Sulfate-dependent acetate oxidation under extremely natron-alkaline conditions by syntrophic associations from hypersaline soda lakes

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So far, anaerobic sulfate-dependent acetate oxidation at high pH has only been demonstrated for a low-salt-tolerant syntrophic association of a clostridium ‘Candidatus Contubernalis alkalaceticum’ and its hydrogenotrophic sulfate-reducing partner Desulfonatronum cooperativum. Anaerobic enrichments at pH 10 inoculated with sediments from hypersaline soda lakes of the Kulunda Steppe (Altai, Russia) demonstrated the possibility of sulfate-dependent acetate oxidation at much higher salt concentrations (up to 3.5 M total Na+). The most salt-tolerant purified cultures contained two major components apparently working in syntrophy. The primary acetate-fermenting component was identified as a member of the order Clostridiales forming, together with ‘Ca. Contubernalis alkalaceticum’, an independent branch within the family Syntrophomonadaceae. A provisional name, ‘Ca. Syntrophonatronum acetoxidans’, is suggested for the novel haloalkaliphilic clostridium. Two phylotypes of extremely haloalkaliphilic sulfate-reducing bacteria of the genus Desulfonatronospira were identified as sulfate-reducing partners in the acetate-oxidizing cultures under extreme salinity. The dominant phylotype differed from the two species of Desulfonatronospira described so far, whilst a minor component belonged to Desulfonatronum thiodismutans. The results proved that, contrary to previous beliefs, sulfate-dependent acetate oxidation is possible, albeit very slowly, in nearly saturated soda brines.

INTRODUCTION

Soda lakes are naturally occurring highly alkaline and saline habitats containing sodium carbonate at high concentrations, maintaining a stable high pH of the brines. Despite these extreme conditions, soda lakes usually have high primary production. This, together with high sulfate concentrations, is the reason why the microbiological sulfur cycle is highly active in soda lakes. Soda lake sediments usually contain millimolar concentrations of free sulfide and FeS in the top 20 cm (Sorokin et al., 2011a). Detailed studies of the reductive sulfur cycle in soda lakes, including measurements of sulfate reduction rates, revealed active sulfate, thiosulfate and sulfur/poly-sulfide reduction mostly stimulated by formate/H2 (Gorlenko et al., 1999; Kulp et al., 2006, 2007; Sorokin et al., 2004, 2010a). However, only sulfur reduction was apparently active with acetate as electron donor in a relatively short-term incubation experiment. This was corroborated by the results of a cultivation approach, which demonstrated a domination of lithotrophic sulfate-reducing bacteria (SRB) using H2/formate, lactate and ethanol as electron donors and disproportionating thiosulfate/sulfite, such as in the genera Desulfonatronum, Desulfonatronovibrio and Desulfonatronospira (Pikuta et al., 1998, 2003; Zhilina et al., 1997, 2005a; Sorokin et al., 2008, 2011b). Heterotrophic SRB isolated from soda lakes are either ‘incomplete oxidizers’, producing acetate as a final product during oxidation of simple organic compounds (Desulfobotulus alkaliphilus and Desulfobulbus alkaliphilus; Sorokin et al., 2010b, 2012) or ‘complete oxidizers’, which cannot use external acetate (Desulfonatronobacter...
Acidovorans; Sorokin et al., 2012). However, a specific group of acetate- and propionate-oxidizing sulfur/polysulfide respiring nanophosphates able to grow in concentrated soda brines can be isolated easily from hypersaline soda lakes (Desulfurispira natronophila; Sorokin & Muyzer, 2010).

So far, there has been only a single report on the possibility of indirect sulfate-dependent acetate oxidation at high pH by a syntrophic association of a novel obligate syntrophic endospore-forming clostridium Candidatus Contubernalis alkalaceticum and a hydrogenotrophic SRB, Desulfitonatronospira cooperativum (Zhilina et al., 2005b). The association was enriched from the low-salt alkaline lake Haydin in Tuva Republic (Russia) as a part of a low-salt anaerobic cellulose-degrading community (total Na⁺ ~0.25 M, pH 10) selected by using acetate as electron donor and sulfate as electron acceptor (Kevbrin et al., 1999). The association oxidized ~25 mM acetate in 30 days, producing up to 20 mM sulfide. However, neither the pH nor salt ranges were reported for the acetate-oxidizing association. Meanwhile, the question of the salt limit for the low-energy-yielding anaerobic acetate oxidation with sulfate as final electron acceptor is fundamental for understanding thermodynamic limits of microbial life. Growth at high salt demands high extra energy input for the synthesis of osmolytes and, therefore, low-energy-generating metabolism is compromised under these conditions (Oren, 2011). So far, the salt limit for direct oxidation of acetate by halophilic SRB is 2 M NaCl, which is not surprising taking into account the very low energy yield of the conversion: CH₃COO⁻ + SO₄²⁻ → HS⁻ + 2 HCO₃⁻ [ΔG"=−56 kJ (pH 7; Schink & Stams, 2006); ΔG"=−47 kJ (pH 10; Oren, 2011)].

