to Gerhardt *et al.*, 1994. Transmission electron microscopy was performed with a model JEM-100 electron microscope (JEOL, Tokyo, Japan). For negative staining of whole cells we used a 1% solution of phosphotungstic acid. Ultrathin sections of glutaraldehyde fixed cells were prepared using uranyl acetate and lead citrate for poststaining as described previously (Frasca & Parks 1965, Bryantseva *et al.*, 1999).

Growth condition and substrate spectrum

Temperature, pH and NaCl concentration ranges were determined by growing cells in the BEM with thiosulfate as the electron acceptor instead of polysulfide. The influence of temperature on growth was studied by incubation in a gradient thermostat at temperatures between 30-70°C for 12 hours. Salt concentration varied between 0 and 10% at optimum pH. The pH range for growth was determined at 58°C. The pH was adjusted by adding 1M HCl after adding NaHCO_3 (pH between 6.0 – 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) and thiosulfate (20 mM) as

the electron acceptor, the following substrates were tested as electron donors at a concentration of 0.2% (w/v): ribose, mannose, fructose, sucrose, lactose, galactose, maltose, , trehalose, arabinose, raffinose, cellulose, cellobiose, glycogen, pectin , starch, pullulan, xylan, xylose, glycerol, mannitol, xylitol, acetate, formate, fumarate, malate, lactate, pyruvate, methanol, purine, glycine, alanine, yeast extract, malt extract, peptone, soytone peptone, tryptone peptone, casamino acids, H₂.

Dissimilatory inorganic compound reduction

Utilization of various electron acceptors was tested on the BEM with peptone (2 g/l) and 100 mg/l yeast extract for strain PAOHA – 1 and for *A. horikoshii* and with glucose for *A. gottschalkii*. Cysteine hydrochloride (0.25g/l) was also added to the medium in all experiments. Electron acceptors were added at the following concentrations : sulfate, 10mM; thiosulfate, 10mM; sulfite, 4mM; polysulfide (H₂S₄) 20 mM; nitrate, 5mM; fumarate, 5mM; Se(IV) as sodium selenate, 0.5mM; Mn(IV) as manganese dioxide, 10mM; Fe(III) in the form of ferric citrate, 10mM and Fe(III) in the form of insoluble ferric hydroxide Fe(OH)₃ (prepared by boiling iron hydroxide made with 0.5mL of 2M FeCl₃, 1.5 mL of 5M NaOH, centrifuged and washed 3 times for a final concentration of ~18mM per 40 mL culture). Elemental sulfur was also tested at a concentration of 1% (w/v). Serum bottles containing anaerobic medium at pH 9.5 were incubated at 58°C .

Chemical analyses

Analytical methods.

Growth was estimated by measuring the turbidity of the medium at 650 nm with an HP-5342 spectrophotometer. Iron reduction was determined by measuring Fe(II) using the ferrozine method (Stookey, 1970). Formation of magnetic Fe –product was detected using its positive reaction to the magnetic field of a stationary magnet. Reduction of manganese was detected visually by observing the color change as Mn(IV) solid was reduced to colorless and soluble Mn(II). 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). Selenide reduction was determined visually, when the red amorphous precipitate of colloidal elemental Se was biologically reduced to soluble colorless selenide (after exposure to oxygen, samples containing selenide were rapidly oxidized to elemental Se and re-precipitated). Ammonium content was measured using a kit (Merck, Germany).

Amplification and sequencing of 16S rRNA genes.

PCR was performed on whole cells obtained from pure cultures. The 16S rRNA genes were selectively amplified using primers 5'-GTTTGATCCTGGCTCAG-3' (forward) and 5'-ACGGYTACCT-TGTTACGACTT-3' (reverse). PCR products were cloned using a TA cloning kit (Invitrogen, Carlsbad, California). Sequencing was performed on a Licor by MWG Biotech (High Point, North Carolina).

16S rRNA gene sequence analysis.

The 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 *Proteobacteria*.

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 Jukes & Cantor method (Jukes and Cantor, 1969). A 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.

DNA isolation and determination of G+C content

DNA was isolated in the exponential growth phase by the method of Marmur (Marmur, 1961). Guanine plus cytosine (G+C) content was estimated from renaturation rates (De Ley et al., 1970)

DNA-DNA hybridization

DNA was isolated by the known method (Sambrock et al., 1989). For DNA-DNA hybridization the DNAs (1 µg/ml) obtained from the all cultures were labelled with the ³H-dNTP by using "Nick translation Kit" № 5500 (Amersham Pharmacia Biotech). DNA (1 mg) was immobilized on 0.22 µm nylon membranes ("Hiiu Kalur", Estonia) and hybridized to various radioactive labeled probes (specific activity 5x10⁶ dpm/µg). The hybridization mixture (Sambrock et al., 1989) was incubated for 24 h at optimal conditions: $T_{inc} = 0,51 GC\% + 0,47 - 0,61 \cdot (\% \text{ formamide})$.

