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ORIGINAL PAPER

Haloalkaliphilic spore-forming sulfidogens from soda lake sediments and description of *Desulfitispora alkaliphila* gen. nov., sp. nov.

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Abstract An anaerobic enrichment with pyruvate as electron donor and thiosulfate at pH 10 and 0.6 M Na^+ inoculated with pasteurized soda lake sediments resulted in a sulfidogenic coculture of two morphotypes of obligately anaerobic haloalkaliphilic endospore-forming clostridia, which were further isolated in pure culture. Strain AHT16 was a thin long rod able to ferment sugars and pyruvate and to respire H_2, formate and pyruvate using thiosulfate and fumarate as electron acceptors and growing optimally at pH 9.5. Thiosulfate was reduced incompletely to sulfide and sulfite. The strain was closely related (99% sequence similarity) to a peptolytic alkaliphilic clostridium *Natronincola peptidovorans*. Strain AHT17 was a short rod with a restricted respiratory metabolism, growing with pyruvate and lactate as electron donor and sulfite, thiosulfate and elemental sulfur as electron acceptors with a pH optimum 9.5. Thiosulfate was reduced completely via sulfite to sulfide. The ability of AHT17 to use sulfite explained the stability of the original coculture of the two clostridia—one member forming sulfite from thiosulfate and another consuming it. Strain AHT17 formed an independent deep phylogenetic lineage within the Clostridiales and is proposed as a new genus and species *Desulfitisporum alkaliphilum* gen. nov., sp. nov. (=DSM 22410^T = UNIQEM U794^T).

Keywords Clostridia · Haloalkaliphilic · Soda lakes · Sulfidogenic · Sulfite · Thiosulfate

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Nucleotide sequence accession numbers: the GenBank/EMBL accession number of the 16S-rRNA gene sequence of strains AHT 16 and AHT 17^T are FJ788524 and FJ788525.

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Introduction

Soda lakes are extreme habitats with a stable pH between 9 and 11 due to a presence of free sodium carbonate/bicarbonate, which can reach saturation (4 M alkalinity). Despite these harsh conditions, the element cycling in soda lakes, driven by a fully structured haloalkaliphilic prokaryotic community, is very active, especially at low to moderate salt concentrations (Zavarzin 2007). The sulfur cycle is one of the most active microbial cycles in soda lakes, even at hypersaline conditions, which is evident from the usually high acid-labile sulfide content in the sediments, the high-sulfate reduction rates, and the high viable numbers of sulfur-oxidizing bacteria (Gorlenko et al. 1999; Sorokin et al. 2004, 2006; Foti et al. 2007; Kulp et al. 2006).

In contrast to sulfur oxidation (Sorokin et al. 2006), sulfidogenesis at extremely haloalkaline conditions is not well understood. Until now, three haloalkaliphilic sulfate-reducing bacteria (SRB), members of the order

Desulfovibrionales (*Deltaproteobacteria*), have been cultivated from soda lake sediments, including the genera *Desulfonatronovibrio* (Zhilina et al. 1997), *Desulfonatronum* (Pikuta et al. 1998, 2003; Zhilina et al. 2005) and *Desulfonatronospira* (Sorokin et al. 2008a). Recently, it was extended to representatives of the order *Desulfobacterales* (Sorokin et al. 2010). Culture-independent studies of sulfate-reducing bacteria (SRB) in Mono Lake, California (Scholten et al. 2005), Wadi Natrun, Egypt (Mesbah et al. 2007) and Kulunda Steppe, Russia (Foti et al. 2007) also revealed mostly a presence of the deltaproteobacterial SRB lineages from *Desulfovibrionales* and *Desulfobacterales*. Although many of the sequences were grouped within the class Clostridia, their phylogenetic affiliation with the Gram-positive SRB could not be established with confidence.

