RESEARCH ARTICLE

Sulfidogenesis in hypersaline chloride–sulfate lakes of Kulunda Steppe (Altai, Russia)

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Abstract

The activity and culturable diversity of sulfidogens were investigated in anoxic sediments of four hypersaline lakes with pH 7.6–8.2 in the Kulunda Steppe (Altai, Russia). Sulfate reduction rates were low, varying from 0.1 to 6.0 nmol $HS^-/(cm^3\ h)$ with a maximum in the top 10 cm layer. Potential sulfidogenic rates with thiosulfate and sulfur as the *e*-acceptors were higher than with sulfate and were stimulated by formate, lactate, and acetate. Sulfidogenesis was optimal at salt concentrations below 2 M NaCl. Cultivation at 2 M NaCl resulted in the isolation of several strains of moderately halophilic SRB, but no growth of SRB was observed at 4 M NaCl. At lithotrophic conditions (i.e., with formate or H_2 as *e*-donors), several closely related alkalitolerant strains belonging to the genus *Desulfonatronovibrio* were isolated. Enrichments at heterotrophic conditions with lactate, propionate, acetate, or butyrate using sulfate or thiosulfate as *e*-acceptors yielded isolates related to *Desulfosalsimonas propionica*, *Desulfohalobium utahense*, and *Desulfocella halophila*. Sulfur-reducing enrichments at 2 M NaCl with ethanol produced a member of the genus *Halanaerobium*, while enrichments at 4 M NaCl with acetate were dominated by archaea, demonstrating for the first time such type of catabolism in haloarchaea.

Introduction

Hypersaline intra-continental (*athalassohaline*) salt lakes represent a final salt trap for minerals leached out of the solid phase and carried by the ground waters into small depressions, whereby evaporative concentration leads to the formation of inland salt lakes. The main salts in these lakes are sodium chloride and sodium sulfate. In contrast to the sea-derived hypersaline habitats (*thalassic*), such as solar sea salterns, the Mg concentrations of continental salt lakes are much lower. These lakes belong to an extreme type of habitats where halophilic and extremely halophilic prokaryotes are a dominant form of life (Oren, 2002).

However, it must be stressed that so far extremely halophilic aerobic and heterotrophic archae dominating in the saturated salt brine received most scientific attention, while not much information is yet available on the sediments and inorganic biogeochemical cycles there. Recently we demonstrated an unexpectedly high diversity of halophilic chemolithoautotrophic sulfur-oxidizing bacteria in hypersaline habitats among which five novel genera of the *Gammaproteobacteria* were found, including two extremely halophilic groups (Sorokin, 2008).

In general, the few available data indicate a clear depression of the sulfidogenic activity (SA) at salt-saturating conditions. In particular, sulfate reduction rates in the anoxic sediments of Eliat solar saltern ponds (Israel) and in the Great Salt Lake (Utah) sharply decreased at salinity above 2 M NaCl (Brandt *et al.*, 2001; Sørensen *et al.*, 2004; Waldron *et al.*, 2007). On the other hand, one report communicated maximal sulfate reduction rates at salt-saturating conditions in a solar saltern with the rates comparable to eutrophic marine sediments (Porter *et al.*, 2007). This is difficult to understand, as no pure culture of known halophilic sulfate-reducing bacteria (SRB) belongs to extreme halophilic type, i.e., growing optimally at salt-saturating conditions. It is also necessary

to keep in mind that the measurements of sulfate reduction rates with ^{35}S-sulfate could be subjected to a major error because of the very high concentrations of dissolved sulfate reaching molar concentrations. The few SRB species isolated in pure culture from hypersaline habitats are all moderate halophiles, growing optimally at NaCl concentrations below 2 M. They include members belonging to the genera *Desulfohalobium*, *Desulfovermiculus* (order *Desulfovibrionales*), and *Desulfocella* (Ollivier *et al.*, 1991; Brandt *et al.*, 1999; Belyakova *et al.*, 2006; Jakobsen *et al.*, 2006) belonging to the 'incomplete oxidizers' (forming acetate as a final product), while the recently described genus *Desulfosalsimonas* (*Desulfobacteraceae*) is apparently a 'complete oxidizer', although it can not utilize external acetate (Kjeldsen *et al.*, 2010).

