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Abstract:	Two novel haloalkaliphilic bacteria with dissimilatory sulfidogenic metabolism were recovered from syntrophic associations obtained from anaerobic sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). Strain ASO3-2T was a member of a sulfidogenic syntrophic association oxidizing acetate at extremely haloalkaline conditions, and was isolated in pure culture using formate as electron donor and sulfate as electron acceptor. It was identified as a new member of the genus Desulfonatronospira within the Deltaproteobacteria. In contrast to the two known species of this genus, the novel isolate was able to grow with formate as electron donor and sulfate, as well, as with sulfite as electron acceptor. Strain Acr1 was a minor component in a soda lake syntrophic association converting benzoate to methane and acetate. It became dominant in a subculture fed with crotonate. While growing on crotonate, Acr1 formed unusually long cells filled with PHA-like granules. Its metabolism was limited to fermentation of crotonate and pyruvate and the ability to utilize thiosulfate and sulfur/polysulfide as e-acceptor. Strain Acr1 was identified as a new member of the genus Desulfitispora in the class Clostridia. Both isolates were obligately haloalkaliphilic with extreme salt tolerance. On the basis of phenotypic and phylogenetic analyses, the novel sulfidogenic isolates from soda lakes are proposed to form two new species: Desulfonatronospira sulfatiphila sp. nov. (ASO3-2T = DSM 100427= UNIQEM U993T) and Desulfitispora elongata sp. nov. (Acr1T = DSM 29990 = UNIQEM U994T).

Desulfonatronospira sulfatiphila sp. nov., and *Desulfitispora elongata* sp. nov., the two novel haloalkaliphilic sulfidogenic bacteria from soda lakes

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Running title: *Desulfonatronospira sulfatiphila* sp. nov., *and Desulfitispora elongata* sp. nov.

The GenBank/EMBL/DDBJ accession number for the 16S-rRNA gene sequences of strains ASO3-2^T and Acr1^T are KP223255 and KP657487; the numbers of DsrB gene/protein sequences of strains ASO3-2^T and Acr1^T are KF835251 and KP939039.

Two novel haloalkaliphilic bacteria with dissimilatory sulfidogenic metabolism were recovered from syntrophic associations obtained from anaerobic sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). Strain ASO3-2^T was a member of a sulfidogenic syntrophic association oxidizing acetate at extremely haloalkaline conditions, and was isolated in pure culture using formate as electron donor and sulfate as electron acceptor. It was identified as a new member of the genus *Desulfonatronospira* within the *Deltaproteobacteria*. In contrast to the two known species of this genus, the novel isolate was able to grow with formate as electron donor and sulfate, as well, as with sulfite as electron acceptor. Strain Acr1 was a minor component in a soda lake syntrophic association converting benzoate to methane and acetate. It became dominant in a subculture fed with crotonate. While growing on crotonate, Acr1 formed unusually long cells filled with PHA-like granules. Its metabolism was limited to fermentation of crotonate and pyruvate and the ability to utilize thiosulfate and sulfur/polysulfide as *e*-acceptor. Strain Acr1 was identified as a new member of the genus *Desulfitispora* in the class *Clostridia*. Both isolates were obligately haloalkaliphilic with extreme salt tolerance. On the basis of phenotypic and phylogenetic analyses, the novel sulfidogenic isolates from soda lakes are proposed to form two new species: *Desulfonatronospira sulfatiphila* sp. nov. (ASO3-2^T = DSM 100427= UNIQEM U993^T) and *Desulfitispora elongata* sp. nov. (Acr1^T = DSM 29990 = UNIQEM U994^T).

Our recent research into syntrophic oxidation of volatile fatty acids (VFA) at extremely haloalkaline conditions in anaerobic sediments of hypersaline soda lakes resulted in discovery of several highly enriched associations oxidizing VFA either with sulfate as electron acceptor and forming sulfide (Sorokin *et al*., 2014) or without sulfate at methanogenic conditions (Sorokin *et al*., 2016). An association, oxidizing acetate in presence of sulfate as *e*-acceptor at extreme salinity of up to 3.5 M total Na^+ was purified to two components consisting of a novel lineage of acetate-oxidizing clostridium '*Ca*. Syntrophonatronum acetioxidans' and its sulfate-reducing partner, strain ASO3-2, identified as a member of the genus *Desulfonatronospira*. This genus of extremely haloalkaliphilic SRB has previously been found in hypersaline soda lakes and is characterized by its ability to grow chemolithoautotrophically by dismutation of sulfite and thiosulfate, while growth with sulfate was only possible in presence of organic *e*-donors, such as lactate (Sorokin *et al*., 2008). Another syntrophic association obtained from soda lakes, along with two dominant organisms, participating in benzoate conversion to methane and acetate (Sorokin *et al*., 2016), also contained a minor bacterial component with unusually long cells. This organism was apparently feeding on some intermediates of benzoate conversion and was finally isolated using crotonate as substrate.

This paper is describing the properties of the novel isolates from the soda lake syntrophic associations and suggest to place them into two new species within the two genera of sulfidogenic bacteria, *Desulfonatronospira* and *Desulfitispora*, previously found in soda lakes.

The two syntrophic associations which served as the source of novel isolates were obtained from anoxic sediments in hypersaline soda lakes in the Kulunda Steppe (south-western Siberia, Altai, Russia; sampled in July 2010 and 2011) (Sorokin *et al*., 2015; 2016). The brines had salinities from 120 to 300 g l^{-1}, a pH from 10.1 to 10.4 and a total soluble carbonate alkalinity from 0.8 to 3.4 M.

The mineral sodium carbonate-based medium with pH 10 and 0.6 M-4 M total Na^+ used for the enrichment and growth experiments, the anaerobic cultivation technique, and the measurements of pH/salinity growth profiles was similar to those described previously (Sorokin *et al*., 2011). The incubation temperature was 30^{o}C. Electron donors were used at concentration of 10-50 mM and electron acceptors at concentrations of 5 (nitrate, nitrite, selenite, selenate, arsenate, arsenite) or 20 (sulfate, thiosulfate, sulfur, fumarate, ferrihydrite) mM. The analysis of sulfur compounds, VFA, PLFA and microscopy methods was performed as described previously (Sorokin *et al*., 2008; 2011).

Strain ASO3-2^T was isolated from an acetate-oxidizing sulfidogenic syntrophic association obtained from hypersaline soda lake Bitter-1 in Kulunda Steppe at pH 10 and 2 M total Na^+ (Sorokin et al., 2014). First, a subculture was established at 2 M Na^+ and pH 10, using formate as *e*-donor and sulfate as acceptor, followed by several dilution to extinction series until the 16S-rRNA gene-based DGGE showed a single band with a sequence identical to those present in the binary culture. The cells were nonmotile rod to coma shaped (**Fig. 1 a**). It grew with formate+sulfate at pH 10 in carbonate-based medium at salinity from 1 to 4 M (optimum at 2 M). At optimal salinity it showed an obligately alkaliphilic profile, growing within the pH range from

9 to 10.3 with an optimum at 9.7-10. The growth rate with formate+sulfate even at optimal salt-pH conditions was extremely low (0.003 h^{-1}). Substrate profiling showed that, apart from formate-sulfate pair, it can use the following donor-acceptor combinations: formate+sulfite, lactate+sulfate, EtOH+sulfate, pyruvate+sulfate, BuOH+sulfate, sulfite alone (disproportionation). Surprisingly, no growth was observed when thiosulfate was used either as electron acceptor with formate or alone in disproportionation mode. Likewise, no growth was achieved with H_2 and either sulfate or sulfite as *e*-acceptors. With sulfate as the acceptor, ASO3-2 was unable to grow with acetate, propionate, butyrate, malate, succinate, and fumarate. When formate was used as the electron donor, no growth was observed with the following acceptors: sulfur, ferrihydrite, arsenate, selenate, nitrate, nitrite, fumarate.