RESULTS AND DISCUSSION

Isolation

Serum bottles containing anaerobic (BEM) medium with soytone peptone (0.02%) and yeast extract (0.01%) as carbon sources and thiosulfate 16 mM as an electron acceptor at pH 9.5 were inoculated with water and biofilm material collected from beds of Paoha springs at temperatures ranging from 45 to 94°C. The inoculated bottles were incubated at 60°C. After 12 hours thin, long, sometimes curved rod shaped bacteria (Fig. 1) were found. After purification by serial dilution on Gel-Grotm media and re-growth, the isolated strains were used for future investigation. The sequences of 16sRNA genes for two strains, PAOHA-1 and PAOHA-2, isolated from hot springs with temperatures of 92°C and 84°C, respectively, were obtained. The physiological properties of the PAOHA-1 strain were investigated in greater detail.

Cellular characteristics

Morphology and cell construction

Cells of *Anaerobranca californiensis* strain PAOHA-1 are 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 with only single cells (Fig. 1) exhibiting mobility in young cultures. PAOHA-1 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. Although the cells were stained as Gram negative in both the exponential and stationary phases of growth, electron microscopy of ultrathin

sections revealed a Gram-positive type thin cell wall (Fig. 2). The outer layers of the cell wall were irregular and had high electron density and seemed to be protein-like in nature. The cytoplasm was granular, and sometimes had large inclusions of an as yet unidentified low electron density material. Cell wall composition and cell size of *Anaerobranca californiensis* is similar to *A. horikoshii* (Engle et al., 1995) but differs from *A. gottshalkii*, which has larger cells with thick cell walls (Prowe & Antranikian, 2001) (Table 1).

Growth conditions

Anaerobranca californiensis PAOHA-1 is an obligate anaerobe that grows without addition of reductants such as sulfide and dithionite. The cells were not sensitive to oxygen and could be stored under aerobic conditions for several months at room temperature without losing viability.

Temperature and pH

The pH range for growth and production of sulfide on the medium with peptone (2 g/l), yeast extract (0.1 g/l) and thiosulfate (20 mM) at 58°C was from 8.6 – 10.4 with optimum 9.0 – 9.5 at 25°C (Fig. 3). There was practically no growth at pH 7 and 10.3. Using polysulfide (20mM) as an electron acceptor, the cell growth and the formation of sulfide was possible in a very narrow pH range between 8.5 and 10.3. Thus the bacterial strain *Anaerobranca californiensis* PAOHA-1 is considered alkaliphilic. *Anaerobranca californiensis* PAOHA-1 is a moderate thermophile with a temperature range for growth and sulfide formation on polysulfide of 45 – 67°C with an optimum of 58°C (Fig. 4). Very slow growth was detected at 35°C and 70°C (data not shown).

Effect of NaCl and total Na⁺

The dependence of sulfide formation by strain PAOHA-1 at pH 9.5 and temperatures between 47°C and 58°C on NaCl concentration had a broad optimum from 5g/l (85mM) to 60g/l (1M) (Fig . 5). However, the dependence of sulfide formation on NaCl concentration at 70°C had a sharp maximum at 2.5% (430mM) NaCl. The total Na⁺ concentration (NaCl + sodium carbonates) for optimal growth was between 310 to 775 mM depending on temperature. For comparison the optimal growth of *A. horikoshii* was observed at 8.5 mM Na⁺ and at 230mM for *A. gotschalkii* (Prowe & Antranikian, 2001).

Substrate for fermentation and growth

PAOHA-1 is proteolytic microorganism exhibiting growth on a variety of substrates with a preference for peptides. The best carbon sources are peptone, tryptone, soytone peptone, yeast extract, malt extract, and casamino acids (Table 2). Slow growth (and sulfide production from thiosulfate) is seen on fructose, sucrose, maltose, starch, glycogen, dextrose, cellobiose, and glycerol in the presence of yeast extract (0.01%) as a growth factor (Table 2, Fig. 6). Strain PAOHA-1 is unable to utilize cellulose, glucose, lactate, or acetate, but can utilize pyruvate as a carbon source. Thus the metabolism of *Anaerobranca californiensis* is close to the metabolism of *A. horikoshii*.