The results described in this paper present, to the best of our knowledge, the first proof of anaerobic acetate oxidation with sulfate as electron acceptor by nanophosphates syntrophic associations at much higher salt concentrations than believed previously. The acetate-oxidizing association consisted of a member of a novel branch within the class Clostridia and its extremely nanophosphates lithoautotrophic SRB partner from the genus Desulfonatronospira, which, working together, oxidized acetate in nearly saturated soda brines.

**METHODS**

**Samples.** Anaerobic sediment cores (5–15 cm depth) were obtained from the following soda lakes of the Kulunda Steppe (south-eastern Siberia, Altai, Russia): (i) the moderately saline soda lake Cock Soda Lake (salinity 70 g L⁻¹, pH 10.1, carbonate alkalinity 0.7 M), and (ii) the hypersaline soda lakes Tanatar-5 (salinity 170 g L⁻¹, pH 9.9, carbonate alkalinity 1.9 M) and Bitter-1 (salinity 400 g L⁻¹, pH 10.65, carbonate alkalinity 4.4 M) in July 2010, and Bitter-1 in July 2011 (salinity 400 g L⁻¹, pH 10.1, carbonate alkalinity 4.0 M).

**Enrichment and cultivation conditions.** Anaerobic acetate-dependent sulfate-reducing cultures were enriched from soda lake sediments at 30 °C in a mineral medium based on sodium carbonate/bicarbonate buffer with stable pH 10 containing in total 0.6–3 M Na⁺, 0.1–0.3 M NaCl and 1 g KH₂PO₄ L⁻¹. After sterilization, the medium was supplemented with 4 mM NH₄Cl, 1 mM MgSO₄, 20 mg yeast extract L⁻¹ and 1 ml L⁻¹ of each solution of acidic trace metals and vitamins (Pfenning & Lippert, 1966), and 1 ml basic filter-sterilized Se/W solution (Plugg, 2005). Acetate and sulfate at 20 mM were used as electron donor and electron acceptor, respectively; 1 mM HS⁻ was added as a reductant. The SRB partners were sub-cultured from the acetate-utilizing association in a medium specific for Desulfitonatronospira, containing 3 M total Na⁺ with formate and sulfite (Sorokin et al., 2008). Routine cultivation was performed in 18 ml Hungate tubes with 10 ml medium made anoxic by several cycles of flushing with argon and evacuation. Growth was monitored by sulfide production and measurements of OD₆₀₀. When the sulfide concentration in the enrichments exceeded 5 mM, the cultures were transferred into new medium at 1:100 dilution. After two or three successful transfers, the enrichments were serially diluted up to 10⁻¹⁰. Growth on solid medium was not observed. One of the probable reasons was a problem with clarity and solidification of agar at high sodium carbonate concentrations.

The pH dependence was examined at 2 M Na⁺ using the following filter-sterilized buffers: for pH 7–8, 0.1 M HEPES and NaCl; for pH 8.5–11, a mixture of sodium bicarbonate/sodium carbonate. Final pH values were taken to indicate a suitable range for growth, because of the pH shift at pH extremes. To study the influence of salt concentration on growth, mineral sodium carbonate bases at pH 10 containing 0.6 and 4.0 M total Na⁺ were mixed in different proportions.

**Analyses.** Sulfide was precipitated in 10 % (w/v) zinc acetate and analysed by the methylene blue method after separation from the supernatant as ZnS (Trüper & Schlegel, 1964). The amount of cell protein was measured by the Lowry method (Lowry et al., 1951) after removal of interfering FeS from the cell pellet by a double wash with 1 M NaCl, pH 4. Acetate in the supernatant was analysed by GC after removal of sulfide and acidification to pH 5 (Chromotrec-Crystall 5000.2; column Sovpol-5, 1 m, 180 °C; detector PID, 30 °C). Phase-contrast photomicrographs were obtained with an Axioplan Imaging 2 microscope (Zeiss).