So far, non-sulfate-reducing sulfidogens from alkaline habitats are represented by the sulfur-reducing *Desulfurispirillum alkaliphilum*, which was isolated from a bioreactor (Sorokin et al. 2007) and by two sulfur/thiosulfate reducers, *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus,* isolated from soda lakes (Sorokin et al. 2008b). The latter, together with the alkali-tolerant clostridium *Desulfitibacter alkalitolerans* isolated from an anthropogenic habitat (Nielsen et al. 2006) are the only known culturable haloalkaliphilic Gram-positive sulfidogens.

In an attempt to investigate the presence of endospore-forming *Desulfotomaculum–Desulfosporosinus*-like SRB in soda lakes cultivation was performed after pasteurization of the inoculum, a mixture of sediment samples. In this paper, properties of two haloalkaliphilic non-sulfate-reducing sulfidogenic clostridia completely reducing thiosulfate to sulfide in concert action are described.

Methods

Samples

Surface sediment samples (2–10 cm) were obtained from ten soda lakes in south-eastern Kulunda Steppe (Altai, Russia) in July 2008. The pH of the brines varied from 9.5 to 10.6, the total salt concentration from 40 to 500 g l^{-1}, and the total soluble carbonate alkalinity from 0.3 to 4.0 M. The samples from individual lakes were pooled in equal proportions, vigorously homogenized by vortexing, and subjected to a short low-speed centrifugation to remove coarse particles. The resultant inoculum consisted of a fine colloidal fraction enriched with cells (according to fluorescent microscopy check). Before inoculation, the sample was heat-treated at 85°C for 30 min and added to the medium at 1% (v/v).

Cultivation

The anaerobic enrichment and routine cultivation were performed at 30°C on a mineral medium containing sodium carbonate/bicarbonate buffer (0.5 M total Na^+, pH 10), 0.1 M NaCl, and 0.5 g l^{-1} of K_2HPO_4. After sterilization, the medium was supplemented with 4 mM NH_4Cl, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mL l^{-1} each of acidic (Pfennig and Lippert 1966) and alkaline (Plugge 2005) trace metal solutions and 20 mg l^{-1} yeast extract. The medium was reduced by 1 mM of Na_2S/0.1 mM dithionite, dispensed into the Hungate tubes or 100 mL serum bottles and made anoxic by five cycles of evacuation/flushing with argon gas. The electron donors were supplied at 10 mM for sugars and 50 mM for formate. Electron acceptors that were tested included Na_2SO_4 (20 mM), $Na_2S_2O_3$ (20 mM), KNO_3 (10 mM), KNO_2, Na_2SO_3, sodium selenate and selenite, sodium arsenate, DMSO (5 mM each), sodium fumarate (20 mM; alone or with 2 mM acetate as carbon source), and freshly prepared ferrihydrite (50 mM) prepared according to Kostka and Nealson (1998). Elemental sulfur was sterilized as a 50% (w/v) water suspension at 110°C for 60 min and used at final concentration 2 g l^{-1}. Growth at microoxic conditions was tested in the absence of the reductant with an oxygen concentration in the gas phase of 2%. Solid alkaline media with a final salt concentration of 0.5 M Na^+ was prepared by 1:1 mixing of 4% (w/v) washed agar and 1 M Na^+ reduced anaerobic mineral medium at 50°C with doubled sulfide concentration. The plates were immediately placed in closed jars under the stream of argon with an oxygen-scavenging catalyzer (Oxoid) and incubated for up to 1 month before visible colonies appeared.

The pH dependence was examined at Na^+ content of 0.6 M, using the following filter-sterilized mineral media: for pH 6–8, 0.1 M HEPES and NaCl; for pH 8.5–11.5, a mixture of sodium bicarbonate/sodium carbonate containing 0.1 M NaCl. Growth and sulfidogenesis resulted in a shift of initial pH values, especially at the pH extremes. Therefore, final pH values were taken to indicate the suitable range for growth and activity. The influence of salt concentration on growth and activity was investigated in sodium carbonate buffer containing 0.1–3.0 M of total Na^+ at pH 10.

