Desulfonatronum cooperativum sp. nov., a novel hydrogenotrophic, alkaliphilic, sulfate-reducing bacterium, from a syntrophic culture growing on acetate

Tatjana N. Zhilina,¹ Daria G. Zavarzina,¹ Jan Kuever,² Anatoly M. Lysenko¹ and Georgy A. Zavarzin¹

Correspondence
Tatjana N. Zhilina
zhilinat@mail.ru

¹S. N. Winogradsky-Institute of Microbiology, Russian Academy of Sciences, Prospect 60-letiya Oktyabrya 7/2, 117312, Moscow, Russia

²Bremen Institute for Materials Testing, Department of Microbiology, Paul-Feller Str. 1, Bremen, D-28199, Germany

A novel alkaliphilic, sulfate-reducing bacterium was isolated from a syntrophic acetate-decomposing community enriched from samples of the soda lake Khadin, Tuva, Russia; the isolate was designated strain Z-7999^T. Cells of strain Z-7999^T were vibrioid, Gram-negative, 0.4–0.5 × 1.0–2.5 µm and motile by means of a polar flagellum. The temperature range for growth was 15–40 °C, with an optimum of 35–38 °C. The pH range for growth was 6.7–10.3, with an optimum of pH 8.0–9.0. The NaCl concentration range for growth was 1–80 g l⁻¹. The novel isolate was obligately anaerobic, was alkaliphilic with a broad pH range and had an obligate requirement for carbonate ions in the growth medium. In the presence of sulfate as electron acceptor, it grew with hydrogen, formate and lactate. It was not able to ferment sugars, organic acids, amino acids or peptides. During growth on formate, strain Z-7999^T reduced sulfite and thiosulfate to sulfide. It was able to grow lithoheterotrophically with sulfate and formate when acetate was added as a carbon source for biosynthesis of biomass. The G + C content of the genomic DNA of strain Z-7999^T was 56.5 mol%. Results of comparative 16S rRNA gene sequence analyses revealed that strain Z-7999^T was part of the *δ-Proteobacteria* and clustered with other members of the genus *Desulfonatronum* (similarity values of 95.2 and 95.3 % to *Desulfonatronum lacustre* and *Desulfonatronum thiodismutans*, respectively). DNA–DNA hybridization with *D. lacustre* was 37 %. On the basis of physiological and phylogenetic data, it is proposed that strain Z-7999^T (= DSM 16749^T = VKM B-2329^T) should be placed in the genus *Desulfonatronum* as a representative of a novel species, *Desulfonatronum cooperativum* sp. nov.

The anaerobic decomposition of organic matter is performed by fermenting bacteria and typically leads to acetate and H₂ as major products. In alkaliphilic microbial communities, several micro-organisms that utilize molecular hydrogen are described, including sulfate-reducing bacteria, which often dominate in alkaliphilic environments where the sulfur cycle is well developed (Zavarzin *et al.*, 1996; Gorlenko *et al.*, 1999). In contrast to hydrogen-utilizing micro-organisms, very little is known about micro-organisms that consume acetate in soda lakes or other alkaline habitats (Zavarzin & Zhilina, 2000). The first evidence for acetate utilization coupled to sulfate reduction

was observed in cellulose-decomposing enrichment cultures obtained from such habitats (Kevbrin *et al.*, 1999). Sulfidogenesis on acetate occurred rapidly, although no specialized acetate-consuming, alkaliphilic sulfate reducers have been isolated so far. Members of the genus *Desulfobacter*, which show a rapid turnover of acetate and are found in brackish, marine and hypersaline habitats, seem to be absent from these habitats.

An acetate-utilizing, sulfate-reducing enrichment culture obtained from deposits of the local soda lake Khadin, located near Kysil-city, Tuva, Russia, was found to contain several types of straight rods and vibrios. The rods present in the enrichment were unable to grow on acetate and showed no reduction of sulfate. Therefore, it was concluded that utilization of acetate might be carried out by a syntrophic association, i.e. an acetate-oxidizing bacterium

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that produced molecular hydrogen and a hydrogenotrophic sulfate-reducing bacterium producing H₂S. To verify this hypothesis, it was necessary to isolate the hydrogenotrophic member of this association in pure culture.

Here, the isolation and characterization of a novel hydrogenotrophic, alkaliphilic, sulfate-reducing species, *Desulfonatronum cooperativum* sp. nov., that is able to participate in acetate decomposition in syntrophic co-culture are described. In addition, this novel species is able to grow with lactate as an electron donor. For isolation of alkaliphilic, sulfate-reducing bacteria, samples of anoxic deposits and water from the soda lake Khadin were used. The pH of water at the sampling site was 9.5, salinity was 10 g l⁻¹ and alkalinity (Na₂CO₃ + NaHCO₃) was 450 mg l⁻¹.