**Genetic and phylogenetic analysis.** DNA was extracted from the cells using the UltraClean Microbial DNA Isolation kit (Mobio Laboratories) following the manufacturer’s instructions. The nearly complete 16S RNA gene was obtained from finally diluted associations by standard molecular cloning procedures using general bacterial primers 11f/1492r (Lane, 1991). The PCR products were purified using the Wizard SV-gel and PCR Clean-Up System (Promega). The purified fragments were ligated into plasmids using the pGEM-T Easy Vector System (Promega) and the plasmids were then electroporated into the competent cells of Escherichia coli strain DH10B. DNA from positive clones (n=25) was extracted with Wizard MiniPreps (Promega). Community analysis of syntrophic associations was performed by using 16S-rRNA-gene-based and dsrB-based denaturing gradient gel electrophoresis (DGGE) according to Schäfer & Muyzer (2001). For the 16S rRNA analysis, the primer pair was bacterial 341F+GC clamp/907R and the gel gradient was from 20 to 70 %. For the dsrB analysis, the primer pair was DSR2606F+GC clamp/DSR4r (Geets et al., 2006; Wagner et al., 1998) with the gel gradient from 30 to 65 %.

For the phylogenetic analysis of the 16S rRNA gene, the obtained sequences were first compared to all sequences stored in GenBank using the BLAST algorithm and were consequently aligned using CLUSTALW. The phylogenetic trees were reconstructed using TREECON and the neighbour-joining algorithm. Phylogeny of the dsrB fragments was reconstructed using ARB software. The sequences were aligned using CodonCode Aligner. Sequences were aligned with complete-length sequences of closest relatives from the order Desulfovibrionales and the family Desulfo bacteraceae obtained from the updated dsrAB database (Loy et al., 2009) using the ARB fast-
RESULTS AND DISCUSSION

Enrichment and isolation of syntrophic acetate-oxidizing sulfidogenic associations from soda lake sediments

In contrast to our previous negative short-term experiments (Sorokin et al., 2010a), prolonged incubations of sediment slurries amended with acetate and sulfate showed sulfide formation after 3–6 months in samples taken in 2010 at 0.6–2.0 M total Na\(^+\), pH 10. After several subcultures, a maximum of 10 mM sulfide was produced in 3 months. In serial dilutions, cultures with reduced morphological diversity were obtained from three lakes. The final diluted culture at the lowest salinity was dominated by two clearly different phenotypes (Fig. S1a, available in the online Supplementary Material), whilst the other two cultures only included short rods (Fig. S1b, c). In 2011, the experiment with the most saline soda lake, Bitter-1 (sample 3KL-011), was repeated to determine the salt limit of the process by slurry incubations at 2–4 M Na\(^+\), pH 10. Sulfide formation was observed at sodium carbonate concentrations up to 3 M total Na\(^+\) after 6 months of incubation, with the purified culture dominated by short rods (Fig. S1d). None of the acetate-oxidizing cultures obtained contained endospore-forming clostridia observed previously in the low-salt alkaliphilic acetate-oxidizing association (Zhilina et al., 2005a, b). These results demonstrated the extraordinary possibility of a very slow, but reproducible microbial sulfate-dependent oxidation of acetate in soda lake sediments at moderate to extremely high salt concentrations.

Growth dynamics and influence of sodium on acetate-dependent sulfidogenesis in cultures from the hypersaline soda lake Bitter-1

In maximally purified culture from 3KL-011 grown at 2 M total Na\(^+\), pH 10, full oxidation of 18 mM acetate was observed in 3 months (Fig. 1a). The oxidation was accompanied by biomass growth and formation of 16 mM sulfide, which corresponded well to the eight-electron stoichiometry 1:1 of the donor (acetate) and acceptor (sulfide) assuming that part of the acetate was assimilated. The experimental maximum specific growth yield was 1.2 mg cell protein (mmol acetate)\(^{-1}\). Cultures from both 2010 and 2011 were obligately alkaliphilic, growing within a range from pH 8.7 to 10.5 with an optimum around pH 10 (Fig. 1b). Both cultures grew in sodium carbonate brines containing at least 1 M total Na\(^+\) with an optimum at 1.5–2 M. There was a slight difference between the upper salt limit, which was higher for the culture from 2011 which grew in nearly saturated sodium carbonate brines containing 3.5 M Na\(^+\) (Fig. 1c). However, full oxidation of 18 mM acetate under such extreme conditions was at least three times slower than under the optimal salt concentration.