Sulfidogenesis is not limited to sulfate reduction. Other sulfur species with intermediate valence, such as thiosulfate and elemental sulfur, which are stable intermediates in the natural sulfur cycle, might be as important as sulfate in electron accepting function (Jørgensen, 1990; Bonch-Osmolovskaya, 1994). No published records, however, could be found concerning reduction in thiosulfate and elemental sulfur in hypersaline habitats with neutral pH.

In this study, we investigated sulfidogenesis with sulfate, thiosulfate and elemental sulfur as electron acceptors in sediments of four chloride–sulfate hypersaline lakes located in south-western Siberia (Altai, Russia). The results indicated low sulfidogenic rates with all three electron acceptors at salt-saturating conditions and the presence of moderately halophilic SRB.

Materials and methods

Samples and chemical analyses

Sampling was performed in July 2010, in four hypersaline chloride–sulfate lakes located in the southern part of the Kulunda Steppe, south-western Siberia (Altai, Russia). The chemical details of the brines of the studied lakes are summarized in Table 1. Samples for *in situ* experiments and for microbiological investigations were collected from the top 20–30 cm of the sediment with a plastic corer of an inner diameter of 30 mm. Laboratory measurements of potential rates of sulfidogenesis were carried out with samples from the top 10 cm sediment diluted with two parts of the near-bottom brines. All samples were stored and transported at 8 °C to the laboratory and kept at 4 °C in the laboratory before they were processed. The pH and salinity of the brines were measured with a field pH-conductometer. The acid-labile sulfides ($H_2S/HS^- + FeS$) content of the sediments was determined after cold distillation of the Zn-acetate fixed samples with 1 M HCl according to Trüper & Schlegel (1964). The same method was used to determine sulfide production in pure cultures of halophilic sulfidogens. Sulfate concentrations of the brines and the sediment pore waters were determined by non-suppressed anion chromatography after neutralization and dilution in Milli-Q water using a Biotronic, column BT11AN, detector refractometer, and 1 mM Na_2CO_3 and 1.2 mM $NaHCO_3$ as the eluent with a flow rate of 1.5 mL min^{-1}.

Sulfidogenic activity

The *in situ* sulfate reduction activity within the top 20–30 cm sediment layer was measured with $^{35}S\text{-}SO_4^{2-}$ in 5 mL syringes as described previously (Foti *et al.*, 2007). The samples were incubated at ambient temperature in the field and fixed 48 h with 1 mL of anoxic 10 M KOH. The potential SA was determined in the laboratory using sediment slurries containing 2 cm^3 of sediment and 4 mL of near-bottom brines in 10 mL serum bottles incubated at anoxic conditions (achieved by several cycles of argon flushing-evacuation) in duplicates. Electron donors and acceptors were added at final concentrations of 2 and 5 mM, respectively. To study the effect of salinity, the pore brines were replaced by 0.2–4 M NaCl solutions buffered at pH 7.8 by 50 mM potassium phosphate. The bottles were incubated in the dark at 25 °C for 2–7 days with periodic sampling for sulfide analyses. The sulfidogenic rates were calculated from the linear part of the plot.

