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‘*Candidatus Desulfonatronobulbus propionicus*’: a first haloalkaliphilic member of the order *Syntrophobacterales* from soda lakes

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Abstract Propionate can be directly oxidized anaerobically with sulfate as *e*-acceptor at haloalkaline conditions either incompletely to acetate (an example is *Desulfobulbus alkaliphilus*), or completely (for example by the members of genus *Desulfonatronobacter*). An enrichment with propionate at methanogenic conditions (without sulfate) inoculated with mixed sediments from hypersaline soda lakes in Kulunda Steppe (Altai, Russia) resulted in a domination of a new member of *Syntrophobacteraceae* (*Deltaproteobacteria*) in a consortium with the haloalkaliphilic lithotrophic methanogen *Methanocalculus alkaliphilus*. Transfer of this culture to a medium containing propionate as *e*-donor and sulfate as *e*-acceptor resulted in a disappearance of the methanogen and sulfide formation by the bacterial component, finally isolated into a pure culture at these conditions. Strain APr1 formed a distinct phylogenetic lineage within the family *Syntrophobacteraceae*, being equally distant

from its members at the genus level. Phenotypically, strain APr1 resembled the species of the genus *Syntrophobacter* with substrate spectrum restricted to propionate and propanol utilized with sulfate, sulfite and thiosulfate as the *e*-acceptors. Propionate is oxidized incompletely to acetate. It is a moderately salt-tolerant (max. 1.2 M Na⁺) obligate alkaliphile (pH opt. 10). The isolate is proposed to be classified as a new candidate genus and species ‘*Candidatus Desulfonatronobulbus propionicus*’.

Keywords Syntrophic · Propionate · Soda lakes · Haloalkaliphilic · *Syntrophobacterales* · Sulfate-reducing

Introduction

Intensive microbiological and molecular ecological investigation of microbial sulfur cycle in soda lakes during last decades allowed to obtain comprehensive information on functional-structural composition of the microbial players in its oxidative and reductive branches (Sorokin et al. 2011, 2013, 2014a, 2015a). In the reductive cycle, the microbiological and molecular ecology studies identified 3 groups of lithotrophic alkaliphilic SRB from the order *Desulfovibrionales* dominating in soda lakes, including moderately salt-tolerant genera *Desulfonatronum* and *Desulfonatronovibrio* and an extremely salt-tolerant genus *Desulfonatronospira* (last reviewed in Sorokin et al. 2015a). However, the *dsrB* clone libraries also indicated a presence in soda lakes of heterotrophic SRB belonging to the order *Desulfobacterales* (Foti et al. 2007), which, later on, has been confirmed by isolation of the VFA-oxidizing haloalkaliphilic SRB belonging to the genera *Desulfonatronobacter* (unique for soda lakes), *Desulfobulbus* and *Desulfobotulus* (Sorokin et al. 2010, 2012, 2014b, 2015b).

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Nucleotide sequence accession number GenBank/EMBL accession numbers of the 16S rRNA and *dsrA* gene sequences determined in this study are KU681311 and KX756667, respectively.

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Furthermore, oxidation of acetate at soda lake conditions has been shown for two syntrophic associations, whereby their hydrogenotrophic sulfate-reducing partners were either *Desulfonatronum* (Zhilina et al. 2005) at low salinity or *Desulfonatronospira* at moderate to extremely high salinity (Sorokin et al. 2014b). Our recent investigation of the VFA oxidation in soda lakes at methanogenic conditions produced a binary culture consisting of a propionate-oxidizing bacterium and its lithotrophic methanogenic partner *Methanocalculus alkaliphilus*, which converted propionate to acetate and methane at moderate salinity and pH 9–10 (Sorokin et al. 2015c, d, 2016). Eventually, it was possible to cultivate the propionate oxidizer separately using sulfate as *e*-acceptor. The obtained pure culture, strain APr1, is a member of the family *Syntrophobacteraceae*, where, so far, no haloalkaliphilic representatives were found. In this paper the properties of this organism are described.

Methods

Isolation source

The source for the isolation was a syntrophic enrichment culture oxidizing propionate to acetate at methanogenic conditions at pH 9.5 and 0.6 M Na⁺ and inoculated with mixed anaerobic sediment sample from hypersaline soda lakes in Kulunda Steppe, Altai, Russia (Sorokin et al. 2016). The chemical parameters of the lake brines are described previously (Sorokin et al. 2015d).