Analysis of the composition of acetate-oxidizing syntrophic cultures

DGGE analysis of the 16S rRNA genes of three acetate-oxidizing cultures from the 2010 samples showed a domination of two closely related clostridial phylotypes (Fig. 2a); one presented at a low-to-moderate salinity (3KL-010 and 7KL-010) and another presented at high salinity (5KL-010). Together with the two clones detected directly in anaerobic sediments of one of the Bitter lake systems (T. P. Tourova, unpublished), they formed a novel lineage within the family Syntrophomonadaceae (class Clostridia) distantly related to ‘Ca. Contubernalis alcalaceticum’ (Fig. 3). The SRB partner at the lowest salinity was identified as a member of the genus Desulfonatronum and the SRB partner at the highest salinity was identified as a novel representative of the extremely natronophilic genus Desulfanatronospora (Sorokin et al., 2008). A more specific analysis of the functional gene dsrB showed the presence of a novel Desulfanatronospora in moderate- and high-salt cultures, and confirmed the presence of Desulfanatronum in the low-salt culture (Fig. 2b, Table 1). Several Bacteroidetes phylotypes present in cultures
at low dilutions (Fig. 2a) disappeared at high dilutions, indicating their irrelevance for the main process.

The comparison of the enrichment culture from Bitter-1 lake (2011) at 3 M Na\(^+\) with the ‘AAS1’ culture from the same lake (2010) showed the presence of the same key players, i.e. a novel member of the class *Clostridia* and a novel *Desulfonatronospira* (Fig. 4). Subculturing on a medium specific for *Desulfonatronospira* with formate and sulfite resulted in the domination of a rod-shaped organism and, in addition, a spiral-shaped *Desulfonatronospira proliferated in the offshoots of the 3KL-011 culture. Both types were highly enriched and identified by cloning. The rod-shaped phylotype was distant from the described *Desulfonatronospira* by its full-length 16S rRNA gene and probably represents a novel species within this genus, whilst the spiral morphotype belonged to the type species *Desulfonatronospira thiodismutans* (Fig. 5a). It seems likely that the novel rod-shaped *Desulfonatronospira* may have been selected in the high-salt acetate-oxidizing syntrophic cultures on the basis of its better adaptation to grow with sulfate as electron acceptor than the species described previously (Sorokin et al., 2008).

Interestingly, in the 3KL-011 culture, another SRB was detected that belonged to the order *Desulfobacterales* with *Desulfonatronobacter acidivorans* as the closest relative (Figs 4 and 5b, Table 1). The latter is the only known ‘complete oxidizing’ natronophilic SRB found so far in soda lakes, but it cannot utilize external acetate and cannot grow lithotrophically with formate. As this organism was absent in the 2010 culture, we can only conclude that it was not present in the culture at the time of sampling.

![Figure 2](image-url)
essential for the syntrophic acetate oxidation. The same applies to the presence of ‘Halanaerobium hydrogenoforsans’ in the formate + sulfite offshoots from the 3KL-011 culture. This haloalkaliphilic clostridium is a fermentative saccharolytic (Brown et al., 2011). However, it must be pointed out that some members of the order Halanaeribiales have the potential to use sulfur-dependent respiration as an additional metabolism (Sorokin et al., 2011a).

Attempts to find other electron donors for the highly purified syntrophic cultures from Bitter-1 lake were complicated by the fact that Desulfonatronospira is more diverse physiologically than the SRB partner (genus Desulfonatronum) in the low-salt association described previously. The latter was reported to be able to oxidize 1- and 2-propanol, iso-butyrate, serine and fructose, apart from acetate (Zhilina et al., 2005a). The high salt cultures actively grew with ethanol, propanol and butanol, the substrates also utilized by Desulfonatronospira alone. Other donors, positive for the ‘Ca. Contubernalis’/Desulfonatronum association, did not support growth of the high-salt cultures, as well as the range of substrates tested (negative for Desulfonatronospira alone), including propionate, malate, fumarate, succinate, valerate, caproate and peptone. Also, the acetate-oxidizing clostridium alone did not grow on substrates employed commonly for the cultivation of syntrophs (McInerney et al., 2008; Stams & Plugge, 2009), such as fumarate, pyruvate, crotonate and ethylene glycol.

In summary, the results presented here demonstrate anaerobic acetate oxidation with sulfate as electron acceptor at extremely natron-alkaline conditions of hypersaline soda lakes. This process is driven by syntrophic interaction between a novel and apparently obligately syntrophic clostridial lineage in the family Syntrophomonadaceae and a novel member of the extremely natronophilic lithotrophic SRB of the genus Desulfonatronospira. The clostridial member is suggested to be accommodated into a novel candidate taxon ‘Candidatus Syntrophonatronum acetioxidans’. Despite a very slow growth, the mere fact that such a low-energy-yielding catabolic reaction can support growth at extreme salt and pH is an important finding for understanding the limits of microbial life and its biogeochemical role. That such a process has been detected so far only in hypersaline soda lakes, and not in hypersaline habitats with neutral pH (Oren, 2011), indicates that sodium carbonates may create an environment fundamentally different from the conditions present in a high-NaCl world.