Enrichment and isolation of halophilic sulfidogens

Halophilic sulfidogens were enriched either directly from the sediments or, in some cases, from the sediment slurries that exhibited elevated SA with sulfate, thiosulfate, or sulfur as *e*-acceptors. The basal medium included 2–4 M NaCl, 50 mM HEPES (pH 7), 1 g L^{-1} K_2HPO4, and 5 mM NH_4Cl. After sterilization, the medium was supplemented with (final concentration) the following: 5 mM $NaHCO_3$ (from 1 M filter-sterilized stock, pH 8), $MgCl_2$ (5 mM), acidic trace metals (1 mL L^{-1}) and vitamins (1 mL L^{-1}) (Pfennig & Lippert, 1966), basic Se/W solution (1 mL L^{-1}) (Plugge, 2005) and yeast extract (20 mg L^{-1}). Electron donors were supplied at concentrations of 10–50 mM, sulfate and thiosulfate at 20 mM, sulfite at 5 mM and elemental sulfur concentration at 2 g L^{-1}. The medium was dispensed either into 30 mL serum bottles (20 mL medium) or into 15 mL Hungate tubes (10 mL medium). Finally, oxygen was removed by five cycles of evacuation/flushing with argon, and 1 mM Na_2S was added as a reductant. Pure cultures were isolated by several rounds of serial dilutions to extinction.

Table 1. Chemical parameters of brines and sediments and rates of $^{34}S\text{-}SO_4^{2-}$ reduction (SRR) in hypersaline lakes of Kulunda Steppe

Lake	Layer	pH	Salinity (g L^{-1})	Sulfate (mM)	Acid-labile sulfides* (mM)	SRR nmol (cm^3 $h)^{-1}$
Cock Saline Lake (52.16°N/79.28°E)	nbb	7.6	320	542		
	S 0–5 cm			1468	17.20	4.9
	S 6–10 cm			1148	4.62	0
	S 10–15 cm			910	2.24	3.3
Chicken Lake (52.12°N/79.29°E)	nbb	8.1	125	587		
	S 0–5 cm			779	0.31	5.2
	S 6–10 cm			166	0.28	1.75
	S 11–15 cm			195	0.03	0.1
Lake Lomovoe (51.42°N/79.42°E)	nbb	7.9	325	427		
	S 0–10 cm			125	1.22	3.75
	S 11–20 cm			78	1.02	0.21
Hummocky Lake (51.40°N/79.46°E)	nbb	8.2	330	354		
	S 0–10 cm			286	8.98	2.91
	S 11–18 cm			138	0.25	1.21
	S 19–25 cm			229	0.12	0.42

nbb, near-bottom brine; S, sediments.
*H_2S/HS^- + FeS.

Phylogenetic analyses of pure cultures

Genomic DNA was extracted from the cells using lysis in 1% SDS/0.2 M NaOH at 60 °C and purified with the Wizard Preps kit (Promega). The nearly complete 16S rRNA gene was amplified from pure cultures with the general bacterial primers 11f and 1492r (Lane, 1991). The sequences were aligned with sequences from the GenBank using CLUSTAL W, and the phylogenetic tree was reconstructed using neighbor-joining algorithm in the TREECONW program package (van de Peer & de Wachter, 1994).

Results and discussion

Sulfate reduction at *in situ* conditions

Despite the presence of very high concentrations of HS^-/FeS in the top sediments of at least two of the four hypersaline lakes investigated, in all of them, the measured *in situ* rates of sulfate reduction were very low in comparison with the values reported for, for example, marine shelf sediments (50–150 nmol cm^3 day^{-1}; Lein *et al.*, 2002) and soda lakes in the same area (3–420 nmol cm^3 day^{-1}; Foti *et al.*, 2007; Sorokin *et al.*, 2010). However, in comparison with the other hypersaline habitats (few nmol cm^3 day^{-1}), the detected values are of similar magnitude (Brandt *et al.*, 2001; Sørensen *et al.*, 2004; Kulp *et al.*, 2007). In most of the cases, the maximal rates were observed within the top 5–10 cm sediment layer (Table 1).

Potential SA in sediment slurries

The potential rate measurements of sulfidogenesis in top 10 cm sediments from the four hypersaline lakes clearly indicated the lowest SA with sulfate and the highest values with elemental sulfur, similar to what we recently observed in soda lakes located in the same area (Sorokin *et al.*, 2010). From the tested *e*-donors, the most stimulating were formate, acetate (mostly with elemental sulfur), and lactate, while propionate and butyrate supported sulfidogenesis only in Chicken Lake with the lowest salinity (Fig. 1).