Strain Acr1^T was obtained from a syntrophic methanogenic association enriched from Kulunda Steppe soda lakes on benzoate (Sorokin et al., 2016) at pH 10 and 0.6 M total Na^+. In an attempt to grow the benzoate-fermenting syntroph alone, a subculture was made using crotonate as a single substrate in presence of bromethane sulfonate to inhibit methanogens. However, instead of the syntroph, a minor bacterial component still present in the association became dominating, and it was further purified by dilution series to homogeneity. While growing with crotonate, the culture was dominated by long rods filled with PHA-like refractive granules and motile with peritrichous flagella (**Fig. 1 b, c**). However, cells grown with pyruvate lack the inclusions. At pH 10 it was able to grow at salinity range from 0.4 to 3 M total Na^+ with an optimum at 0.6-1.0 M. It was obligately alkaliphilic, growing at 1 M Na^+ within the pH range from 8.3 to 10.5 (optimum at 9.3-9.5). From the tested *e*-donors, strain Acr1^T was only able to grow with crotonate (C4) and pyruvate (C3). Both were

fermented. Crotonate was fermented to a mixture of acetate and butyrate with trace amount of H_2 in the gas phase, while the only detectable product of pyruvate fermentation was acetate. Furthermore, in presence of thiosulfate anaerobic growth on crotonate and pyruvate was accompanied by sulfide production. Acr1^T reduced both sulfur atoms of thiosulfate to sulfide (maximum production - 7 mM). On the other hand, elemental sulfur was onlyreduced in presence of pyruvate, with intermediate formation of polysulfide (maximum total sulfane accumulation 9.5 mM). In the presence of both acceptors the final growth yield of the culture increased by 10-15% in comparison to fermentation. Moreover, the addition of thiosulfate to crotonate culture resulted in product shift: H_2 was completely absent, while the amount of acetate increased two times in parallel to a corresponding decrease in butyrate formation. On the other hand, no thiosulfate-dependent changes in the products was observed in the pyruvate culture. Similar to elemental sulfur, sulfite was also utilized as *e*-acceptor only in case of pyruvate, but it was toxic already at concentration 5 mM and the final amount of produced sulfide was two times lower than in case of thiosulfate. None of the other donors and acceptors (mentioned above as tested for strain ASO3-2) supported growth of Acr1.

The PLFA profile of strain ASO3-2^T was dominated by two saturated species 16:0 with i15:0 with two unsaturated compounds 18:1ω7 and 16:1ω7 in less abundance (**Supplementary table S1**). The profile was clearly different from the two closely related species from the same genus (see below). In strain Acr1^T the PLFA profile was more diverse with a domination of C16-C18 unsaturated species, such as 16:1ω7c, 16:1ω9c and 18:1ω7, while a single dominant among the saturated species was represented by 16:0 (**Supplementary table S2**). In general, the profile was

similar to the closest relative (see below), except for a presence/absence of two unsaturated compounds in each.

High molecular weight genomic DNA was extracted by the phenol-chloroform method (Marmur, 1961) and its G + C content was analyzed by the thermal denaturation/reassociation technique (Marmur & Doty, 1962) using *Escherichia coli* as a standard (details are in Supplementary data). The G + C content of genomic DNA for strains ASO3-2^T and Acr1^T was 51.1 and 40.3 mol%, respectively.

The DNA for molecular analysis was extracted using the UltraClean Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA). The nearly complete 16S rRNA gene was obtained with general bacterial primers 11f-1492r (Lane 1991). The *dsr*AB genes were amplified with the primers DSR1F/DSR4R [ACGCCACTGGAAGCACG/GTGTAGCAGTTACCGCA] (Wagner *et al*., 1998). The PCR mix was incubated for 5 min at 94ºC, followed by 34 cycles of 20 s at 93ºC, 45 s 55ºC, and 190 sec at 72ºC, with the final extension at 72ºC for 10 min. The PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen, the Netherlands). The sequences were aligned to the related *dsr*B sequences using CLUSTAL W. The phylogeny was inferred using the Neighbor-Joining (NJ) method and the trees were constructed by using the MEGA-6 package (Tamura *et al*. 2013).

The phylogenetic analysis of 16S rRNA gene demonstrated that strain ASO3-2^T is a member of the genus *Desulfonatronospira* (family *Desulfohalobiaceae*, *Deltaproteobacteria*) accommodating extremely salt tolerant alkaliphilic sulfate-reducing bacteria from hypersaline soda lakes (Sorokin *et al*., 2008) (**Fig. 2a**), while strain Acr1^T falls into the genus *Desulfitispora* (*Clostridia*), so far containing a single

haloalkaliphilic species of sulfidogenic haloalkaliphile from soda lakes (Sorokin *et al*., 2010) (**Fig. 3a**). Both had 98% sequence similarity to the type species of the corresponding genera. The DNA-DNA hybridization (according to De Ley *et al*., 1970; detailes in Supplementary data) between strain ASO3-2 and *Desulfonatronospira thiodismutans* ASO3-1 showed 41% homology, while strain Acr1 had 32% homology to *Desulfitispora alkaliphila* AHT17.

The amplification of *dsr*AB was positive for both organisms and phylogenetic analysis based on DsrB showed a close relation between the type species and Acr1 (**Fig. 3 b**). In case of ASO3-2, however, the DsrB phylogeny was less obvious and the clustering order depended on the algorithm used for the tree calculation. While the NJ method placed ASO3-2 sequence into the cluster of *Desulfonatronospira-Desulfohalophilus* (Supplementary fig. S1), in the ML-calculated tree ASO3-2 DsrB formed a deep lineage at the root of *Desulfonatronospira-Desulfohalophilus-Desulfonatronovibrio* clade (**Fig. 2 b**).

Overall, the two novel sulfidogens from soda lakes, although being clearly members of the know haloalkalphilic genera, are sufficiently different from the type species both (phylo)genetically and phenotypically (the comparison is given in **Table 1**) to be suggested as two novel species *Desulfonatronospira sulfatiphila* ASO3-2^T and *Desulfitispora elongata* Acr1^T.

Description of *Desulfonatronospira sulfatiphila* sp. nov.

[sul.fa.ti′phi.la. N.L. masc. n. *sulfas, sulfatis*, sulfate; Gr. adj. *philos* loving; N.L. fem. adj. *sulfatiphila* loving sulfate]

Cells are Gram-negative nonmotile rod to coma shaped, 0.7-0.8 x 1.5-3 μm. Lyzes at salt concentrations below 0.5 M. The dominant PLFA include 16:0, i15:0, 18:1ω7 and 16:1ω7 (in order of abundance). Obligately anaerobic, utilizing formate, EtOH, lactate, pyruvate and BuOH as energy source and sulfate and sulfite as electron acceptor. Sulfite can be disproportionated. Extremely salt-tolerant with a salinity range for growth (as sodium carbonates) from 1 to 4 M total Na^+ (optimum at 2 M) and obligately alkaliphilic with a pH range for growth between 9 and 10.3 (optimum at pH 9.7-10). The growth temperature maximum is 40ºC (optimum 33-35ºC). The G + C content of the DNA is 51.1 mol% (T_m). Isolated from sediments of a hypersaline soda lake Bitter-1 in the south-western Siberia (Altai, Russia). The type strain is ASO3-2^T (DSM 100427= UNIQEM $U993^T$). The 16S-rRNA gene sequence accession number is KP223255.

Description of *Desulfitispora elongata* sp. nov.

[e.lon.ga′ta. L. fem. part. adj. *elongata* elongated]

Cells are Gram-positive long rods, 0.8-1.0 x 3.0-25 μm, motile with peritrichous flagella and forming multiple inclusions of PHA granules. The dominant PFLA include 16:1ω7c, 16:1ω9c and 18:1ω7 and 16:0. Obligately anaerobic, utilizing only crotonate and pyruvate as carbon and energy source by fermentation or by facilitated fermentation in presence of thiosulfate, sulfite or elemental sulfur as electron acceptor. Thiosulfate is reduced completely to sulfide. Moderately salt-tolerant with a salinity range for growth from 0.4 to 3 M total Na^+ (optimum at 0.6-1.0 M) and obligately alkaliphilic with a pH range for growth between 8.3 and 10.5 (optimum at pH 9.3-9.5). The growth temperature maximum is at 41ºC (optimum 35-37ºC). The G + C content of the DNA is 40.3 mol% (T_m). Isolated from sediments of soda lakes in

south-western Siberia (Altai, Russia). The type strain is Acr1^T (DSM 29990 = UNIQEM U994^T). The 16S-rRNA gene sequence accession number is KP657487.