Sulfidogenic activity of resting cells

Cultures were grown in 1 L volume with pyruvate in the presence of an electron acceptor to induce the sulfidogenic capacity. The cells were harvested by centrifugation, washed with anoxic 0.5 M sodium carbonate buffer, pH 10, containing 1 mM HS^- and resuspended in the same buffer at concentration 0.2 mg protein mL^{-1}. 2 mL

portions of the suspension was dispensed in 7 ml serum bottles, supplemented with sulfur electron acceptors and made anaerobic by evacuation/argon flushing. The reaction was started by injection of 20 mM pyruvate. The bottles were incubated at 30°C with periodic sulfide analysis in 0.1 mL sample.

Analytical procedures

Sulfide/polysulfide-sulfane was precipitated in 10% (w/v) Zn acetate and analyzed by the methylene blue method after separation from the supernatant (Trüper and Schlegel 1964). Thiosulfate and sulfite were analyzed by iodimetric titration after sulfide removal as ZnS. Cell protein was measured by the Lowry method (Lowry et al. 1951) after removal of interfering FeS from the cell pellet by a double washing with 0.5 M NaCl acidified with HCl to pH 4. Organic compounds (glucose and carbonic acids) in neutralized supernatants were detected by HPLC [column HPX-87-H (BioRad) at 60°C; detector UV/IR; carrier 5 mM H_2SO_4 at 0.6 mL/min].

Phase-contrast microphotographs were made with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). For electron microscopy of total preparations, the cells from 2 mL of the exponentially growing liquid cultures were collected, resuspended in 0.5 M NaCl, fixed with glutaraldehyde (final concentration of 3% v/v) and contrasted with 1% (w/v) neutralized phosphotungstic acid. Cellular fatty acids were extracted with a mixture of methanol–chloroform and analyzed by GC–MS according to Zhilina et al. (1997).

Genetic and phylogenetic analysis

Isolation of genomic DNA and determination of the G+C content of the DNA from pure cultures was performed according to Marmur (1961) and Marmur and Doty (1962). For the 16S-rRNA gene sequencing, the DNA was extracted from the cells using alkaline SDS lysis at 60°C (Birnboim and Doly 1979) and purified with the Wizard Preps kit (Promega, USA). The nearly complete 16S rRNA gene was obtained from the pure cultures using general bacterial PCR primers GM3f (5′-AGAGTTTGATCCTG GCTCAG-3′) and GM4r (5′TACGGTTACCTTGTTA-CGACTT-3′) (Schäfer and Muyzer 2001). The PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen, The Netherlands). The sequences were first compared with sequences stored in GenBank using the BLAST algorithm. Subsequently, the sequences were imported into the ARB software program (Ludwig et al. 2004), automatically aligned, and added to a phylogenetic tree using the Quick-add tool. The SILVA SSU_reference database version 1.4 was used to select for sequences of other taxa. The alignment created by ARB was curated manually. Sub-trees were then built using the neighbour-joining algorithm with automatic selected correction settings.

Results and discussion

Enrichment and isolation of pure cultures of spore-forming sulfidogens from soda lakes

Initial enrichment cultures with pasteurized sediments as inoculum were set with sulfate as electron acceptor and lactate, ethanol, methanol and CO (20% v/v) as electron donors at pH 10 and 0.6 M total Na^+. From these, only the combination ethanol/sulfate produced sulfide during the first two 1:100 transfers and then stopped. From the primary ethanol/sulfate enrichment additional transfers were made with ethanol/sulfite, pyruvate/sulfate and pyruvate/thiosulfate. Only the latter combination yielded a stable sulfidogenic mixed culture consisting of two different morphotypes of endospore-forming bacteria. A numerically more dominant morphotype represented by long thin rods was separated from less abundant short thick rods by repetitive dilution to extinction in liquid medium with pyruvate/thiosulfate. It was designated strain AHT16 (Fig. 1a). The second morphotype, forming mixed colonies with AHT16, produced much more spores on solid medium than strain AHT16, which favored the isolation in pure culture after heat treatment of the colony material. The treated material was further used in dilution series on liquid medium with pyruvate/thiosulfate, eventually resolving the second member of the consortium in pure culture, strain AHT17 (Fig. 1b, c).