Enrichment, isolation and cultivation conditions

Anaerobic enrichment with propionate as *e*-donor (10 mM) producing methane was obtained from anaerobic sediments taken from 5 hypersaline soda lakes in Kulunda Steppe in July 2013. The enrichment contained 5 cm³ sediments in 80 ml medium at pH 9.5 and salinity 0.6 M total Na⁺ and was incubated at 30 °C. The basal sodium carbonate-based mineral media containing from 0.3 to 2 M total Na⁺ and buffered at pH 9.5–10 were prepared as described previously (Sorokin et al. 2015c, d). Routine cultivation was performed in 15 ml Hungate tubes with 10 ml medium. For the large scale cultivation, 100–500 ml serum bottles capped with butyl rubber stoppers and filled to 75 % volume were employed. The electron donors were (with sulfate as acceptor) used at concentrations from 10 to 50 mM and the electron acceptors (with propionate as *e*-donor) at concentrations from 5 to 20 mM. The pH dependence of growth and activity of washed cells was examined at 0.6 M total Na⁺ using 0.1 M HEPES/NaCl/NaHCO₃ for the pH 6–8 and a mixture of sodium bicarbonate/sodium carbonate containing 0.1 M NaCl for the pH 8.5–11. The final pH

values were taken to indicate a suitable range for growth and activity. To study the influence of Na⁺ concentration on growth and activity, sodium carbonate-based buffers with pH 9.5 containing 0.3–2.0 M of total Na⁺ were mixed in different proportions.

Analyses

Sulfide was precipitated in 10 % (w/v) Zn acetate and analyzed by the methylene blue method after separation from the supernatant (Trüper and Schlegel 1964). Acetate was detected by HPLC anionic chromatography, as described previously (Sorokin et al. 2012). The cell growth was monitored by measuring OD₆₀₀. Membrane polar lipids for the PLFA analysis were extracted from freeze-dried biomass by acidic methanol and their fatty acid composition examined with GC–MS according to Zhilina et al. (1997). Phase contrast photomicrographs were obtained with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). For the whole cell electron microscopy, the cells were separated from sodium carbonates by centrifugation and resuspended in 0.3 M NaCl. The cell suspension was applied onto the copper electron microscopy grid coated with formvar film, stained in 2 % (w/v) uranyl acetate for 1 min and washed briefly in 0.1 M NaCl before drying. The cells were inspected under the JEOL-100 transmitting electron microscope (Japan).

Genetic and phylogenetic analysis

Isolation of genomic DNA and determination of the G + C content of the DNA from pure cultures was performed according to Marmur (1961) and Marmur and Doty (1962). For molecular analysis, the DNA was extracted from the cells using the UltraClean Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. The nearly complete 16S rRNA gene was amplified and sequenced with general bacterial primers 11f-1492r (Lane 1991). The *dsrAB* genes were amplified and sequenced with the primer pair DSR1F/DSR4R [AC(GC)CACTGG AAGCACG/GTGTAGCAGTTACCGCA] according to Wagner et al. (1998). The PCR mixture was incubated for 5 min at 94 °C, followed by 34 cycles of 20 s at 93 °C, 45 s 55 °C, and 190 s at 72 °C, with the final extension at 72 °C for 10 min. The PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen, the Netherlands). The sequences were first compared to all sequences stored in GenBank using the BLAST algorithm and were consequently aligned using CLUSTAL W. The evolutionary history was inferred using the ML method and the trees were constructed in the MEGA-6 package (Tamura et al. 2013).

Results and discussion

Isolation of strain APr1

A sub-enrichment with 10 mM propionate and 20 mM sulfate from the methanogenic syntrophic culture was first done in 1:10 dilution which resulted in the formation of 9 mM sulfide in 2 months period. After 3 consecutive 1:10 transfers, the enrichment was serially diluted up to 10^{-10} using propionate as *e*-donor and sulfate as *e*-acceptor. The growth was observed up to 10^{-8} , however, the isolation of the dominant organism into pure culture proved to be impossible by direct transfers and the failure to obtain growth in solid medium. The culture was contaminated with a bacterium with cell size at least 5 times smaller than the target organism, which made the biomass domination of the latter insufficient for the direct dilution to extinction approach. On the other hand, the large cells of the target were much heavier than of the small cells of contaminant, allowing to use several rounds of a low speed centrifugation (in sterile 2 ml Eppendorf tubes at a minimal speed of 2000 rpm for 1–3 min) cell sorting to enrich the bigger cells and, finally, to purify the propionate-oxidizing strain APr1. The purity of the isolate was checked by microscopy, by the absence of growth on rich media (1 g/l of yeast extract) without electron acceptors and by the 16S-rRNA gene sequencing.

Cell morphology of strain APT3

The cells of strain APr1 were coccoid rods, $1.2\text{--}1.4 \times 1.5\text{--}3\text{ }\mu\text{m}$, motile (while actively growing) with a single polar flagellum and, occasionally, with gas vacuoles evident mostly in the stationary growth phase (Fig. 1).