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Table 1. Comparison of properties of novel sulfidogenic isolates from soda lakes with their closest relatives from the genera *Desulfonatronospira* and *Desulfitispora*

Property	**ASO3-2^T**	*Desulfonatronospira thiodismutans* ASO3-1^T	**Acr1^T**	*Desulfitispora alkaliphila* AHT17^T
Cell morphology	short rods to comma	vibrio to spirilla	long rods	short rods
Motility	-	+ single polar flagellum	+ multiple peritrichous flagella	+ single subpolar flagellum
Endospores	-	-	-	+
PHA granules	-	-	+	-
Dominant fatty acids in membrane polar lipids	16:0, i15:0, 18:1ω7, 16:1ω7	i15:0, i17:1, 16:0	16:1ω7c, 16:1ω9c, 18:1ω7, 16:0	16:1ω7c, 16:1ω5c, 18:1ω7, 16:1ω9c
Metabolism	anaerobic respiration, disproportionation		fermentation, anaerobic respiration	
Electron donors	formate, lactate, pyruvate, EtOH, BuOH	H_2, formate, lactate, pyruvate, EtOH, BuOH	crotonate, pyruvate	lactate, pyruvate
Electron acceptors	sulfate, sulfite	sulfate, sulfite, thiosulfate	thiosulfate, sulfite, sulfur	
Salt range (optimum), M Na^+	1.--4.0 (2.0)	1.5-4.0(2.0-2.5)	0.4-3.0 (1.0-1.5)	0.1-1.4 (0.4)
pH range (optimum)	9.0-10.3 (9.7-10.0)	8.5-10.6 (9.5-10.0)	8.3-10.5 (9.3-9.5)	8.3-10.5 (9.5)
G + C, mol%	51.1	50.4	40.1	37.9
Habitat	Hypersaline soda lakes, south-western Siberia, Russia			

Legend to the figures

Fig. 1 Cell morphology of strains ASO3-2^T (**a)** and Acr1^T (**b**, **c)** grown at pH 10 with formate+sulfate and crotonate, respectively. (**a-b)**, phase contrast microphotographs; (**c**), electron microphotographs of cells stained with phosphotungstic acid.

Fig. 2 Phylogenetic position of novel haloalkaliphilic sulfidogenic isolate ASO3-2^T within the *Deltaproteobacteria* based on 16S rRNA gene (**a**) and DsrB (**b**) sequence analysis. The trees were reconstructed from evolutionary distances by using the maximum likelihood (ML). The percentage of bootstraps was derived from 500 resamplings. Values greater than 50 % were considered as significant.

Fig. 3 Phylogenetic position of novel haloalkaliphilic sulfidogenic isolate Acr1^T within the order *Peptococcales* (*Clostridia*) based on 16S rRNA gene (**a**) and DsrB (**b**) sequence analysis. The trees were reconstructed from evolutionary distances by using the ML algorithm. The percentage of bootstraps was derived from 500 resamplings. Values greater than 50 % were considered as significant.

Figure1

Click here to download Figure fig1.ppt

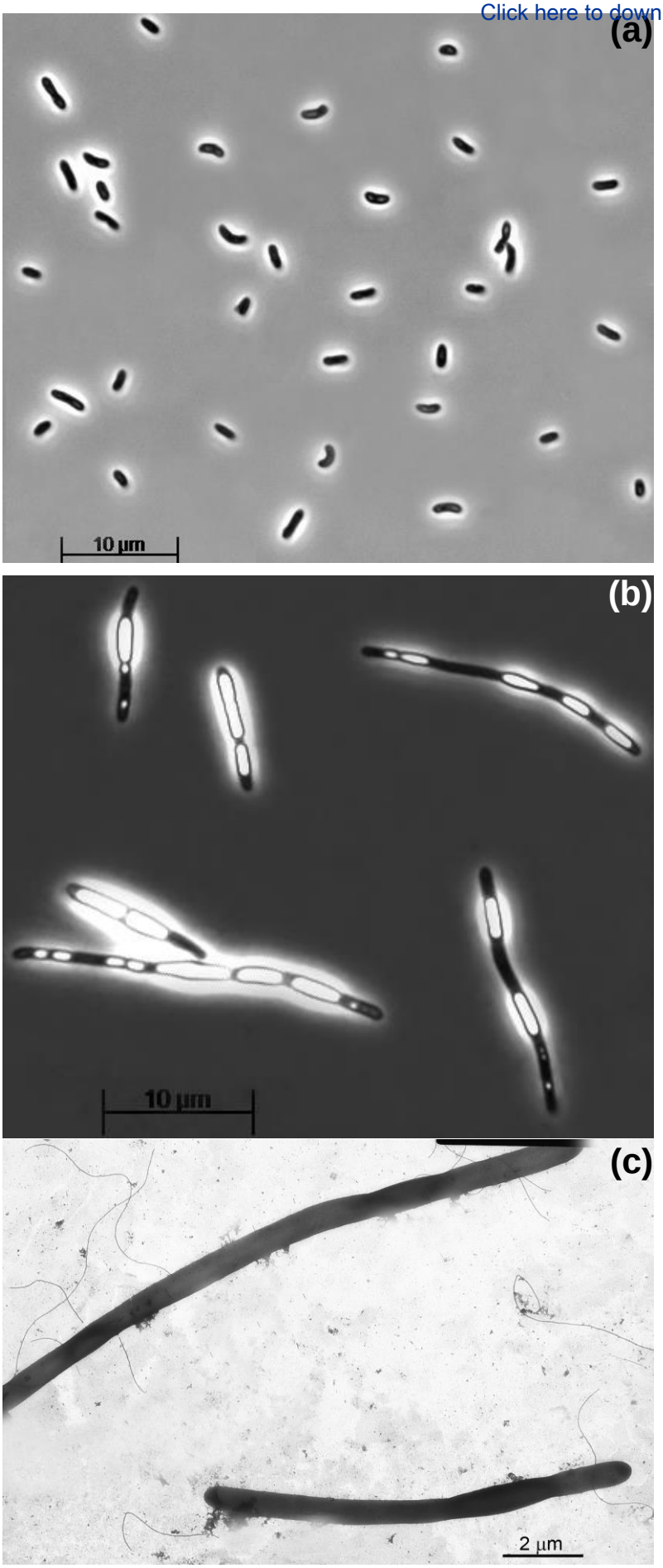
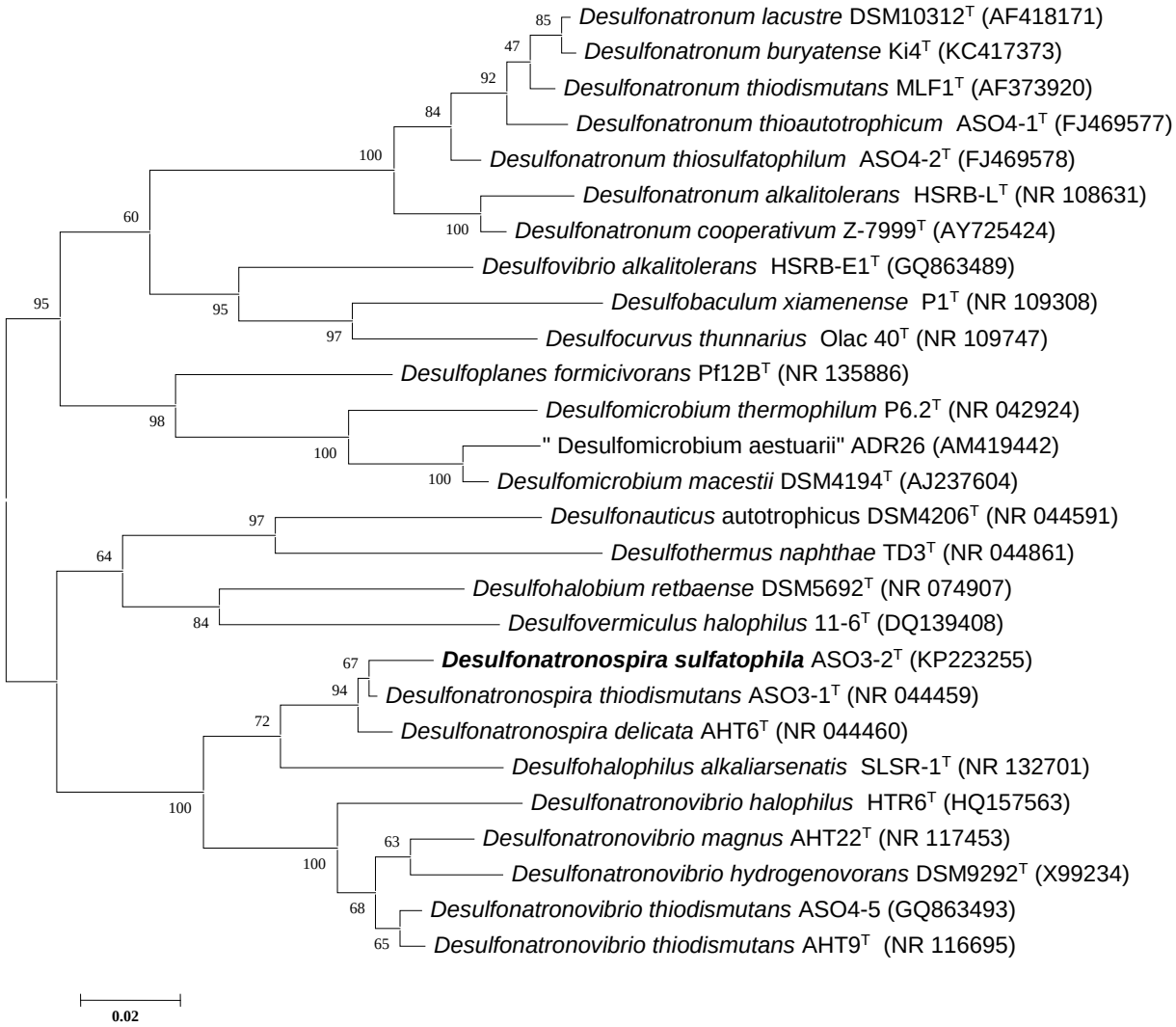