Identification of the isolates

Phylogenetic analysis, based on 16S rRNA gene sequencing, placed both isolates in the order Clostridiales, but with different affiliations (Fig. 2). Strain AHT16 is a member of the clostridial cluster XI (Wiegel et al. 2006) closely related (99.4% sequence similarity) to *Natronincola peptidovorans* Z-7031, which was isolated from a soda lake and described as an obligate fermentative peptolytic alkaliphile (Zhilina et al. 2009). However, it must be pointed out that apparently, both strains Z-7031 and AHT16 are more closely related to the genus *Anaerovirgula*, a moderate haloalkaliphilic versatile fermentative bacterium from hypersaline alkaline Owens Lake (Pikuta et al. 2006), than to the genus *Natronincola*. The sequence of *Anaerovirgula* has not been included into the phylogenetic analysis by Zhilina et al. (2009) which probably resulted in the wrong identification. The strain has been deposited in DSMZ

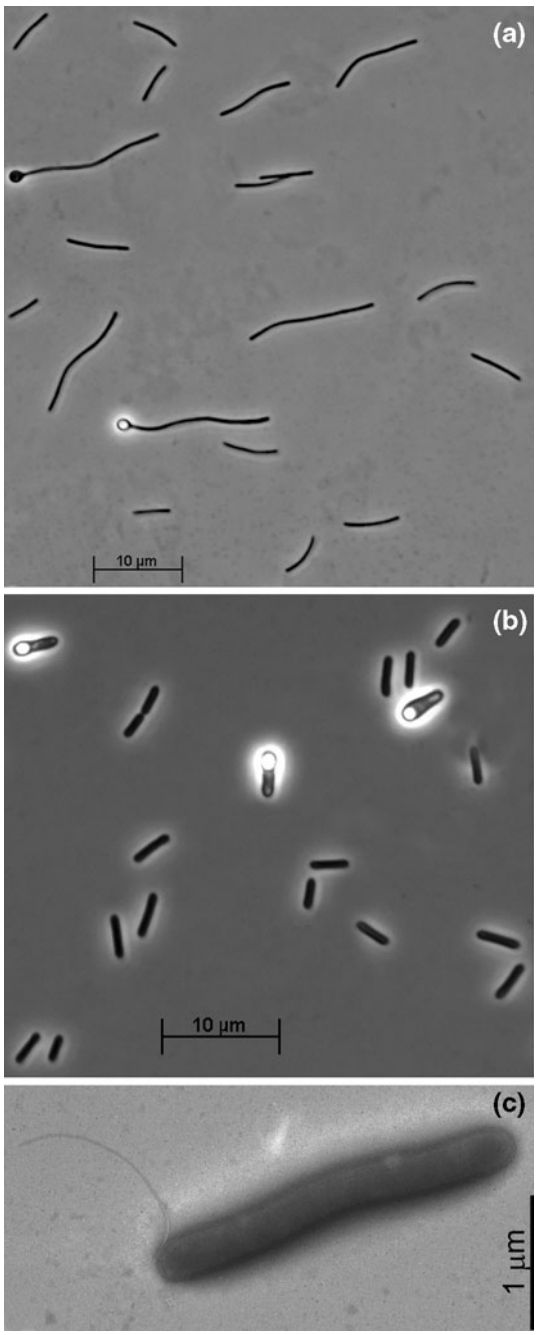

Fig. 1 Cell morphology of strains AHT16 (**a**), and AHT17 (**b**, **c**). **a**, **b** Phase-contrast microphotographs, **c** total electron microphotographs of positively stained cell

(Germany) under the number DSM22429 and in UNIQEM (Moscow) as U 793.