The initial enrichment was obtained using acetate as the only carbon source in an anaerobically prepared basal medium of the following composition (g l⁻¹): NH₄Cl, 0.5; KH₂PO₄, 0.2; MgCl₂·6H₂O, 0.1; KCl, 0.2; yeast extract (Difco), 0.2; Na₂CO₃, 6.5; NaHCO₃, 3.25; NaCl, 4.18; Na₂SO₄, 3.0; sodium acetate, 3.0; and Na₂S·9H₂O, 0.5. The medium also contained a trace element solution (1 ml l⁻¹; Kevbrin & Zavarzin, 1992). The pH was maintained at 10.0 with a sodium carbonate/sodium bicarbonate buffer before autoclaving. The medium was dispensed into 120 ml flasks with screw caps and the headspace (70 ml) was filled with N₂ at atmospheric pressure. Inoculated flasks were incubated at 35 °C.

Light and electron microscopy were carried out as described previously (Zhilina *et al.*, 1997). For determination of utilization of various compounds, formate in the medium was replaced by the following: organic growth substrates (peptides and organic acids) at a concentration of 3.0 g l⁻¹ (w/v); alcohols (5.0 ml l⁻¹); or molecular hydrogen (100 % gas phase). The ability to ferment sugars was tested using the basal medium without sulfate and formate. Utilization of various electron acceptors (sulfate, thiosulfate, sulfite, dithionite, nitrate, nitrite, fumarate and elemental sulfur) was tested as described previously (Zhilina *et al.*, 1997). Lithotrophic growth with molecular hydrogen was tested on medium with the same mineral composition in the absence of yeast extract with acetate (0.16 g l⁻¹) as carbon source. Cultivation was performed in 60 ml screw-capped flasks containing 10 ml medium. Headspace (50 ml) was filled with 100 % hydrogen.

Temperature, pH and NaCl concentration requirements were tested using the basal medium with formate as electron donor and sulfate as electron acceptor. The pH and NaCl ranges for growth were determined at 35 °C as described previously (Garnova *et al.*, 2003). To determine whether sodium carbonates were required for growth, carbonates were replaced with NaCl (15 g l⁻¹) and a pH of 9.0 was obtained with 20 mM Tris/base buffer (pK_a of 9.0).

Bacterial growth was monitored by direct cell counting under a phase-contrast microscope (Reichert Zetopan),

by measuring sulfide in the growth medium (Trüper & Schlegel, 1964) and by estimating the increase in OD at 600 nm (Spectol-10; Carl Zeiss). DNA was isolated and purified as described by Marmur (1961). The G + C content was determined by the thermal denaturation method (Owen *et al.*, 1969); *Escherichia coli* K-12 DNA was used as a standard. For DNA–DNA hybridization with the type species *Desulfonatronum lacustre* Z-7951^T, DNA was obtained by a 'nick-translation' reaction based on [³H]cytidine (Rigby *et al.*, 1977). 16S rRNA gene amplification, sequencing and sequence analyses were done as described previously (Kuever *et al.*, 2001). The sequence was loaded into the 16S rRNA sequence database of the Technical University of Munich, Germany, using the ARB program package (Ludwig *et al.*, 2004). The tool ARB_ALIGN was used for sequence alignment, visually inspected and corrected manually. Tree topologies were evaluated by performing maximum-parsimony, neighbour-joining and maximum-likelihood analyses using a termini filter. Only sequences with at least 1200 nt were used for calculations. The tree contains all sequences of species with validly published names of the genera *Desulfovibrio*, *Bilophila*, *Lawsonia* and *Desulfomicrobium*. Species designations and GenBank/EMBL/DDBJ nucleotide sequence accession numbers (type strains) that were not included in the ARB database are as follows: *Desulfonatronovibrio hydrogenovorans*, X99234; '*Desulfothermus naphthae*' (strain TD3), X80922; *Desulfonauticus submarinus*, AF524933; *Desulfonatronum thiodismutans*, AF373920; and *Desulfonatronum lacustre*, AF418171.

After 30 days incubation, utilization of acetate coupled to sulfide production was obtained. Several types of straight rods (some of them spore-forming) and small vibrioid cells were observed in the medium. After several successive transfers in acetate-containing basal medium, the enrichment was serially diluted in roll-tubes using the same medium solidified with Bacto-agar (2.0 g l⁻¹). Several types of colonies appeared after 10 days incubation and were transferred to the liquid basal medium, but none of them grew with acetate. This phenomenon could be explained by syntrophic oxidation of acetate by the alkaliphilic community, which includes at least one sulfate-reducing bacterium, possibly serving as a hydrogen or formate scavenger. The enrichment was serially diluted in roll-tubes with the same medium in which acetate was substituted by formate (3 g l⁻¹). Single, slightly lens-shaped, rose colonies, 0.1–0.2 mm in diameter, appeared after 7 days incubation and were transferred to the liquid basal medium with formate as electron donor. All isolates obtained had the same phenotypic characteristics. One of them, designated strain Z-7999^T, was selected for detailed characterization.