Fig.1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Desulfonatronum buryatense Ki4T (KC417373)
Desulfonatronum thiodismutans MLF1T (AF373920)
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Desulfonatronum thiosulfatophilum ASO4-2T (FJ469578)
Desulfonatronum alkalitolerans HSRB-LT (NR 108631)
Desulfonatronum cooperativum Z-7999T (AY725424)
Desulfovibrio alkalitolerans HSRB-E1T (GQ863489)
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Desulfocurvus thunnarius Olac 40T (NR 109747)
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Desulfonatronovibrio magnus AHT22T (NR 117453)
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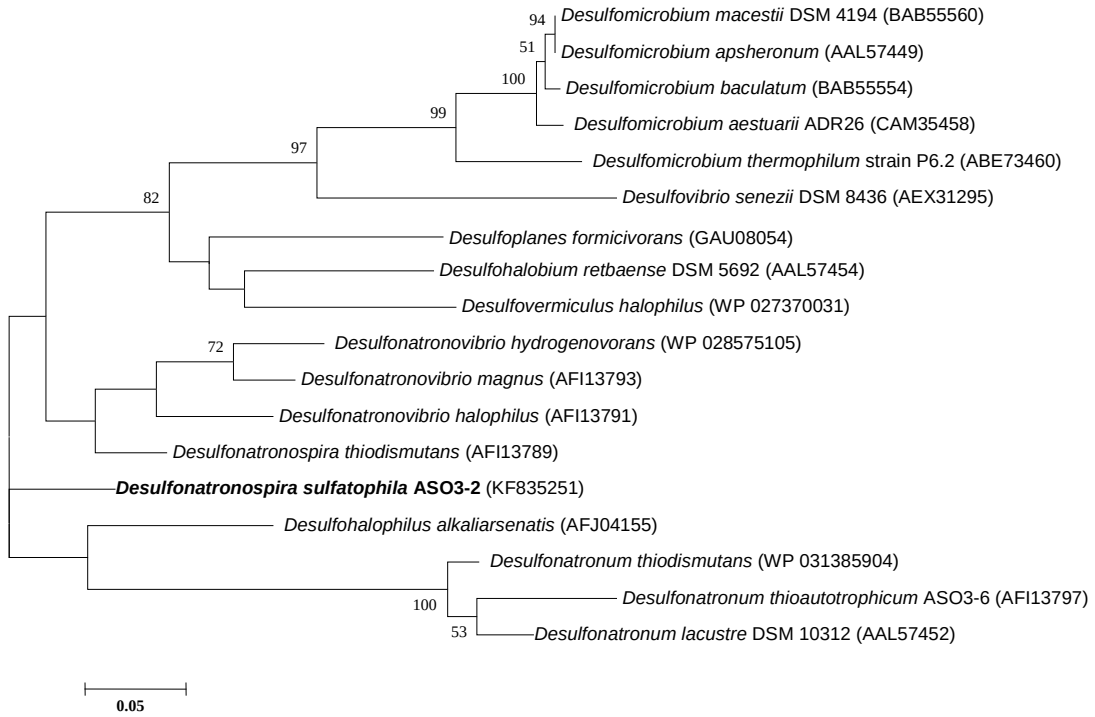
Fig. 2a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Desulfoplanes formicivorans (GAU08054)
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Desulfovermiculus halophilus (WP 027370031)
Desulfonatronovibrio hydrogenovorans (WP 028575105)
Desulfonatronovibrio magnus (AFI13793)
Desulfonatronovibrio halophilus (AFI13791)
Desulfonatronospira thiodismutans (AFI13789)
***Desulfonatronospira sulfatophila* ASO3-2** (KF835251)
Desulfohalophilus alkaliarsenatis (AFJ04155)
Desulfonatronum thiodismutans (WP 031385904)
Desulfonatronum thioautotrophicum ASO3-6 (AFI13797)
Desulfonatronum lacustre DSM 10312 (AAL57452)
0.05