The effect of salinity on the SA with three different *e*-acceptors had a common trend for all four lakes: the activity significantly dropped at salinities above 2 M NaCl, indicating a domination of moderately halophilic sulfidogenic populations similar to what was observed for SRB from salterns (Sørensen *et al.*, 2004) and the Great Salt Lake (Brandt *et al.*, 2001). Within the interval of moderate salinity (i.e., below 2 M NaCl), in several cases, complex profiles were observed, probably due to the presence of populations with different salt adaptation, such as the SRB population in 11KL and thiosulfate reducers in 2KL. The experiments also indicated that sulfur-reducing populations were more halophilic than SRB and thiosulfate reducers (Fig. 2).

Overall the activity tests demonstrated that, in contrast to the aerobic part of the sulfur cycle, which is active up to saturated salt concentrations in hypersaline chloride–sulfate habitats (Sorokin, 2008), sulfidogenesis is hampered at hypersaline conditions and, therefore, the whole sulfur cycle may be uncoupled, unless a short variant

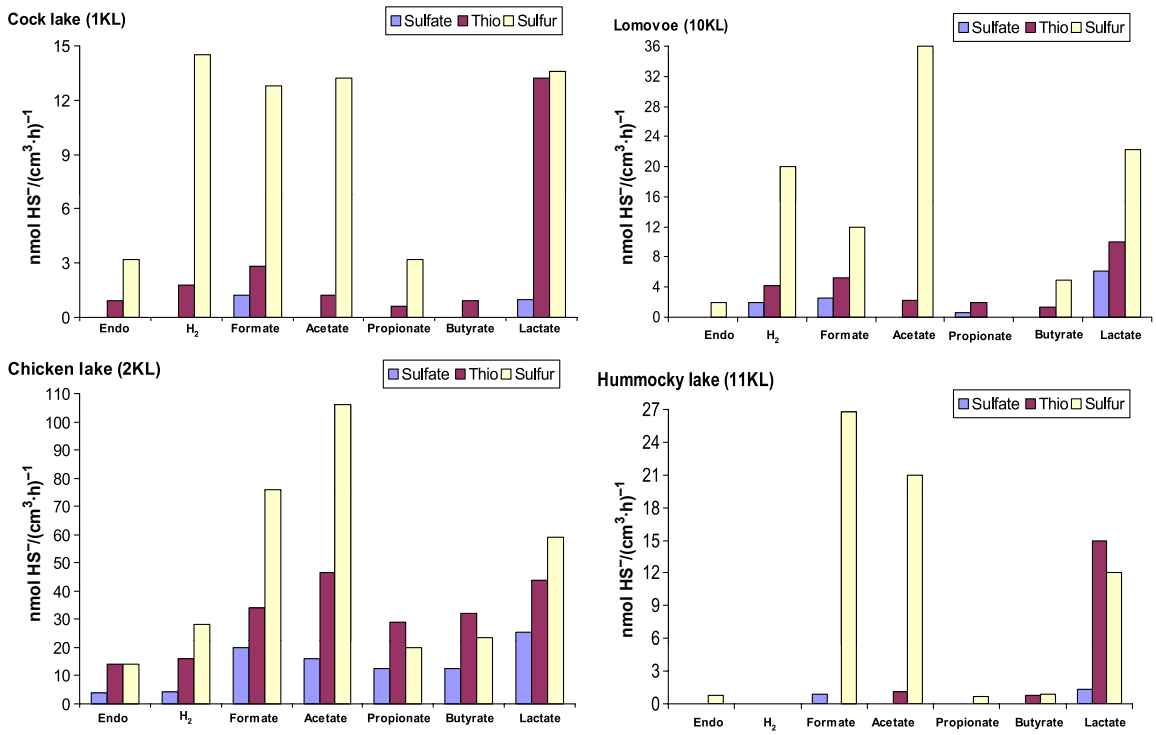

Fig. 1. Influence of electron donors on potential SA with three different sulfur electron acceptors in sediment slurries from hypersaline lakes in Kulunda Steppe. The average results from two independent experiments with deviations below 15%.