Strain AHT17 represented an independent deep lineage within the family *Peptococcaceae* with spore-forming alkali-tolerant sulfidogen *Desulfitibacter alkalitolerans* as a closest cultured relative (87.4% sequence similarity). Both neighbor-joining with Olsen correction, and maximum likelihood algorithms clustered AHT17 with *Desulfitobacter alkalitolerans* with high bootstrap values (92%). Other members of this family distinguished by their dissimilatory sulfidogenic metabolism include the genera *Desulfotomaculum*, *Desulfosporosinus*, *Desulfitobacterium* and *Desulfurispora*.

Cellular fatty acid analysis

Three dominant fatty acids (16:1ω7c, 16:0 and 14:0) were identified in the membrane lipids of strain AHT16 comprising more than 70% of the total. In AHT17 there were two obviously dominating fatty acids, 16:1ω5 and 16:1ω7c, which made up of 50% from the total (see Supplementary Table).

Metabolic properties

The two isolates were very different in their metabolic profiles (summarized in Table 1). Strain AHT16 is a fermentative bacterium, utilizing a number of hexoses (glucose, fructose, galacturonic acid, galactose, glucuronic acid, ramnose, and lactose) and xylose. It also can ferment pyruvate. Apart from fermentation, the bacterium was capable of anaerobic respiration with thiosulfate and fumarate as electron acceptors using H_2 and formate (with acetate as C source) for lithotrophic growth and pyruvate for heterotrophic growth. Chemolithoautotrophy was not observed. The maximum amount of sulfide formed during thiosulfate reduction was 15 mM HS^-/13 days. Thiosulfate was reduced incompletely to sulfide and sulfite, which was not surprising because the latter could not serve as the electron acceptor for this bacterium. The addition of thiosulfate markedly influenced the growth pattern on fermentable substrates, such as pyruvate and glucose, in strain AHT16. In the presence of thiosulfate, the growth yield on pyruvate was doubled and with glucose even tripled. A switch to anaerobic respiration was accompanied by a dramatic increase in the formation of the end product formate (Table 2).

Strain AHT17 is a strictly respiratory anaerobe (except for the ability to ferment pyruvate, which is common for sulfate-reducing bacteria) with a very restricted metabolism (see Table 1). It used pyruvate and lactate as electron donors with thiosulfate, sulfite and elemental sulfur as electron acceptors. Utilization of sugars, fermentation and lithotrophic growth with H_2 or formate was not observed. In contrast to AHT16, thiosulfate respiration was slow with a maximum HS^- formation of 5 mM/13 days, but it was a complete reduction of both sulfane and sulfone atoms of thiosulfate to sulfide. The latter is explained by the ability of this bacterium to use sulfite as the electron donor, albeit at relatively low concentrations (concentrations above