Fig. 2b

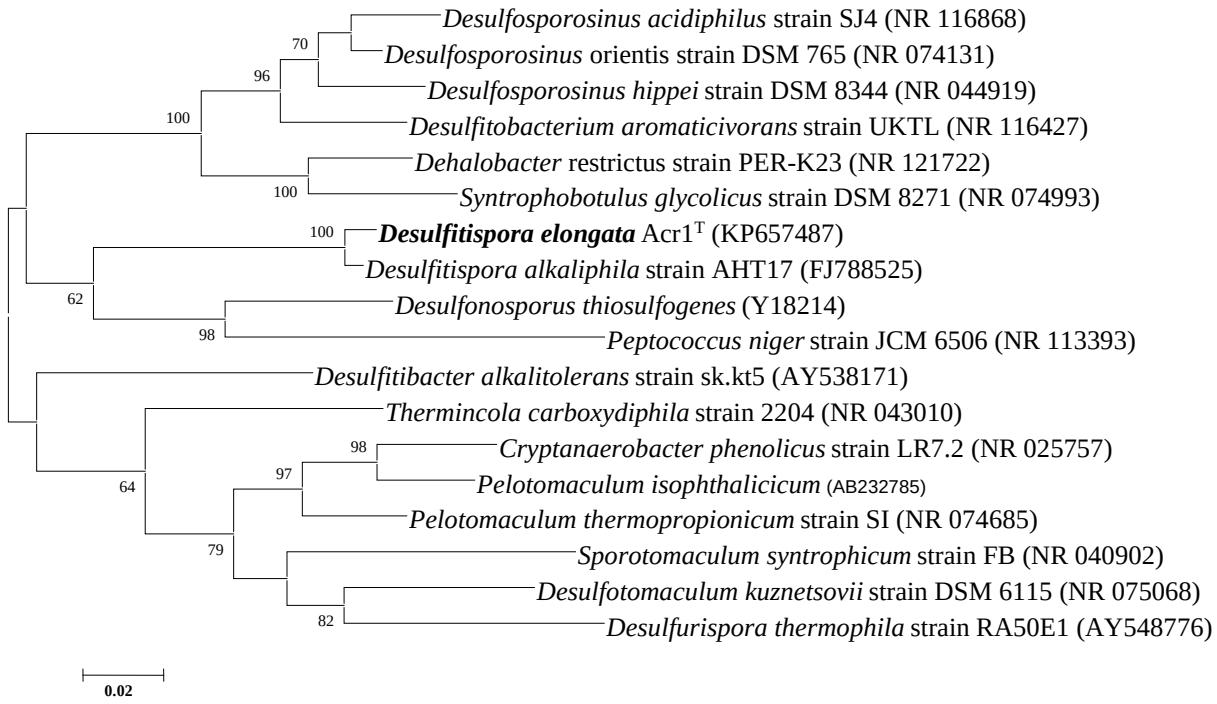
Desulfosporosinus acidiphilus strain SJ4 (NR 116868)
Desulfosporosinus orientis strain DSM 765 (NR 074131)
Desulfosporosinus hippei strain DSM 8344 (NR 044919)
Desulfitobacterium aromaticivorans strain UKTL (NR 116427)
Dehalobacter restrictus strain PER-K23 (NR 121722)
Syntrophobotulus glycolicus strain DSM 8271 (NR 074993)
Desulfitispora elongata Acr1^{T} (KP657487)
Desulfitispora alkaliphila strain AHT17 (FJ788525)
Desulfonosporus thiosulfogenes (Y18214)
Peptococcus niger strain JCM 6506 (NR 113393)
Desulfitibacter alkalitolerans strain sk.kt5 (AY538171)
Thermincola carboxydiphila strain 2204 (NR 043010)
Cryptanaerobacter phenolicus strain LR7.2 (NR 025757)
Pelotomaculum isophthalicicum (AB232785)
Pelotomaculum thermopropionicum strain SI (NR 074685)
Sporotomaculum syntrophicum strain FB (NR 040902)
Desulfotomaculum kuznetsovii strain DSM 6115 (NR 075068)
Desulfurispora thermophila strain RA50E1 (AY548776)
70
96
100
100
100
62
98
98
97
64
79
82
0.02

Fig. 3a

Figure3b

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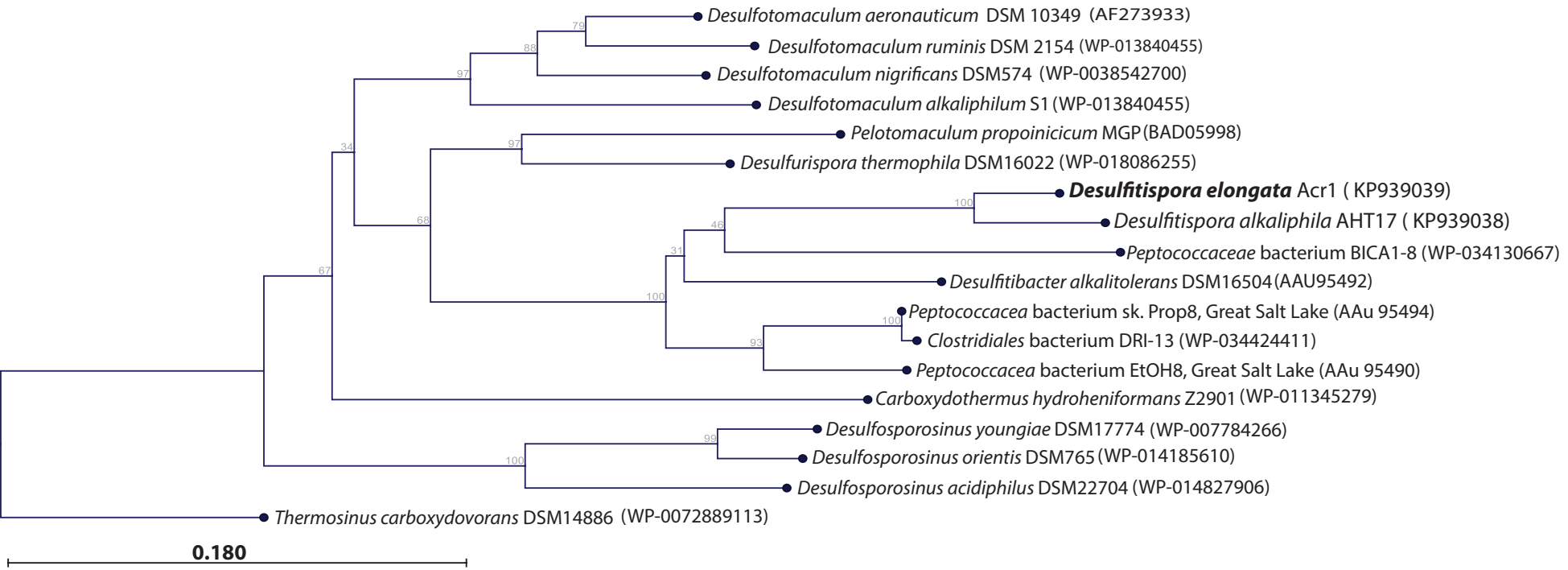


Supplementary files to:

Desulfonatronospira sulfatiphila sp. nov., and *Desulfitispora elongata* sp. nov., the two novel haloalkaliphilic sulfidogenic bacteria from soda lakes

Dimitry Y. Sorokin, Nikolai A. Chernyh

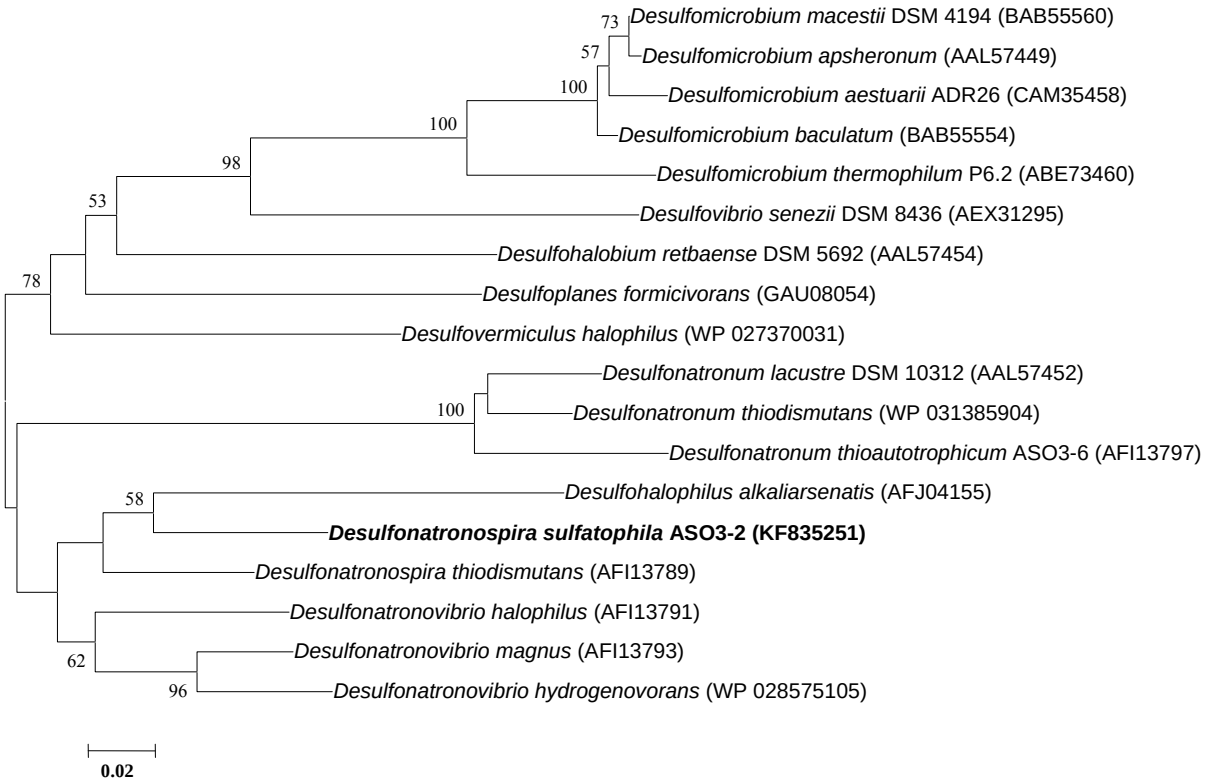
Supplementary Table S1
Polar lipids fatty acid composition of strain ASO3-2 in comparison with the *Desulfonatronospira* species (grown at 2 M Na^+, pH 10, 30°C).
Only species above 0.5% are shown, the values above 5% are in bold