Reduction of inorganic compounds

No dissimilatory fumarate, sulfate, sulfite or nitrate reduction was detected. Sulfide was detected during the growth of strain PAOHA-1 in the presence of sulfur compounds such as polysulfide, sulfur and thiosulfate. The maximal final concentration

of sulfide was 40 mM when *A. californiensis* strain PAOHA-1 was grown on media containing peptone (2g/l), yeast extract (0.5 g/l), and sulfur as the electron acceptor. Sulfite was also formed in an equal ratio to sulfide during reduction of thiosulfate (Fig. 7). Thus, thiosulfate reduction by *A. californiensis* involves only sulfane sulfur as has been shown for *Proteus vulgaris* (Barret & Clark, 1987). Sulfite is stable in alkaline media, and in our experiments was not oxidized to sulfate. We showed that *A. horikoshii* and *A. gotschalkii* are also able to reduce thiosulfate with concomitant production of sulfide using tryptone-peptone (for *A. horikoshii*) or glucose (for *A. gotschalkii*) as carbon sources. Thiosulfate reduction has been shown for many anaerobic thermophilic bacteria performing the fermentative metabolism, but there is not much information about the products of thiosulfate reduction and enzymatic mechanism of this process (Ravot, *et al.*, 1995, Fardeau *et al.*, 2000).

In addition to reducing sulfur compounds during growth on peptides and yeast extract, PAOHA-1 was able to reduce Se(IV), Fe(III), and Mn(IV) (Tables 3 and 4). The reduction of selenite (Na_2SeO_3) led to the formation of intermediate elemental Se^0 or polyselenites and finally Se(II) as sodium selenide (Na_2Se).

We found that *A. californiensis* strain PAOHA-1 reduces Fe (III)-citrate very actively compared to its reduction of elemental sulfur or thiosulfate. In the chemical control (without microorganisms) only a small amount of reduced iron was found, which confirms the biological nature of the process. Extracellular magnetic material (possibly magnetite) was one of the end products of Fe (III) reduction. We showed that *A. horikoshii* and *A. gotschalkii* also have the ability to reduce ferric citrate (Tables 3 and 4). *A. californiensis* strain PAOHA-1 can also reduce hydrous ferric oxide during growth on heterotrophic media containing tryptone-peptone (2 g/l) and yeast extract (1g/l). Thus, for the first time thermoalkaliphilic obligately anaerobic bacteria capable of reducing these inorganic acceptors have been isolated. The possible role of bacteria in transformation of sulfur compounds, metals and oxidized salts of toxic metals in these specific alkaline and highly mineralized thermal springs of Paoha island is a question of a strong interest. Minerals produced by these bacteria in alkaline and high temperature conditions can differ from minerals produced by bacteria with a similar metabolism, but in environments with the pH close to neutral.

Phylogenetic and taxonomic position.

The complete (comprising 1527 nucleotides) sequences of the 16S rRNA gene of two strains, PAOHA-1 and PAOHA-2, were determined. In the initial analysis, the 16S rRNA sequence of these strains was compared with the corresponding sequences from RNA data base. This analysis revealed that the new isolates, PAOHA-1 and PAOHA-2, were members of *Clostridium/Bacillus* subphylum of Gram-positive bacteria.

Additional sequence alignment and phylogenetic analyses were performed with a set of the validly published related species of this subphylum. Positions of sequence and alignment uncertainties were omitted, and a total of 1214 nucleotides were used in the analysis. According to this analysis, strains PAOHA-1 and PAOHA-2 belong to the *Anaerobranca*-genus cluster with a maximum level (100) bootstrap value (Fig. 8).

The 16S rRNA sequences of strains PAOHA-1 and PAOHA-2 were almost identical (99.3%) and showed some differences with *A. horikoshii* (98.4 - 98.8%) and *A. gotschalkii* (97.0-97.2%) (Table 5 and Fig. 8).

The DNA G+C content of *Anaerobranca californiensis* PAOHA-1 is 30.3 mol% , which is similar to that of *Anaerobranca gotschalkii* LBS3^T (30.9 mol%), but differs from the G + C content of *Anaerobranca horikoshii* (33 - 34.3 mol%). (Table 1)

DNA-DNA hybridization of *A. californiensis* strain PAOHA-1 with type strain of *A. horikoshii* showed only 38% relatedness and with type strain of *A. gottschalkii* - 29% (Table 6). Homology between type strains of *A. horikoshii* and *A. gottschalkii* according to our data was 51%.

These data demonstrate clearly that the new isolate is a new species within the genus *Anaerobranca*. This fact is also supported by the physiological and morphological data presented in Table 1. The main differences between these three species of the *Anaerobranca* genus are: cell wall properties, salt tolerance, ability to ferment glucose and ability to use fumarate as an electron acceptor.

We suggest the name for newly isolated bacteria *Anaerobranca californiensis* sp. nov.

Description of *Anaerobranca californiensis* sp. nov.

Anaerobranca californiensis (ca.li.for.ni.en.sis, from California).