($H_2S \gg S_8 \gg H_2S$) is replacing the 'long' cycle ($H_2S \gg SO_4^{2-} \gg H_2S$). Another possibility is that there might be a temporal (seasonal) and spatial (freshwater springs) decrease in salinity activating the near-dormant sulfate reduction.

Culturable diversity of halophilic SRB in Kulunda salt lakes

Positive enrichment cultures with sulfate and thiosulfate as *e*-acceptors and several *e*-donors were only obtained at a salinity of 2 M NaCl, but not at a salinity of 4 M NaCl. The enrichment cultures resulted in the isolation of 12 pure cultures of SRB (Table 2). Four closely related vibrio-shaped isolates (with a 99% similarity of their 16S rRNA genes), which grew best with formate as an *e*-donor, belonged to the genus *Desulfonatronovibrio* (Fig. 3). So far, representatives of this SRB genus have been only found in soda lakes and contained only obligately alkaliphilic species (Zhilina *et al.*, 1997; Sorokin *et al.*, 2011a). All four isolates from the salt lakes, however, grew best at near-neutral pH in NaCl solutions up to 2 M with a pH optimum around 8–8.5 and only tolerated a high pH up to 9.4. Therefore, they may be considered as a novel halophilic and alkalitolerant species of the genus *Desulfonatronovibrio*.

Another two representatives of the order *Desulfovibrionales* were obtained from the enrichments with thiosulfate (Fig. 3). Strain HTR7, isolated with formate as *e*-donor and acetate as C-source, was closely related to *Desulfovibrio halophilus* (99% of 16S rRNA gene sequence similarity) from Solar Lake in Israel (Caumette *et al.*, 1991), despite its obvious morphological difference from the type strain (long thin rods). The second organism, strain HTR8, was related to *Desulfohalobium utahense* (96% of 16S rRNA gene sequence similarity) from Great Salt Lake (Utah) isolated with EtOH and sulfate (Jakobsen *et al.*, 2006; Kjeldsen *et al.*, 2007). Strangely, strain HTR8 was enriched and isolated with acetate as *e*-donor and C-source, while for *D. utahense*, it was specifically stressed as not capable of acetate dissimilation (also it was confirmed with a specific test recently; K. Ingvorsen, personal communication). Furthermore, acetate oxidation is not a characteristic for the order *Desulfovibrionales*, which accommodates mostly so-called incomplete oxidizing SRB (Kuever *et al.*, 2005). However, the acetate-dependent growth of strain HTR8 in pure culture, in contrast to the enrichment, was very irregular and observed only with thiosulfate as *e*-acceptor, but not with sulfate. Despite that a possibility for growth by thiosulfate disproportionation in this organism was ruled out, and direct dependence of sulfide