code	**ASO3-2**	*Dnsp. thiodismutans*	*Dnsp. delicata*
13:1	3.0		
14:0	4.0	0.70	0.14
15:0	1.8		
i15:0	**16.7**	**45.62**	**48.86**
a15:0	2.2	1.78	3.87
16:0	**40.0**	**11.34**	**4.65**
i16:0	0.6	1.57	3.92
16:1ω7	**8.6**	0.98	0.28
i16:1	0.6	0.87	0.95
10Me16			**12.5**
17:0	1.7	0.56	1.49
i17:0		**6.28**	**11.80**
17:1 ω8	1.9		
a17:0		0.94	**4.52**
i17:1	**5.5**	**14.93**	0.26
a17:1		1.43	0.93
18:0	4.0	**6.49**	2.32
18:1 ω7	**10.8**	**4.26**	0.94
18:1 ω9		0.63	0.14
10Me18:0		1.64	0
i19:0	1.1		
20:1 c1/t11	2.0		

Supplementary Table S2

Polar lipids fatty acid composition of strain Acr1 in comparison with the type strain of the genus *Desulfitispora*. The cultures were grown in 0.6 M total Na^+, pH 10 at 30°C and harvested in late exponential growth phase.

FA	% from total	
	Acr1	*Desulfitispora alkaliphila*
15:1ω8c	1.3	
16:0	**11.9**	**6.9**
16:0 DMA	3.7	
i16:1ω7		1.5
i16:1ω5a		1.8
16:1ω5c	2.1	**19.4**
16:1ω5a		**1.3**
16:1ω5 DMA		1.7
16:1ω7c	**23.1**	**27.3**
16:1ω7c DMA	**5.4**	1.6
16:1ω7a		1.1
16:1ω9c	**11.6**	**8.0**
17:1 ω8c	1.4	
17:1 ω9c	0.6	
18:0	3.1	0.6
18:1ω9c DMA	3.9	
18:1ω7	**13.3**	**10.4**
18:1ω9c	**5.6**	
18:1ω6		**6.9**
i18:1ω6a		1.8
i18:1ω7a		2
18:1ω7c DMA	**9.0**	1.4
10Me18:0	1.0	
18:2		2.1
i19:1	1.7	
20:1ω7c	2.4	
20:4 ω6c	0.8	



Supplementary fig.S1: Phylogenetic tree based on the DsrB aminoacid sequence analysis showing position of strain ASO3-2 within the order *Desulfovibrionales* in the *Deltaproteobacteria*. The tree was reconstructed using the neighbour-joining algorithm. The number on nods indicate the bootstrap values calculated from 500 repetitions with the confidence above 50%.

Isolation of HMW genomic DNA (25-30 kB)

> Washed cells are resuspended in 50 мM TrisHCl/EDTA 50 mM, pH 8.0
> Lyzozyme up to 2 mg/ml, mix, add RNAse up to 0.2 mg/ml and incubate at 37^{o}C, 30 min.
> proteinase K up to 0.4 mg/ml + 0.5 % SDS, incubate at 50 ^{o}C for 30 min, cool to 4^{o}C. At this stage most of the cells were lyzed which was evident from microscopy and increased viscosity.
> Phenol solution for molecular biology (in Tris buffer, pH 8) up to 0.5 V + 0.5 V chloroform kept at 4^{o}C, incubate on the rotary shaker at 20 rpm for 5-10 min.
> Centrifuge in 2 ml epps 10 min at maximal speed, take upper phase, repeat chloroform extraction 2 times to remove traces of phenol add 2 V of cold 96% ethanol; gently mix until the DNA medusa is formed, collect it on to a thin glass tube, put the tube briefly into 70% and finally – 96% ethanol, dry the material briefly and redissolve in 0.1x SSC buffer.
> Measure spectrum and ratio 230-260-280-320 nm.
> Check the molecular weight in 0,8% agarose gel with 25 kB marker.

Determination of the G + C by DNA midpoint melting temperature (Tm)
(according to Marmur&Doti, 1962)
The HMW DNA was dissolved in 0.1 SSC buffer and subjected to thermal denaturation spectrophotometry with the rate of 0.5 ^{o}C/min using Pye-Unicam SP1800 instrument (Cambridge, UK). The standard DNA from *E. coli* strain K12 with the G +C value of 50 was used as a control. DNA base composition, expressed as was calculated from the equation 2.44*(Tm-69.4).

DNA-DNA hybridization by thermal denaturation-reassociation method.
(De Ley et al., 1970)
The HMW DNA dissolved in 0.1 SSC buffer was sonicated at 0.4 mA for 2-3 min on the ice bath under N_2 atmosphere which resulted in DNA shearing into fragments of an approximately 0.5 kB size. 2 ml preparations with the DNA concentration of 100 μg/ml (OD_{260}=2.0) were denatured at 100^{o}C for 5 min. 3 cuvetts containing 0.2 ml x20 SSC were preheated to the optimal reassociation temperature calculated from the G + C content value [0.51* (G + C mol%) + 47] and 0.8 ml of the DNA solutions A, B and C [A:B (1:1)} were added and rapidly mixed. The renaturation was followed during for 35-40 min with the recording step of 15 sec using Pye-Unicam SP1800 instrument. The results were calculated from the equation

$$\%H = \frac{4\,Vc - (V_A + V_B)}{V_A + V_B} \times 100$$

Desulfonatronospira sulfat~~o~~iphila sp. nov., and *Desulfitispora elongata* sp. nov., the two novel haloalkaliphilic sulfidogenic bacteria from soda lakes

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Running title: *Desulfonatronospira sulfat~~o~~iphila* sp. nov., *and Desulfitispora elongata* sp. nov.

The GenBank/EMBL/DDBJ accession number for the 16S-rRNA gene sequences of strains ASO3-2^T and Acr1^T are KP223255 and KP657487; the numbers of DsrB gene/protein sequences of strains ASO3-2^T and Acr1^T are KF835251 and KP939039.

Two novel haloalkaliphilic bacteria with dissimilatory sulfidogenic metabolism were recovered from syntrophic associations obtained from anaerobic sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). Strain ASO3-2^T was a member of a sulfidogenic syntrophic association oxidizing acetate at extremely haloalkaline conditions, and was isolated in pure culture using formate as electron donor and sulfate as electron acceptor. It was identified as a new member of the genus *Desulfonatronospira* within the *Deltaproteobacteria*. In contrast to the two known species of this genus, the novel isolate was able to grow with formate as electron donor and sulfate, as well, as with sulfite as electron acceptor. Strain Acr1 was a minor component in a soda lake syntrophic association converting benzoate to methane and acetate. It became ~~a~~ dominant in a subculture fed with crotonate. While growing on crotonate, Acr1 formed ~~an~~ unusually long cells filled with PHA-like granules. Its metabolism was limited to fermentation of crotonate and pyruvate and the ability to utilize thiosulfate and sulfur/polysulfide as *e*-acceptor. Strain Acr1 ~~i~~was identified as a new member of the genus *Desulfitispora* in the class *Clostridia*. Both isolates were obligately haloalkaliphilic with extreme salt tolerance. On the basis of phenotypic and phylogenetic analyses, the novel sulfidogenic isolates from soda lakes are proposed to form two new species: *Desulfonatronospira sulfat~~o~~iphila* sp. nov. (ASO3-2^T = DSM ~~24257~~100427= UNIQEM U993^T) and *Desulfitispora elongata* sp. nov. (Acr1^T = DSM 29990 = UNIQEM U994^T).