Cells are rod shaped and sometimes branching. Cell size is 0.26-0.31 μm wide and 2.4-5.0 μm or more in length. Division occurs by constriction. Colonies are 3-5 mm in diameter, pale-whitish and lens-shaped. Cell walls are thin, Gram-positive type, other membranes are absent, but cells stain as Gram negative. *A. californiensis* is an obligate anaerobe.

Growth temperatures range from 45-67 °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 occurred from 0 to 6% NaCl (w/v) with an optimum of 1-2.5%.

Able to grow heterotrophically on a variety of substrates, but it prefers 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, dextrose, cellobiose and glycerol in the presence of yeast extract (0.01%) as a growth factor. Cannot utilize glycogen, glucose and cellulose. Pyruvate can be used, but acetate and lactate do not support growth. No dissimilatory fumarate reduction, sulfate reduction, sulfite reduction or nitrate reduction was detected.

The growth is very rapid in the presence of polysulfide, sulfur or thiosulfate as an electron acceptor. During growth with sulfur compounds as electron acceptors sulfide was detected. The organism has a high tolerance to sulfide (up to 40 mM). In the presence of thiosulfate (20 mM) about 10 mM sulfide was formed. Besides sulfide, an equal ratio of sulfite (1:1) was formed during reduction of thiosulfate. Along with sulfur compounds capable of using Fe(III)-citrate and Fe(III)-hydroxide, Se(VI) as sodium selenate, and Mn(IV) as manganese oxide as electron acceptors.

Isolated from alkaline hot springs (pH 9.7, salinity 25 g/l, temperature 90°C) located on Mono Lake's Paoha Island (California, USA).

The DNA G+C content is 30.3 mol% for type strain.

DNA-DNA hybridization of *A. californiensis* strain PAOHA-1 with type strain of *A. horikoshii* showed 38% relatedness and with type strain of *A. gottschalkii* - 29%.

Type strain: PAOHA-1 (DSMZ 14826).

GenBank accession number (16S rRNA): AY064217.

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- Fig. 8. Phylogenetic tree for *Anaerobranca californiensis*, strains PAOHA-1 and PAOHA-2 and related organisms based on 16S rDNA sequences. Bar = 5 inferred changes per 100 nucleotides. After Latin name lead the strains number and accessions number of strains in GenBank.