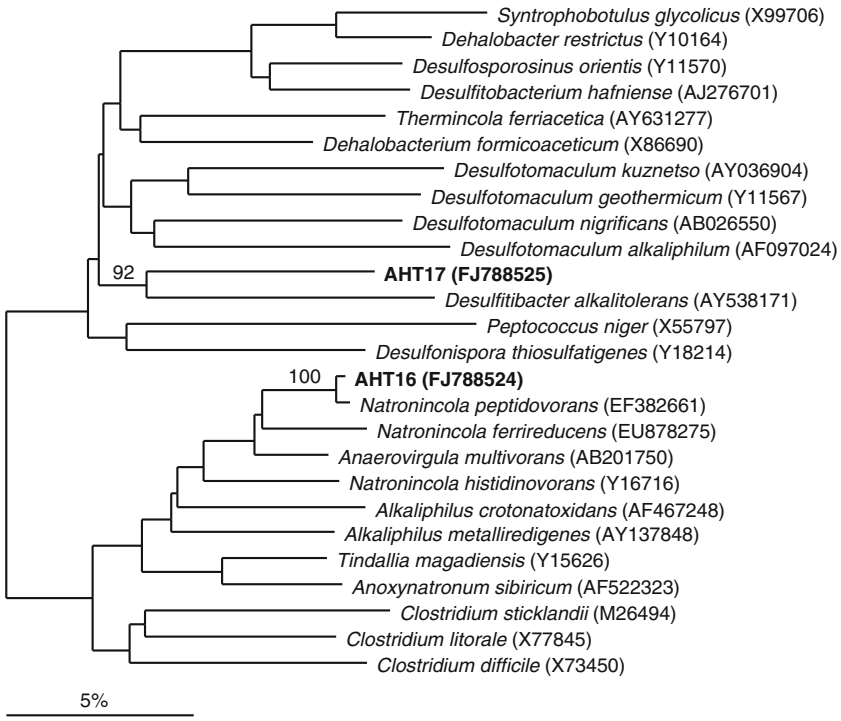

Fig. 2 Phylogenetic position based on 16S rRNA gene sequence analysis of strains AHT16 and AHT17 within the class Clostridia. The tree was constructed using neighbour-joining. The *scale bar* represents five nucleotide changes per 100 nucleotides

Table 1 Comparative properties of the novel haloalkaliphilic sulfidogens and their closest relatives

Property	AHT 16	*Natronincola peptidovorans*	AHT17	*Desulfitibacter alkalitolerans*	*Desulfitibacterium hafniense*
Morphology	Long motile rod with round terminal endospore	Long motile rod with round terminal endospore	Short motile rod with round terminal endospore	Long motile rod with round terminal endospore	Long motile rod with round terminal endospore
Fermentation of:					
Sugars	+	−	−	−	−
Amino acids	−	+	−	+	−
Pyruvate	+	+	+	n.d.	+
Electron donors	H_2, formate, pyruvate	None	Pyruvate, lactate	Formate, lactate, pyruvate, methanol, betain, choline	Pyruvate, tryptophan
Electron acceptors for anaerobic respiration	Thiosulfate, fumarate	None	Thiosulfate, sulfite, sulfur	Thiosulfate, sulfite, sulfur, nitrate, nitrite	Thiosulfate, sulfite, nitrate
Dominant cellular fatty acids	16:1ω7c, 16:0, 14:0	16:1ω7c, 16:0, 18:1ω9, 15:0	16:1ω5, 16:1ω7c	n.d.	n.d.
Alkaliphily	+	+	+	+	−
Salt dependence	+	+	+	−	−
G+C content (mol%)	34.3	35.5	37.9	41.6	47
Habitat	Soda lakes			Municipal water heating system	Municipal sludge

Natronincola peptidovorans (Zhilina et al. 2009); *Desulfitibacter alkalitolerans* (Nielsen et al. 2006); *Desulfitobacterium hafniense* (Christiansen and Ahring 1996)

n.d. no data

5 mM inhibited growth). Maximum sulfidogenic activity with lactate as electron donor was observed with elemental sulfur as acceptor (67 mM sulfane of HS^- + polysulfide/13 days). Therefore, in its essential properties, strain AHT17 represented a typical Gram-positive sulfidogen, similar to *Desulfitobacterium* and

Table 2 Influence of thiosulfate (20 mM) on anaerobic growth of strain AHT16 with pyruvate and glucose (10 mM)

Growth balance	Pyruvate (20 mM)		Glucose (10 mM)	
	$-S_2O_3^{2-}$	$+S_2O_3^{2-}$	$-S_2O_3^{2-}$	$+S_2O_3^{2-}$
Biomass (OD_{600})	0.08	0.150	0.060	0.180
Consumed substrate (mM)	15	20	2.5	6.0
Acetate (mM)	7	1	5	1
Formate (mM)	5	28	2.5	32
HS^- (mM)	–	15.0	–	20.0

Table 3 Sulfidogenic activity [nmol (mg protein h)$^{-1}$] of washed cells grown with pyruvate and thiosulfate (AHT16) or sulfite (AHT17)

Acceptor	AHT16	AHT17
$S_2O_3^{2-}$	380	90
SO_3^{2-}	0	390
S_8	110	840

Electron donor—pyruvate; pH 10 0.6 M total Na^+

Desulfitibacter, which are different from the classical SRB in their inability to activate sulfate.