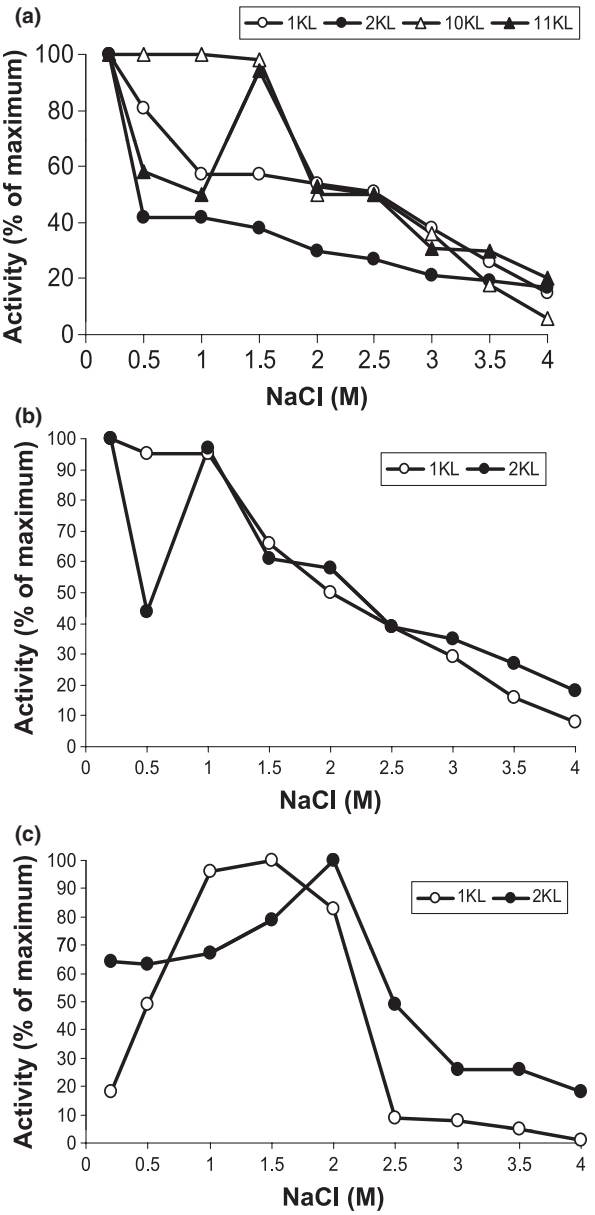

Fig. 2. Influence of NaCl at pH 7.8 on SA in sediment slurries from hypersaline lakes in Kulunda Steppe with formate (5 mM)/lactate (2 mM) as *e*-donors. (a) sulfate reduction; (b) thiosulfate reduction; (c) sulfur reduction. One hundred percent activity of sulfate reduction [nmol HS^- (cm^3 h)$^{-1}$]: 1KL = 27, 2KL = 90, 10KL = 30, 11KL = 58. One hundred percent activity of thiosulfate reduction: 1KL = 76, 2KL = 196. One hundred percent activity of sulfur reduction: 1KL = 495, 2KL = 540.

production from acetate was observed within a concentration range from 1 to 5 mM. This would indicate a genuine growth by acetate oxidation, although this would need additional experiments with ^{14}C-acetate. The best growth was observed with EtOH as *e*-donor at 2 M NaCl, similar to *D. utahense*. In respect to the phylogenetic position of strain HTR8 and *D. utahense*, it must be pointed out that both are quite far away from the type species *Desulfohalobium retbaense* (90–91% 16S rRNA gene sequence similarity) and, most probably, represent a separate genus more closely related to *Desulfovermiculus halophilus* – a halophilic SRB from an oil field (Beliakova *et al.*, 2006), than to the *Desulfohalobium* genus.

Enrichments with volatile fatty acids (VFA) yielded four moderately halophilic heterotrophic SRB isolates, members of the order *Desulfobacterales* (Fig. 3a). Three of them, strains HTR2, HTR3, and HTR12, were closely related (98% 16S rRNA gene sequence similarity) to the recently described halophilic SRB from Great Salt Lake, *Desulfosalsimonas propionicica* (Kjeldsen *et al.*, 2007, 2010). Similar to the latter, the novel isolates seems to belong to 'complete oxidizing' SRB, as they oxidized propionate and butyrate without release of acetate. Despite that and, again, similar to *Desulfosalsimonas*, they were not able to utilize external acetate. So, such a trait seems to be conserved in this group of halophilic SRB. Strain HTR12 was phylogenetically more closely related to HTR2 than to HTR3 (99% and 97% sequence similarity), which also corresponded to the difference in substrate profiles (Table 2). DNA–DNA hybridization showed only 40% similarity between HTR2 and HTR3 indicating that they represent different species. The fourth strain, HTR11, isolated with butyrate and sulfate, was closely related (98% sequence similarity) to *Desulfocella halophila*, also isolated from Great Salt Lake (Brandt *et al.*, 1999).