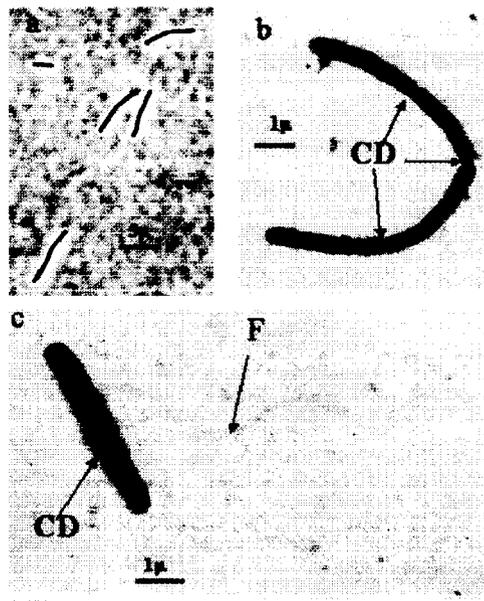


Fig. 1

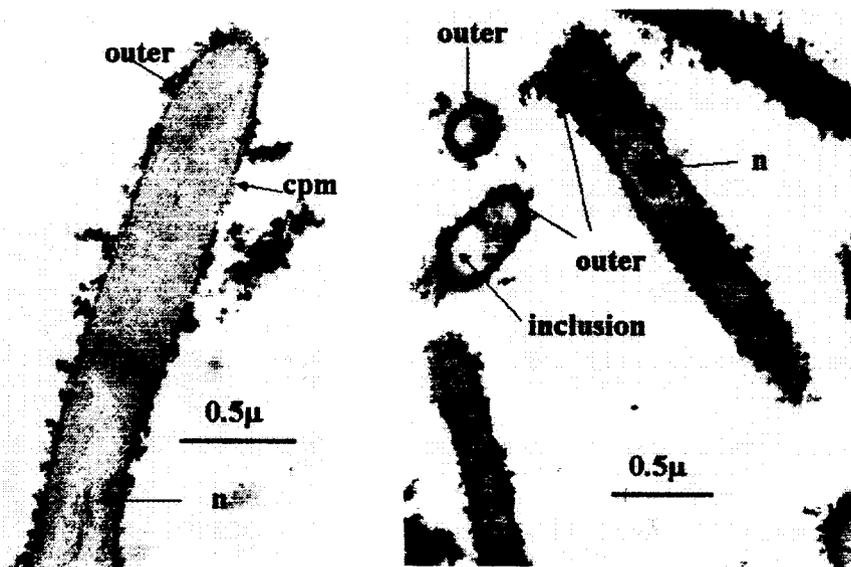


Fig. 2

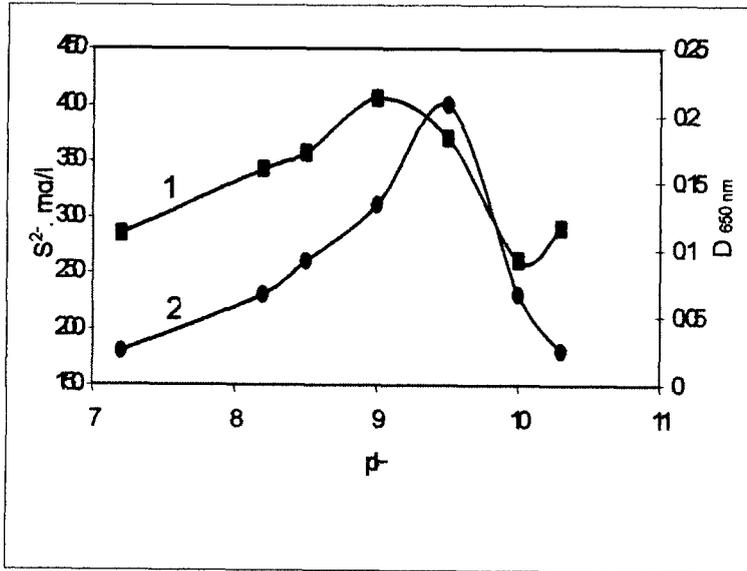


Fig. 3, pH effect on growth

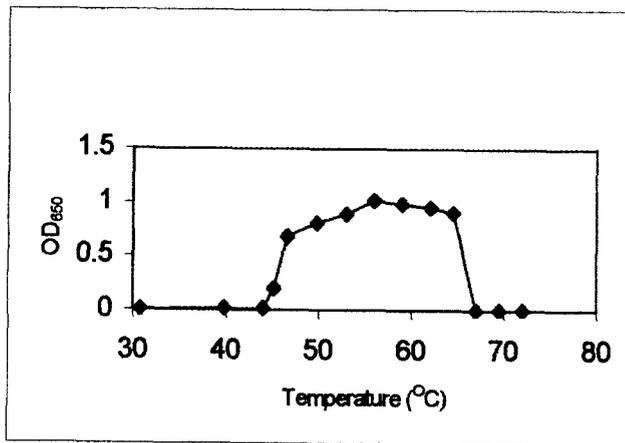


Fig. 4. Effect temperature on growth

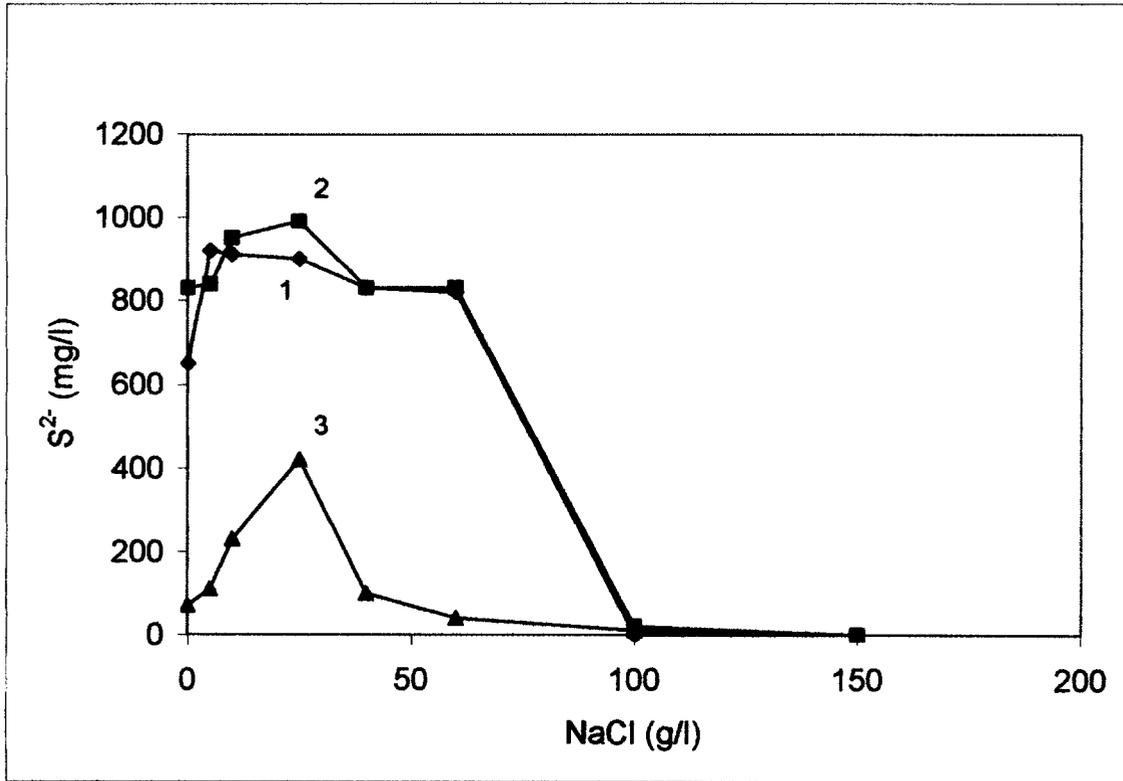