Phylogenetic position and chemotaxonomy of strain APr1

According to the 16S rRNA gene analysis, strain APr1 is a member of the family *Syntrophobacteraceae*, order *Syntrophobacterales* (*Deltaproteobacteria*). The type genus (*Syntrophobacter*) of the family currently includes four validly described species, all of which are dedicated propionate-oxidizing SRB. However, it has been suggested recently that the genus is polyphyletic, with the type species *Syntrophobacter wolinii* positioned outside of the main group containing the other 3 species (Kuever 2014). Phylogenetic analysis based on the 16S rRNA gene showed that APr1 is equally distant from the two clades of *Syntrophobacter* and the other genera currently included in *Syntrophobacteraceae*

Fig. 1 Cell morphology of strains APr1 grown at 0.6 M Na^+ , pH 10 with propionate + sulfate. **a, b** Phase contrast microphotographs; **b** formation of gas vacuoles; **c** electron microphotograph showing flagellation

Fig. 2 Phylogenetic position of strain APr1. **a** Based on 16S rRNA gene sequence analysis within the order *Syntrophobacterales*. **b** Based on DsrA gene sequence analysis. The bootstrap values above 50 % from 500 replicates are shown next to the branches. The evolutionary distances were computed using the neighbor joining and maximum likelihood methods and are in the units of the number of base substitutions per site

(94–95 % sequence similarity) (Fig. 2a). Apparently, this organism has already been previously enriched from the Kulunda Steppe soda lakes but lost during purification process (clone ASP1, Sorokin et al. 2010). Phylogenetic analysis of the functional molecular marker *dsrA* confirmed the association of the novel isolate with the members of *Syntrophobacteraceae* and was also consistent with the 16S rRNA gene phylogeny, indicating that APr1 is more close to *Desulforhabdus* than to the members of *Syntrophobacter* (less than 80 % aa to the closest relative) (Fig. 2b). The phylogenetic divergence was higher for the DsrA than in DsrB (90 % and lower). Such level is, on average, is common for separate genera within *Syntrophobacteraceae*.

The PLFA profile of strain APr1 was dominated by a single unsaturated species 17:1 ω 6c, which can be considered as a specific marker for this organism, and 3 saturated subdominants (15:0, 16:0 and 18:0). The profile was significantly different from the only other species of

Syntrophobacteraceae (*Desulfosoma profundum*, Gregoire et al. 2012) for which the data are available (Supplementary Table 1).

Physiological characteristics of strain APr1

In its catabolic spectrum, APr1 was limited to the oxidation of two C_3 compounds—propionate and 1-PrOH with sulfate, sulfite (up to 10 mM) and thiosulfate as the *e*-acceptor. Propionate was incompletely oxidized to acetate (Fig. 3 a) similar to the members of the genus *Syntrophobacter*. The tested (but not utilized) electron donors with sulfate as acceptor included: H_2 and formate ($\pm$ yeast extract), acetate, EtOH, pyruvate, lactate, butyrate, isobutyrate, 1-butanol, 2-butanol, malate, succinate, fumarate, valerate. The tested electron acceptors (with propionate as donor) included: sulfur, nitrate, nitrite, selenate, arsenate, fumarate, crotonate, ferrihydrite.

Fig. 3 Growth characteristics of strain APr1 with propionate + sulfate. **a** growth dynamics at pH 10 and 0.6 M total Na⁺; **b** influence of pH at 0.6 M total Na⁺ on growth (closed circles) and activity of washed cells (open circles); **c** influence of salt at pH 10 on growth (closed circles) and activity of washed cells (open circles). The data represent mean values from duplicate experiments. OD₆₀₀ optical density at 600 nm, VHS⁻ rate of sulfidogenesis, d days

With propionate + sulfate, strain APr1 was able to grow within the pH range from 8.5 to 10.3 (optimum at pH 10), while sulfidogenic activity of washed cells had a broader pH profile but with similar pH optimum (Fig. 3b). Salt profiling at pH 10 showed that APr1 represents a low

salt-tolerant organism with an optimum at 0.2–0.3 M total Na⁺ and maximum tolerance below 1.5 M (Fig. 3c).

In conclusion, a first salt-tolerant and alkaliphilic member of the family *Syntrophobacteraceae* has been isolated from soda lakes. Despite an obvious physiological similarity to the species of the genus *Syntrophobacter*, the soda lake isolate represents a new genus-level genetic lineage which, together with its unique pH-salt adaptation, separates it from the other genera within the family. However, being primarily associated with hydrogenotrophic partners, such as the haloalkaliphilic methanogens of the genus *Methanocalculus* (Sorokin et al. 2016), in pure culture APr1 grew irregularly and sometimes failed to grow at all even after medium salt-pH optimization. That, so far, prevented it from sustainable accessioning in two culture collection and, therefore, it is now proposed to form a new candidatus genus and species ‘*Candidatus Desulfonatronobulbus propionicus*’. The comparative properties of strain APr1 with selected relatives from the family *Syntrophobacteraceae* are summarized in Table 1.