Fig. 3. Phylogenetic position based on 16S rRNA gene sequence analysis of the acetate-oxidizing clostridial members in syntrophic associations from soda lakes. The clones directly detected in lake Bitter-3 are in bold type. Bar, 5 nt changes per 100 nt. The percentage of bootstraps was derived from 1000 resampling iterations using the neighbour-joining algorithm; only values >70% are indicated. GenBank accession numbers are given in parentheses.
Table 1. Dsr(AB) sequences identified in syntrophic acetate-oxidizing cultures from hypersaline soda lakes (DGGE band sequences)

The reference dsrAB gene sequences of Desulfonatronobacter acidivorans APT2ᵀ have been deposited in GenBank under accession numbers KF835252 and KF835254.

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<th>Salinity (M Na⁺)</th>
<th>Dilution</th>
<th>Substrate</th>
<th>Sequence source*</th>
<th>Closest culturable relative in GenBank</th>
<th>Domination</th>
<th>Similarity of translated amino acids (%)</th>
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*DGGE1 corresponds to Fig. 2(b); DGGE2 corresponds to Fig. 4(b).
Description of ‘Candidatus Syntrophonatronum’

‘Candidatus Syntrophonatronum’ (Syn. tro. pho. natro’ n. um. Gr. prep. syn in company with, together with; Gr. n. trophos one who feeds; L. n. natron soda; M.L. neutr. n. Syntrophonatronum syntrophic natronophile).


Description of ‘Candidatus Syntrophonatronum acetoxidans’

‘Candidatus Syntrophonatronum acetoxidans’ [acet.i. oxi.dan.s. L. n. acetum vinegar; N.L. n. acidum acetici acetic acid; N.L. v. oxido (from Gr. adj. oxus acid or sour and in combined words indicating oxygen) to make acid, oxidize; N.L. part. adj. acetioxidans oxidizing acetate].

Cells are non-spore-forming and non-motile Gram-positive rods, 0.5–2–3 μm. Obligately anaerobic, oxidizing acetate in the presence of a lithoautotrophic sulfate-reducing partner. Other possible substrates are not known. Does not grow alone on pyruvate, crotonate, fumarate or ethylene glycol. Obligately natronophilic with the ability to grow in syntropic culture within the range pH 8.5–10.5 (optimum at pH 10) and sodium carbonate concentrations from 1.0 to 3.5 M total Na⁺ (optimum 1.5–2.0 M). Growth is optimal at 35 °C and possible up to 42 °C.

The type strain ASS1T exists in a syntrophic co-culture with the extremely natronophilic SRB partner from the genus Desulfonatronospira (the GenBank accession number of the 16S rRNA gene sequence is KF588524). It is deposited in the UNIQEM culture collection (Institute of Microbiology, Russian Academy of Sciences) under the

http://mic.sgmjournals.org

Fig. 4. Comparative (a) 16S RNA gene and (b) dsrB DGGE analysis of two extremely natronophilic anaerobic syntrophic cultures oxidizing acetate with sulfate as electron acceptor enriched from hypersaline soda lake Bitter-1. AAS1, final dilution culture from 2010 sample; 3KL-011, final dilution culture from 2011 sample. Left part of the gels shows cultures grown with acetate+sulfate; right part shows the transfer to formate+sulfite. Band identification in (a): 0, Bacteroidetes (87 %); 1, Clostridiales, novel branch; 3, 5, Halanaerobium hydrogenoformans (99 %); 2, 4, Desulfonatronospira spp. (98 %); 6, Desulfonatronospira thiodismutans (99 %); 7, Desulfonatronobacter acidivorans (95 %). Band identification in (b): 3–6, 8, 11, 14, 15, 17–19, 22, 23, 32, Desulfonatronospira thiodismutans (89–92 %); 26, 27, Desulfonatronospira thiodismutans (100 %); 20, 21, 24, 25, 31, Desulfonatronobacter acidivorans (94 %). Band 9 gave a bad quality sequence and is not included.
number U977. The culture was obtained from anaerobic sediments of the hypersaline soda lake Bitter-1 in Kulunda Steppe (Altai, Russia). The GenBank accession number of the 16S rRNA gene sequence of the type strain AAS1T is KF588524.

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