Fig. 5. Effect NaCl on growth at different temperatures

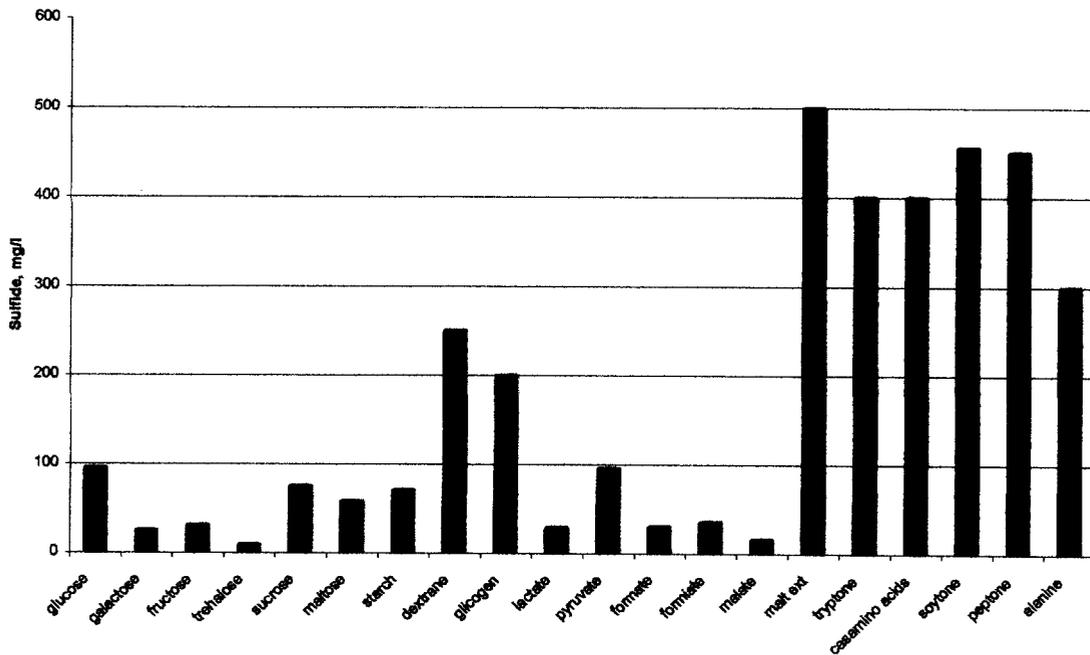


Fig. 6. Effect different substrates on sulfide production by *Anaerobranca californiensis*

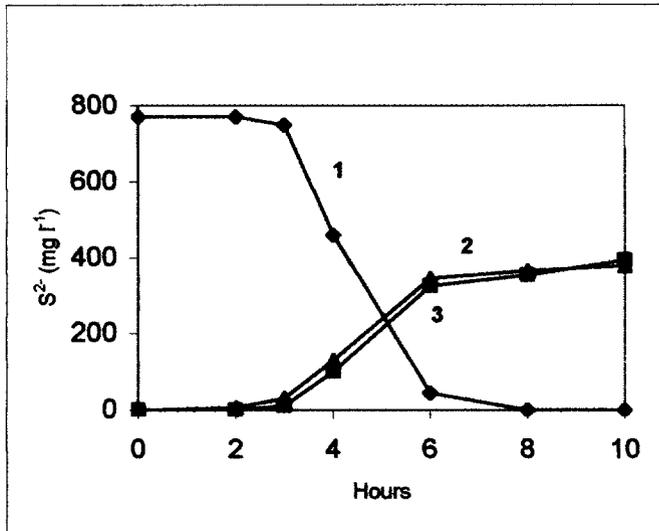


Fig. 7. Reduction of thiosulfate and sulfide formation by *Anaerobranca californiensis*