Our recent research into syntrophic oxidation of volatile fatty acids (VFA) at extremely haloalkaline conditions in anaerobic sediments of hypersaline soda lakes resulted in discovery of several highly enriched associations ~~converting~~ oxidizing VFA either with sulfate as electron acceptor~~to~~ and forming ~~-~~sulfide (Sorokin *et al*., 2014) or without sulfate at methanogenic conditions~~methane~~ (Sorokin *et al*., 2016). An association, oxidizing acetate in presence of sulfate as *e*-acceptor at extreme salinity of up to 3.5 M total Na^+ was purified to two components consisting of a novel lineage of acetate-oxidizing clostridium '*Ca*. Syntrophonatronum acetioxidans' and its sulfate-reducing partner, strain ASO3-2, identified as a member of the genus *Desulfonatronospira*. This genus of extremely haloalkaliphilic SRB has previously been found in hypersaline soda lakes and is characterized by its ability to grow chemolithoautotrophically by dismutation of sulfite and thiosulfate, while growth with sulfate was only possible in presence of organic *e*-donors, such as lactate (Sorokin *et al*., 2008). Another syntrophic association obtained from soda lakes, along with two dominant organisms, participating in benzoate conversion to methane and acetate (Sorokin *et al*., 2016), also contained a minor bacterial component with unusually long cells. This organism was apparently feeding on some intermediates of benzoate conversion and was finally isolated using crotonate as substrate.

This paper is describing the properties of the novel isolates from the soda lake syntrophic associations and suggest to place them into two new species within the two genera of sulfidogenic bacteria, *Desulfonatronospira* and *Desulfitispora*, previously found in soda lakes.

The two syntrophic associations which served as the source of novel isolates were obtained from anoxic sediments in hypersaline soda lakes in the Kulunda Steppe (south-western Siberia, Altai, Russia; sampled in July 2010 and 2011) (Sorokin *et al*., 2015; 2016). The brines had salinities from 120 to 300 g l^{-1}, a pH from 10.1 to 10.4 and a total soluble carbonate alkalinity from 0.8 to 3.4 M.

The mineral sodium carbonate-based medium with pH 10 and 0.6 M-4 M total Na^+ used for the enrichment and growth experiments, the anaerobic cultivation technique, and the measurements of pH/salinity growth profiles was similar to those described previously (Sorokin *et al*., 2011). The incubation temperature was 30°C. Electron donors were used at concentration of 10-50 mM and electron acceptors at concentrations of 5 (nitrate, nitrite, selenite, selenate, arsenate, arsenite) or 20 (sulfate, thiosulfate, sulfur, fumarate, ferrihydrite) mM. The analysis of sulfur compounds, VFA, PLFA and microscopy methods was performed as described previously (Sorokin *et al*., 2008; 2011).

Strain ASO3-2^T was isolated from an acetate-oxidizing sulfidogenic syntrophic association obtained from hypersaline soda lake Bitter-1 in Kulunda Steppe at pH 10 and 2 M total Na^+ (Sorokin et al., 2014). First, a subculture was ~~produc~~established at 2 M Na^+ and pH 10, using formate as *e*-donor and sulfate as acceptor, followed by several dilution to extinction series until the 16S-rRNA gene-based DGGE showed a single band with a sequence identical to those present in the binary culture. The cells were nonmotile rod to coma shaped (**Fig. 1 a**). It grew with formate+sulfate at pH 10 in carbonate-based medium at salinity from 1 to 4 M (optimum at 2 M). At optimal salinity it showed an obligately alkaliphilic profile, growing within the pH range from

9 to 10.3 with an optimum at 9.7-10. The growth rate with formate+sulfate even at optimal salt-pH conditions was extremely low (0.003 h^{-1}). Substrate profiling showed that, apart from formate-sulfate pair, it can use the following donor-acceptor combinations: formate+sulfite, lactate+sulfate, EtOH+sulfate, pyruvate+sulfate, BuOH+sulfate, sulfite alone (disproportionation). Surprisingly, no growth was observed when thiosulfate was used either as electron acceptor with formate or alone in disproportionation mode. Likewise, no growth was achieved with H_2 and either sulfate or sulfite as *e*-acceptors. With sulfate as the acceptor, ASO3-2 was unable to grow with acetate, propionate, butyrate, malate, succinate, and fumarate. When formate was used as the electron donor, no growth was observed with the following acceptors: sulfur, ferrihydrite, arsenate, selenate, nitrate, nitrite, fumarate.

Strain Acr1^T was obtained from a syntrophic methanogenic association enriched from Kulunda Steppe soda lakes on benzoate (Sorokin et al., 2016) at pH 10 and 0.6 M total Na^+. In an attempt to grow the benzoate-fermenting syntroph alone, a subculture was made using crotonate as a single substrate in presence of bromethane sulfonate to inhibit methanogens. However, instead of the syntroph, a minor bacterial component still present in the association became dominating, and it was further purified by dilution series to homogeneity. While growing with crotonate, the culture was dominated by long rods filled with PHA-like refractive granules and motile with peritrichous flagella (**Fig. 1 b, c**). However, cells grown with pyruvate lack the inclusions. At pH 10 it was able to grow at salinity range from 0.4 to 3 M total Na^+ with an optimum at 0.6-1.0 M. It was obligately alkaliphilic, growing at 1 M Na^+ within the pH range from 8.3 to 10.5 (optimum at 9.3-9.5). From the tested *e*-donors, strain Acr1^T was only able to grow with crotonate (C4) and pyruvate (C3). Both were

fermented. Crotonate was fermented to a mixture of acetate and butyrate with trace amount of H_2 in the gas phase, while the only detectable product of pyruvate fermentation was acetate. Furthermore, in presence of thiosulfate anaerobic growth on crotonate and pyruvate was accompanied by sulfide production. Acr1^T reduced both sulfur atoms of thiosulfate to sulfide (maximum production - 7 mM). On the other hand, elemental sulfur was only ~~used as acceptor~~reduced in presence of pyruvate, with intermediate formation of polysulfide (maximum total sulfane accumulation 9.5 mM). In the presence of both acceptors the final growth yield of the culture increased by 10-15% in comparison to fermentation. Moreover, the addition of thiosulfate to crotonate culture resulted in product shift: H_2 was completely absent, while the amount of acetate increased two times in parallel to a corresponding decrease in butyrate formation. On the other hand, no thiosulfate-dependent changes in the products was observed in the pyruvate culture. Similar to elemental sulfur, sulfite was also utilized as *e*-acceptor only in case of pyruvate, but it was toxic already at concentration 5 mM and the final amount of produced sulfide was two times lower than in case of thiosulfate. None of the other donors and acceptors (mentioned above as tested for strain ASO3-2) supported growth of Acr1.

The PLFA profile of strain ASO3-2^T was dominated by two saturated species 16:0 with i15:0 with two unsaturated compounds 18:1ω7 and 16:1ω7 in less abundance (**Supplementary table S1**). The profile was clearly different from the two closely related species from the same genus (see below). In strain Acr1^T the PLFA profile was more diverse with a domination of C16-C18 unsaturated species, such as 16:1ω7c, 16:1ω9c and 18:1ω7, while a single dominant among the saturated species was represented by 16:0 (**Supplementary table S2**). In general, the profile was

similar to the closest relative (see below), except for a presence/absence of two unsaturated compounds in each.

High molecular weight genomic DNA was extracted by the phenol-chloroform method (Marmur, 1961) and its G + C content was analyzed by the thermal denaturation/reassociation technique (Marmur & Doty, 1962) using *Escherichia coli* as a standard. The G + C content of genomic DNA for strains ASO3-2^T and Acr1^T was 51.1 and 40.3 mol%, respectively (the details are given in the Supplementary data).