Sulfur-reducing halophiles from hypersaline lakes

Direct enrichments at 2 M NaCl with sulfur as *e*-acceptor were positive with formate, acetate, and EtOH as *e*-donors, but only the latter resulted in a stable sulfidogenic culture and, eventually, produced a pure culture, strain HSR1. The organism is a slightly bent fat rod with Gram-positive type of the cell wall (3% KOH test). Sulfur respiration was only supported by EtOH and lactate, while it also can ferment pyruvate. Apart from sulfur, no other *e*-acceptors were utilized. The strain was identified as a member of the genus *Halanaerobium* (97% sequence similarity to various species; Fig. 3b). Most of the representatives of the order *Halanaerobiales* are fermentative halophiles, while only for few species, such as *Halanaeobium congolense* (Ravot *et al.*, 1997), *Halobacteroides lacunaris* (Zhilina *et al.*, 1992), and *Natroniella sulfidigena* (Sorokin *et al.*, 2011b), a possibility for anaerobic respiration with sulfur or thiosulfate has been shown.

Table 2. Halophilic SRB isolated from hypersaline lakes of Kulunda Steppe

	Source		Enrichment conditions		Metabolic properties			
Strain	Direct enrichment	Enriched after sediment slurry experiment	*e*-acceptor	*e*-donor*	*e*-donors	*e*-acceptors	NaCl max (M)	Affiliation
HTR1	+		Thiosulfate	Primary- EtOH; secondary – formate	Formate, H_2, pyruvate,	Sulfate, thiosulfate, sulfite	2.0	*Desulfonatronovibrio*
HTR6		+	Sulfate	Formate				
HTR9	+		Sulfate	EtOH				
HTR10		+	Thiosulfate	EtOH				
HTR7	+		Thiosulfate	Formate	Formate, pyruvate, lactate	Sulfate, thiosulfate, sulfite	2.0	*Desulfovibrio halophilus*
HTR8	+		Thiosulfate	Acetate	H_2, formate, acetate, EtOH, propionate	Sulfate, thiosulfate	3.5	*Desulfohalobium utahense*
HTR2	+		Thiosulfate	Propionate	C3-C6 VFA; PropOH, BuOH pyruvate	Sulfate, thiosulfate	2.5	*Desulfosalsimonas propionicica*
HTR3		+	Sulfate	Propionate	Propionate, butyrate, pyruvate		3.0	
HTR12		+	Sulfate	Lactate	C4-C6 VFA; lactate, pyruvate		2.0	
HTR11		+	Sulfate	Butyrate	Butyrate, caproate, lactate	Sulfate, thiosulfate	2.5	*Desulfocella halophila*

*Primary – initial enrichment; secondary – used for the isolation of a pure culture.