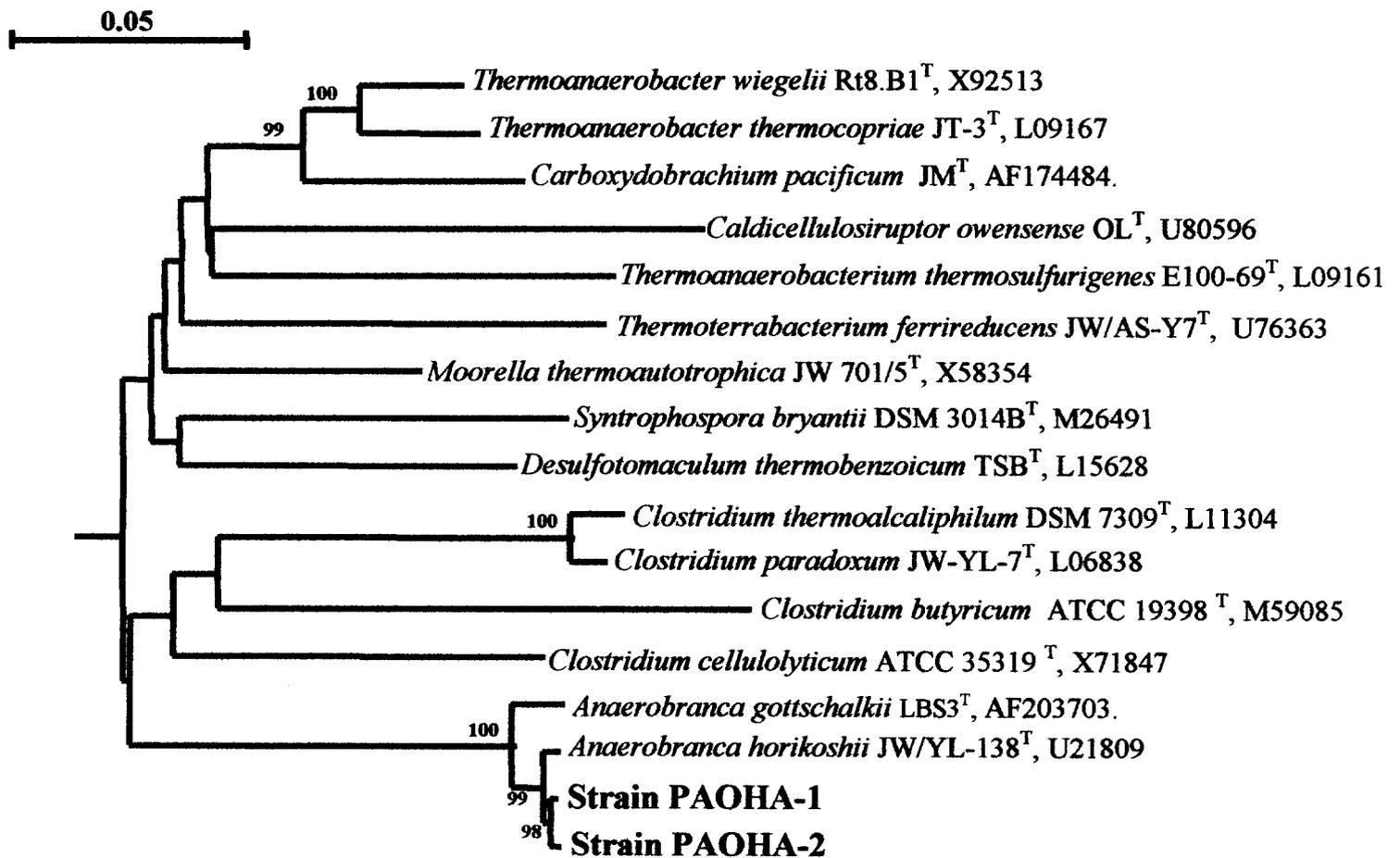


Fig. 8. Phylogenetic tree for *Anaerobranca californiensis*

Table 2. Carbon sources for *A. californiensis* and other two species of genus *Anaerobranca*

Characteristic	<i>Anaerobranca californiensis</i>	<i>Anaerobranca gottschalkii</i>	<i>Anaerobranca horikoshii</i>
Carbon source:			
Glucose	-	++	-
Ribose	-	+	-
Mannose	ND	++	
Fructose	+	++	-
Sucrose	+	++	-
Lactose	ND	+	-
Galactose	+	+	-
Maltose	+	++	-
Trehalose,	-	ND	ND
Arabinose	-	ND	ND
Raffinose	ND		-
Cellulose	-	+	-
Glycogen	+	ND	ND
Pectin	ND	ND	-
Starch	+	++	-
Pullulan	ND	+	
Xylan	ND	-	-
Glycerol	+	+	-
Acetate	-	-	-
Fumarate	+	ND	ND
Malate	+	+	-
Lactate	+	ND	-
Pyruvate	+	ND	-
Methanol	-	ND	-
Glycine	-	ND	-
Alanine	++	ND	ND
Casamino acids	++	ND	-
Yeast extract	++	++	++
Malt extract	+	ND	ND
Soyton peptone	++	ND	ND
Tryptone peptone	++	++	++
Peptone	++	++	++
H ₂ -CO ₂	ND	-	-