Short-term experiments with washed cells grown on pyruvate + thiosulfate (AHT16) or pyruvate + sulfite (AHT17) showed that, despite the absence of growth with sulfur, strain AHT16 was still able to reduce it, although much less actively, than the cells of strain AHT17. It also confirmed the lack of sulfite reductase in AHT16 and showed that the latter is a very active enzyme in AHT17 (Table 3).

The essential physiology of two organisms provided a perfect reason of their selection and stable coexistence in mixed anaerobic enrichment cultures on pyruvate + thiosulfate. Strain AHT16 is a very active thiosulfate reducer, but it produces sulfite, i.e., most of the accepting power is unused. This leaves a place for the second organism, strain AHT17, which was less active with thiosulfate, but used the byproduct of thiosulfate reduction by strain AHT16 making utilization of the electron-accepting potential of thiosulfate complete. A combination of the two pure cultures demonstrated that the consortium was more efficient in utilization of pyruvate with thiosulfate as electron acceptor than the individual strains: the final growth yield of the mixed culture was 75% higher than for the pure culture of strain AHT16 and 60% higher than for the pure culture of strain AHT17.

Influence of pH and salt on growth and sulfidogenic activity

Growth and sufidogenic activity of washed cells were optimal for both strains within the alkaline pH range with a maximum pH 9.5. Both strains were obligately, but moderately alkaliphilic with a lower pH limit above pH 8 and a higher pH limit at 10.3. Sulfidogenic activity of washed cells, however, was still present up to pH 11 (Fig. 3). The salt tolerance, tested in a sodium carbonate/bicarbonate buffer at pH 10, was relatively low in comparison with the salinity of their habitat, with a maximum growth rate and sulfidogenic activity at 0.4 M total Na^+ (Fig. 4). Probably such organisms find micronishes with much lower salinity than the bulk or proliferate only during short spring periods of lake dilution.

In conclusion, we were unable to find a spore-forming SRB, such as *Desulfotomaculum* or *Desulfosporosinus*, in soda lakes, even after elimination of the deltaproteobacterial sulfidogens by pasteurization of the inoculum. Instead, other types of clostridial sulfidogens were selected reducing thiosulfate to sulfide in a concerted action. Strain AHT16 reduced thiosulfate to sulfite, while strain AHT17 subsequently reduced sulfite to sulfide. Strain AHT17 resembled the sulfite-reducing clostridia *Desulfitibacter* an *Desilfitobacterium* but clearly differed from them by distant phylogeny and obligate alkaliphily, and, therefore is proposed here as a novel genus and species *Desulfitispora alkaliphila* gen. nov., sp. nov.

Description of *Desulfitispora* gen. nov.

[*Desulfitispora* (De.sul.fi.ti.spo′ra) L. pref. *de* from, off, away; N. L. n. sulfis-itis sulfite; N.L. fem. n. spora (from Gr. n. *spora*), a seed and in biology a spore; N. L. fem. n. *Desulfitispora* spore-forming bacterium-reducing sulfite].

Gram-positive, endospore-forming rods. Obligately anaerobic with respiratory metabolism. Use sulfur compounds, but not sulfate, as electron acceptor, and short-chain organic acids as electron donors. Obligately alkaliphilic and moderately salt tolerant. A member of the family *Peptococcaceae* within the order Clostridiales. The type species is *D. alkaliphila*. Habitat—soda lakes.

Description of *Desulfitispora alkaliphila* sp. nov.

al.ka.li.phi'la. N. L. n. alkali (from Arabic article *al*, the and Arabic n. *qaliy*, ashes of saltwort), alkali; N.L. adj. *philus* from Gr. adj. *philos* loving; N. L. fem. adj. *alkaliphila*, loving alkaline conditions.