The DNA for molecular analysis was extracted using the UltraClean Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA). The nearly complete 16S rRNA gene was obtained with general bacterial primers 11f-1492r (Lane 1991). The *dsr*AB genes were amplified with the primers DSR1F/DSR4R [ACGCCACTGGAAGCACG/GTGTAGCAGTTACCGCA] (Wagner *et al.*, 1998). The PCR mix was incubated for 5 min at 94^{o}C, followed by 34 cycles of 20 s at 93^{o}C, 45 s 55^{o}C, and 190 sec at 72^{o}C, with the final extension at 72^{o}C for 10 min. The PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen, the Netherlands). The sequences were aligned to the related *dsr*B sequences using CLUSTAL W. The phylogeny was inferred using the Neighbor-Joining (NJ) method and the trees were constructed by using the MEGA-6 package (Tamura *et al*. 2013).

The phylogenetic analysis of 16S rRNA gene demonstrated that strain ASO3-2^T is a member of the genus *Desulfonatronospira* (family *Desulfohalobiacea*e, *Deltaproteobacteria*) accommodating extremely salt tolerant alkaliphilic sulfate-reducing bacteria from hypersaline soda lakes (Sorokin *et al*., 2008) (**Fig. 2a**), while

strain Acr1^T falls into the genus *Desulfitispora* (*Clostridia*), so far containing a single haloalkaliphilic species of sulfidogenic haloalkaliphile from soda lakes (Sorokin *et al*., 2010) (**Fig. 3a**). Both had 98% sequence similarity to the type species of the corresponding genera. The DNA-DNA hybridization (according to De Ley *et al*., 1970; details are in Supplementary data) between strain ASO3-2 and *Desulfonatronospira thiodismutans* ASO3-1 showed 41% homology, while strain Acr1 had 32% homology to *Desulfitispora alkaliphila* AHT17.

The amplification of *dsr*AB was positive for both organisms and phylogenetic analysis based on DsrB showed a close relation between the type species and Acr1 (**Fig. 3 b**). In case of ASO3-2, however, the DsrB phylogeny was less obvious and the clustering order depended on the algorithm used for the tree calculation. While the NJ method placed ASO3-2 sequence into the cluster of *Desulfonatronospira-Desulfohalophilus* (Supplementary fig. S1~~**Fig. 2 b**~~), in the ML-calculated tree ASO3-2 DsrB formed a deep lineage at the root of *Desulfonatronospira-Desulfohalophilus-Desulfonatronovibrio* clade (**Fig. 2 ~~c~~b**).

Overall, the two novel sulfidogens from soda lakes, although being clearly members of the know haloalkalphilic genera, are sufficiently different from the type species both (phylo)genetically and phenotypically (the comparison is given in **Table 1**) to be suggested as two novel species *Desulfonatronospira sulfat~~o~~iphila* ASO3-2^T and *Desulfitispora elongata* Acr1^T.

Description of *Desulfonatronospira sulfat~~o~~iphila* sp. nov.

[sul.fa.t~~o~~i'phi.la. N.L. masc. n. *sulfas, sulfatis*, sulfate; Gr. adj. *philos* loving; N.L. fem. adj. *sulfat~~o~~iphila* loving sulfate]

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Cells are Gram-negative nonmotile rod to coma shaped, 0.7-0.8 x 1.5-3 μm. Lyzes at salt concentrations below 0.5 M. The dominant PLFA include 16:0, i15:0, 18:1ω7 and 16:1ω7 (in order of abundance). Obligately anaerobic, utilizing formate, EtOH, lactate, pyruvate and BuOH as energy source and sulfate and sulfite as electron acceptor. Sulfite can be disproportionated. Extremely salt-tolerant with a salinity range for growth (as sodium carbonates) from 1 to 4 M total Na^+ (optimum at 2 M) and obligately alkaliphilic with a pH range for growth between 9 and 10.3 (optimum at pH 9.7-10). The growth temperature maximum is 40°C (optimum 33-35°C). The G + C content of the DNA is 51.1 mol% (T_m). Isolated from sediments of a hypersaline soda lake Bitter-1 in the south-western Siberia (Altai, Russia). The type strain is ASO3-2^T (DSM ~~24257~~100427= UNIQEM U993^T). The 16S-rRNA gene sequence accession number is KP223255.

Description of *Desulfitispora elongata* sp. nov.

[e.lon.ˈga'ta. L. fem. part. adj. *elongata* elongated]

Cells are Gram-positive long rods, 0.8-1.0 x 3.0-25 μm, motile with peritrichous flagella and forming multiple inclusions of PHA granules. The dominant PFLA include 16:1ω7c, 16:1ω9c and 18:1ω7 and 16:0. Obligately anaerobic, utilizing only crotonate and pyruvate as carbon and energy source by fermentation or by facilitated fermentation in presence of thiosulfate, sulfite or elemental sulfur as electron acceptor. Thiosulfate is reduced completely to sulfide. Moderately salt-tolerant with a salinity range for growth from 0.4 to 3 M total Na^+ (optimum at 0.6-1.0 M) and obligately alkaliphilic with a pH range for growth between 8.3 and 10.5 (optimum at pH 9.3-9.5). The growth temperature maximum is at 41°C (optimum 35-37°C). The G

+ C content of the DNA is 40.3 mol% (T_m). Isolated from sediments of soda lakes in south-western Siberia (Altai, Russia). The type strain is Acr1^T (DSM 29990 = UNIQEM U994^T). The 16S-rRNA gene sequence accession number is KP657487.

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Table 1. Comparison of properties of novel sulfidogenic isolates from soda lakes with their closest relatives from the genera *Desulfonatronospira* and *Desulfitispora*

Property	**ASO3-2^T**	*Desulfonatronospira* ~~(3 strains)~~*thiodismutans* ASO3-1^T	**Acr1^T**	*Desulfitispora alkaliphila* AHT17^T
Cell morphology	short rods to comma	vibrio to spirilla	long rods	short rods
Motility	-	+ single polar flagellum	+ multiple peritrichous flagella	+ single subpolar flagellum
Endospores	-	-	-	+
PHA granules	-	-	+	-
Dominant fatty acids in membrane polar lipids	16:0, i15:0, 18:1ω7, 16:1ω7	i15:0, i17:1, 16:0	16:1ω7c, 16:1ω9c, 18:1ω7, 16:0	16:1ω7c, 16:1ω5c, 18:1ω7, 16:1ω9c
Metabolism	anaerobic respiration, disproportionation		fermentation, anaerobic respiration	
Electron donors	formate, lactate, pyruvate, EtOH, BuOH	H_2, formate, lactate, pyruvate, EtOH, BuOH	crotonate, pyruvate	lactate, pyruvate
Electron acceptors	sulfate, sulfite	sulfate, sulfite, thiosulfate	thiosulfate, sulfite, sulfur	
Salt range (optimum), M Na^+	1.--4.0 (2.0)	1.5-4.0(2.0-2.5)	0.4-3.0 (1.0-1.5)	0.1-1.4 (0.4)
pH range (optimum)	9.0-10.3 (9.7-10.0)	8.5-10.6 (9.5-10.0)	8.3-10.5 (9.3-9.5)	8.3-10.5 (9.5)
G + C, mol%	51.1	50.4	40.1	37.9
Habitat	Hypersaline soda lakes, south-western Siberia, Russia			

Legend to the figures

Fig. 1 Cell morphology of strains ASO3-2^T (**a)** and Acr1^T (**b**, **c)** grown at pH 10 with formate+sulfate and crotonate, respectively. (**a-b)**, phase contrast microphotographs; (**c**), electron microphotographs of cells stained with phosphotungstic acid.

Fig. 2 Phylogenetic position of novel haloalkaliphilic sulfidogenic isolate ASO3-2^T within the *Deltaproteobacteria* based on 16S rRNA gene (**a**) and DsrB (**b**~~–c~~) sequence analysis. The trees were reconstructed from evolutionary distances by using the maximum likelihood (ML) ~~(**a** and **c**) or the neighbor joining (NJ), (**b**) algorithms~~. The percentage of bootstraps was derived from 500 resamplings. Values greater than 50 % were considered as significant.

Fig. 3 Phylogenetic position of novel haloalkaliphilic sulfidogenic isolate Acr1^T within the order *Peptococcales* (*Clostridia*) based on 16S rRNA gene (**a**) and DsrB (**b**) sequence analysis. The trees were reconstructed from evolutionary distances by using the ML algorithm. The percentage of bootstraps was derived from 500 resamplings. Values greater than 50 % were considered as significant.