Table 3. Alternative electron acceptors for *Anaerobranca californiensis* and other two species of genus *Anaerobranca*

Electron acceptors and products of reduction:	<i>Anaerobranca californiensis</i>	<i>Anaerobranca gottschalkii</i>	<i>Anaerobranca horikoshii</i>
Fumarate to succinate	-	-	+
NO ₃ ⁻ to N ₂ O	-	ND	ND
NO ₃ ⁻ to N ₂ O to N ₂	-	ND	ND
NO ₃ ⁻ to NH ₄ ⁻	-	ND	ND
SO ₄ ⁻ to S ²⁻	-	-	-
SO ₃ ⁻ to S ²⁻	-	ND	ND
S ₂ O ₃ ⁻ to S ²⁻ + SO ₃ ⁻	++	+	+
S ⁰ to S ²⁻	++	+	+
S _n ⁻ to S ²⁻	++	-	+
Fe (III)-citrate - to Fe (II)	++	++	++
Fe(III)(OH ₃) - to Fe(II)	+	ND	ND
Mn (IV) - to Mn (II)	+	+	-
Se (IV)O ₃ ²⁻ to Se ⁰ to Se ²⁻	++	++	+

Table 4. Sulfide production from various sulfur compounds and ferric citrate reduction by different *Anaerobranca* species

	<i>Anaerobranca californiensis</i>	<i>Anaerobranca gottschalkii</i>	<i>Anaerobranca horikoshii</i>
	S ²⁻ , mg/l		
Polysulfide	480	10-20	130-185
S ⁰	288	140-185	165
S ₂ O ₃	110	76	66
	Fe ²⁺ , mg/l		
Fe(III)-citrate	210-360	380-420	310-350

Table 5. The level of difference between strains of *A. californiensis* and two other species of genera *Anaerobranca*.

	Level of difference according 16S rRNA data			
	1	2	3	4
1 PAOHA-1				
2 PAOHA-2	0.719	0.000	1.592	2.958
3 <i>A. horikoshi</i>	1.159	1.592	0.000	2.824
4 <i>A. gottschalkii</i>	2.816	2.958	2.824	0.000

Table 6. Levels of DNA-DNA hybridization between different species of *Anaerobranca* genus

Species and strains	Homology, %		
	1	2	3
1. <i>A. californiensis</i> DSMZ 14826 ¹	100		
2. <i>A. horikoshii</i> DSMZ 9786 ¹	38	100	
3. <i>A. gottschalkii</i> DSMZ 13577 ¹	29	51	100

Table 1. General characteristics (properties) of *A. californiensis* and other members of genera *Anaerobranca*

Characteristic	<i>Anaerobranca californiensis</i>	<i>Anaerobranca gottschalkii</i>	<i>Anaerobranca horikoshii</i>
Habitat	Paoha Island's Hot Springs of Mono Lake (USA, California)	Hot inlet of Lake Bogoria, Kenya	Hot spring Old Faithful Hotel (Yellowstone National Park, USA)
Cell's shape	rods	rods	rods
Length, μm	2.4-5	3-5	8-22
Diameter, μm	0.26-0.31	0.3-0.5	0.5-0.65
Branch formation	+	+	+
Spore formation	-	-	-
Gram staining	negative	negative	positive
Cell wall	Gram positive, thin	Gram positive, thin	Gram positive, thick
Motility	+	+	+
Type of flagellation	peritrichous	peritrichous	peritrichous
Growth conditions:			
pH range	8.6-10.4	6.0-10.5	6.5-10.3
Optimum pH	9.0-9.5*	9.5*	8.5**
Temp. range ($^{\circ}\text{C}$)	45-67	30-65	66 (range?)
Temp. optimum ($^{\circ}\text{C}$)	58	50-55	57
NaCl range (% w/v)	0-6	0-4	ND
NaCl optimum (% w/v)	1 - 2.5	1	ND
Strict anaerobe	+	+	+
Type of metabolism	fermenting proteins	fermenting sugars	fermenting proteins
Ammonification	+	ND	ND
G+C content (mol%)	30.3	30.9	34.3

* - pH measured at 20 C. ** - pH measured at 60 C