Cells are Gram-positive rods forming terminal endospores with swollen sporangia, 0.7–0.8 × 2–4 μm, motile with a subterminal flagellum. Strictly anaerobic with respiratory metabolism. Uses thiosulfate, sulfite and elemental sulfur as electron acceptors. Utilizes lactate and pyruvate as both electron donor and carbon source.

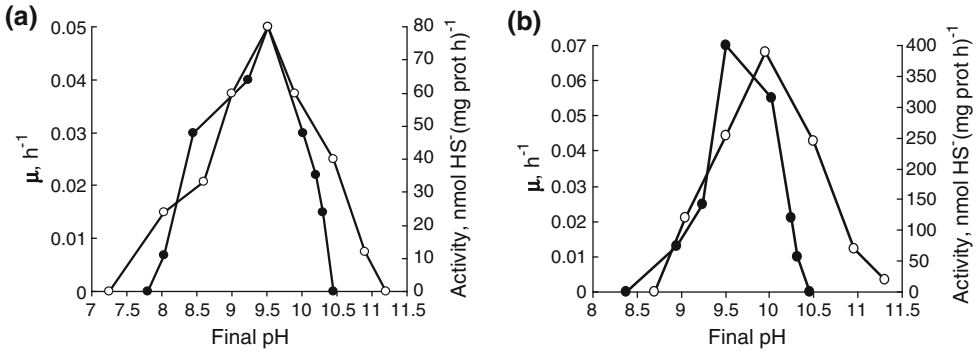

Fig. 3 Influence of pH (at 0.6 M Na^+) on growth (*closed circles*) and sulfidogenic activity of washed cells (*open circles*) in strains AHT16 (**a**) and AHT17 (**b**). Strain AHT16 was grown and the cells were tested with pyruvate and thiosulfate. Strain AHT17 was grown and the cells were tested with pyruvate and sulfite

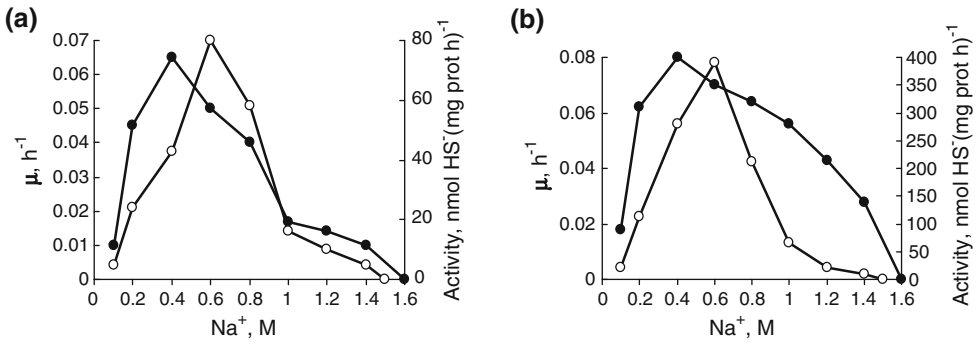

Fig. 4 Influence of salt (at pH 10) in carbonate/bicarbonate buffer on growth (*closed circles*) and sulfidogenic activity of washed cells (*open circles*) in strains AHT16 (**a**) and AHT17 (**b**). Strain AHT16 was grown and the cells were tested with pyruvate and thiosulfate. Strain AHT17 was grown and the cells were tested with pyruvate and sulfite

Pyruvate can be fermented. Obligately alkaliphilic with a pH range for growth between 8.5 and 10.3 and an optimum at pH 9.5 and moderately salt tolerant with a total Na^+ range for growth from 0.1 to 1.4 M (optimum at 0.4 M). Mesophilic, with a maximum temperature for growth at 43°C. The dominant cellular fatty acids include 16:1ω5 and 16:1ω7c. The G+C content of the genomic DNA is 37.9 ± 0.5 mol% (T_m). The type strain is AHT17^T (=DSM 22410 = UNIQEM U794). Isolated from mixed sample of sediments from different hypersaline soda lakes in Kulunda Steppe (Altai, Russia). The GenBank 16S rRNA gene sequence accession number is FJ788525.

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