Description of ‘*Candidatus Desulfonatronobulbus*’

[De.sul.fo.na.tro.no.’bul.bus] L. prep. *de*, from; N. L. pref. *sulfo-*, prefix used for N. L. masc. n. *sulfas*—*atis* sulfate; N. Gr. n. *natron*, arbitrarily derived from the Arabic n. *natrun* or *natron*, soda; L. masc. n. *bulbus*, a bulb, an onion; N.L. masc. n. *Desulfonatronobulbus*, onion-shaped sulfate-reducing bacterium from soda lake.

Obligately anaerobic heterotrophic sulfate-reducing bacteria. Oxidize C₃ fatty acids and alcohols incompletely to acetate with sulfate as terminal electron acceptor and in syntrophy with hydrogenotrophic methanogens. Moderately salt-tolerant and alkaliphilic. Habitat—soda lakes. Member of the family *Syntrophobacteraceae* (*Deltaproteobacteria*).

Description of ‘*Candidatus Desulfonatronobulbus propionicus*’

[pro.pi.o’ni.cus] N.L. n. *acidum propionicum*, propionic acid; N.L. masc. adj. *propionicus*, pertaining to propionic acid.

Cells are Gram-negative coccoid rods, 1.2–1.4 × 1.5–3 μm, motile with a single polar flagellum forming gas vacuoles in the stationary growth phase. The dominant PLFA include 18:1ω7, 16:0 and i14:0. Obligately anaerobic, utilizing propionate and 1-propanol as carbon and energy source with sulfate, sulfite and thiosulfate as electron acceptor in pure culture or in syntrophy with haloalkaliphilic hydrogenotrophic methanogens. The utilized *e*-donors are incompletely oxidized to acetate.

Table 1 Comparative properties of strain APr1 and its closest relatives within *Syntrophobacteraceae* (Kuever 2014)

Property	APr1	<i>Syntrophobacter</i> spp. (4 species)	<i>Desulforhabdus amnigena</i>	<i>Desulfovirga adipica</i>	<i>Desulfosoma profundum</i>
Cell morphology	Rods	Short rods	Rods	Rods	
Motility	–	+	+	+	–
Gas vacuols	+	1 Species	–	–	–
Dominant PLFA	18:1ω7 , 16:0 i14:0	Nd	Nd	Nd	i15:0 , 15:0 16:0
Oxidized substrates	Propionate, PrOH	Propionate, PrOH (1 species), pyruvate (2 species) lactate (1 species)	H ₂ , formate, acetate, propionate, butyrate, isobutyrate, lactate, pyruvate, ethanol, propanol, butanol	C1–C12 VFA and alcohols, adipate, pyruvate, lactate	H ₂ , formate, propionate, butyrate, isobutyrate, valerate, isovalerate, pyruvate, hexadecanoate
Completeness of electron donor oxidation	Incomplete	Incomplete	Complete	Complete	Complete
Electron acceptors	Sulfate, sulfite, thiosulfate	Sulfate, sulfite thiosulfate, fumarate (2 species)	Sulfate, sulfite, thiosulfate	Sulfate, sulfite, thiosulfate, sulfur	Sulfate, sulfite, thiosulfate,
Salt range (opt), M Na ⁺	0.3–4.0 (1.0)	Freshwater	6.0–8.3 (7.0)	6.2–8.1 (6.2–7.4)	57.6
pH range (optimum)	8.5–10.3 (10)	6–8.8 (7.0–7.6)	52.5	60	Hot spring
G + C, mol %	53.5	56.7–60.6			
Habitat	Soda lakes	Methanogenic reactors			

Bold indicates the dominant PLFA

Moderately salt-tolerant with a salinity range for growth from 0.2 to 1.25 M total Na⁺ (optimum at 0.3–0.4 M) and obligately alkaliphilic with a pH range for growth between 8.5 and 10.3 (optimum at pH 10). Maximum growth temperature is 42 °C (optimum 33–35 °C). The G + C content of the DNA is 53.5 mol % (*T_m*). Isolated from anaerobic sediments of soda lakes in south-western Siberia (Altai, Russia). The type strain is AP1^T deposited in the culture collection of Unique Extremophilic Microorganisms (Russian Academy of Sciences, Moscow) under the number UNIQEM U995. The 16S-rRNA gene sequence accession number of AP1^T is KU681311.

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