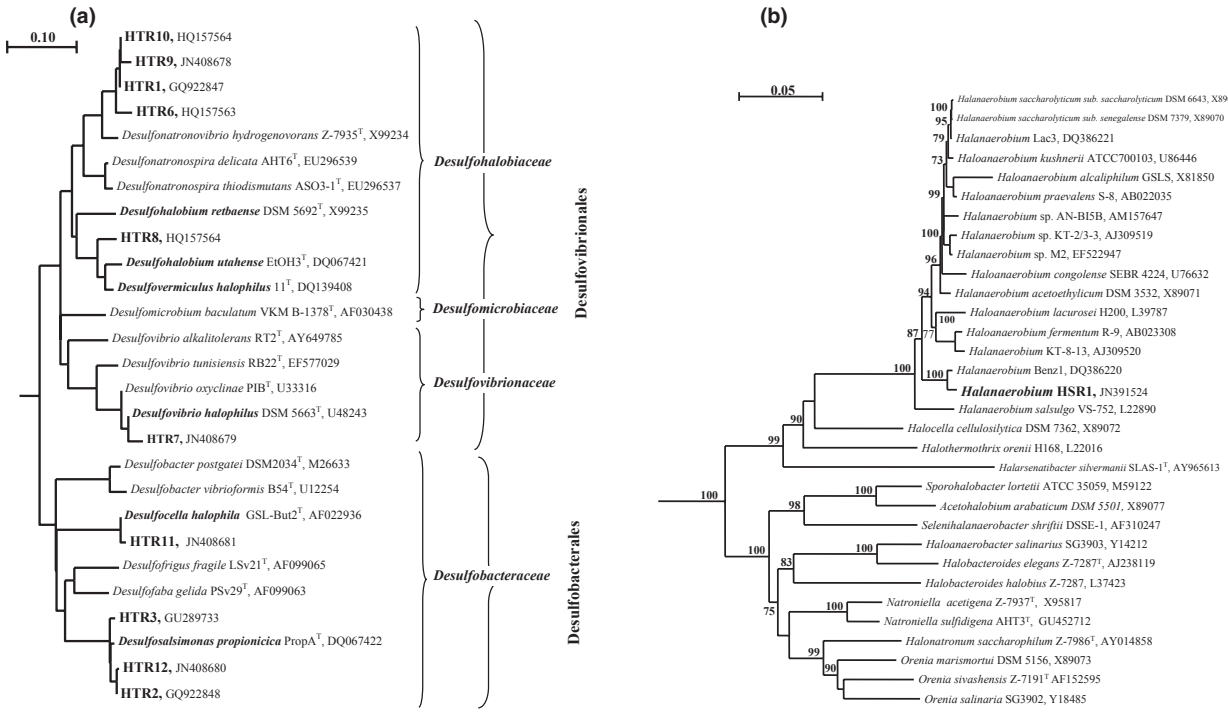

Fig. 3. Phylogenetic position of the SRB (a) and sulfur-reducing (b) isolates from hypersaline lakes in Kulunda Steppe on the basis of the 16S rRNA gene analysis. The tree was constructed using the NJ method. The scale bar represents five nucleotide changes per 100 nucleotides. The percentage of bootstraps was derived from 1000 resampling using neighbor-joining algorithm; only values > 70% are indicated.

The sediment slurry experiments showed stimulation of sulfur reduction by acetate in all four studied lakes (see Fig. 1). However, enrichment cultures at 4 M NaCl started from these experiments by 1 : 100 dilutions only yielded a single stable culture from Hummocky Lake. This culture produced up to 5 mM sulfide within a month of incubation. In maximal serial dilutions small irregular coccoid cells were dominating. Finally, a homogenous culture was obtained designated strain HSR2. A PCR test with bacterial primers did not give any products, while use of a combination of the forward archaeal primer a8f and universal primer 1492r gave a positive result. Sequencing of the product demonstrated that the organism is a novel member of the order *Halobacterales* (< 93% 16S rRNA gene sequence similarity to its culturable representatives; the closest genus is *Halobacterium*; data not shown). This is already a second extraordinary example of a haloarchaeon with the ability of dissimilatory sulfur respiration. The first one is a natronoarchaeon strain AHT32 (related to the genus *Natronolimnobius*), which was isolated recently from hypersaline soda lakes in the same area (Sorokin *et al.*, 2011c). But, in contrast to the halophilic strain HSR2, the natronophile was incapable of anaerobic growth with acetate. These two examples demonstrate for the first time that the metabolism of haloarchaea is not restricted to aerobic respiration (common), denitrification (rare) or fermentation (rare). It also provides a missing link between the Crenarchaea, in which sulfur respiration is a common trait, and the Euryarchae.

Concluding, the *in situ* sulfate- and thiosulfate-reducing activity in surface sediments of hypersaline lakes of south-eastern Siberia was low, but was activated after the reduction in salt concentration. In contrast, sulfur reduction was active at *in situ* conditions and was particularly stimulated by the acetate addition. Pure cultures of sulfate- and thiosulfate-reducing bacteria isolated from the hypersaline lakes were represented by four known genera of moderately halophilic SRB from the orders *Desulfovibrionales* and *Desulfobacterales*, mostly related to the species previously found in Great Salt Lake. Sulfur-reducing organisms isolated in pure culture belonged to *Halanaerobiales* and haloarchae-specific groups of halophiles which employ K^+ as the main compatible solute. Such organisms have a bioenergetic advantage over prokaryotes using organic compatible solutes in case of low energy yield of catabolic reactions (Oren, 1999, 2011). This may explain that only sulfur reduction was highly active at saturated salt concentration in the sediments of studied hypersaline lakes.

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