Cells of strain Z-7999^T were small vibrioids, 0.4–0.5 µm in diameter and 1.0–2.5 µm in length (Fig. 1a). Single cells were observed during the exponential phase of growth. In the stationary phase, cells occurred in chains (three to ten

Fig. 1. Morphology of strain Z-7999^T. (a) Cells as viewed by phase-contrast microscopy (bar, 10 µm). (b) Cell of strain Z-7999^T with polar flagellum (bar, 0.5 µm).

cells per chain). Cells exhibited rapid motility by means of one polar flagellum (Fig. 1b). Formation of spores was not observed. Organisms multiplied by binary fission. Gram-stain was negative.

Strain Z-7999^T was an obligate anaerobe and grew only in anaerobically prepared medium where O₂ was eliminated by boiling and addition of Na₂S.9H₂O. Growth of strain Z-7999^T occurred at temperatures of 15–40 °C, with optimum growth at 35–38 °C. The pH range for growth was 6.7–10.3, with optimum growth at pH 8.0–9.0. Strain Z-7999^T grew in NaCl concentrations of 1–80 g l⁻¹, with optimum growth at 5–15 g NaCl l⁻¹. Carbonate was required for growth.

Strain Z-7999^T grew with sulfate as an electron acceptor and molecular hydrogen, formate and lactate as electron donors. No growth was observed with the following electron donors: malonate, pyruvate, oxalate, malate, aspartate, succinate, glutamate, fumarate, acetate, butyrate, propionate, choline, betaine, methanol, ethanol, Casamino acids and yeast extract. Strain Z-7999^T was not able to grow by a fermentative type of metabolism using sugars, peptone, yeast extract or pyruvate.

On basal medium with formate as the electron donor, strain Z-7999^T reduced sulfate, sulfite and thiosulfate, but did not reduce fumarate, dithionite, elemental sulfur, nitrate or nitrite. Strain Z-7999^T could grow lithotrophically with sulfate and molecular hydrogen as electron donor in the presence of acetate (0.16 g l⁻¹) as carbon source. Lithotrophic growth of strain Z-7999^T was stable and did not decrease after three successive transfers on the same medium. Yeast extract was not required for growth and addition of yeast extract up to 1.5 g l⁻¹ did not stimulate growth of strain Z-7999^T.

The DNA G+C content of strain Z-7999^T was 56.5 ± 1 mol%. The almost-complete sequence of the 16S rRNA gene (1493 nt) of strain Z-7999^T, covering the region between position 33 and 1509 (*E. coli* numbering), was determined. The closest relatives of strain Z-7999^T were *Desulfonatronum thiodismutans* (95.3 % similarity) and *Desulfonatronum lacustre* (95.2 % similarity). A comparative sequence analysis showed that the novel isolate belonged to the genus *Desulfonatronum* (Fig. 2) and the family 'Desulfonatronumaceae' within the order 'Desulfovibrionales' (Kuever *et al.*, 2001). DNA–DNA hybridization of strain Z-7999^T with *Desulfonatronum lacustre* Z-7951^T (= DSM 10312^T) was 35 ± 1 %.

The Gram-negative, alkaliphilic, sulfate-reducing bacteria include two genera: *Desulfonatronovibrio* and *Desulfonatronum* (Zhilina *et al.*, 1997; Pikuta *et al.*, 1998; Kuever *et al.*, 2005). In contrast to monotypic *Desulfonatronovibrio*, the genus *Desulfonatronum* includes the species *Desulfonatronum lacustre* (Pikuta *et al.*, 1998) and *Desulfonatronum thiodismutans* (Pikuta *et al.*, 2003). These organisms oxidize molecular hydrogen, formate and ethanol during sulfate reduction and can also reduce sulfite and thiosulfate. *Desulfonatronum lacustre* has a lithoheterotrophic type of metabolism (Pikuta *et al.*, 1998), whereas *Desulfonatronum thiodismutans* can grow lithoautotrophically using CO₂ as the only carbon source (Pikuta *et al.*, 2003). All of these species are obligate alkaliphiles that cannot grow below pH 8.0.

Phylogenetic analysis indicated that strain Z-7999^T represents a novel species within the genus *Desulfonatronum* (Fig. 2). This is supported by several phenotypic differences between strain Z-7999^T and both described species of the genus *Desulfonatronum*. In contrast to *Desulfonatronum lacustre* and *Desulfonatronum thiodismutans*, cells of strain Z-7999^T are thin and small (see Table 1). Strain Z-7999^T is able to grow over a pH range of 6.7–10.3, whereas the pH range for growth of *Desulfonatronum lacustre* and *Desulfonatronum thiodismutans* is 8.0–10.0 (Table 1). The tolerance to a lower pH and the broader pH range of the novel species might be linked to its ecological function. The ability to utilize organic acids in varying local pH values in microhabitats might require a broader pH tolerance. Strain Z-7999^T utilized molecular hydrogen, formate and lactate, but was unable to use ethanol, unlike the type strains of *Desulfonatronum lacustre* and *Desulfonatronum*

Fig. 2. Phylogenetic tree showing the affiliation of the 16S rRNA gene sequence from strain Z-7999^T to selected reference sequences and families of the δ -Proteobacteria. The tree was calculated by maximum-likelihood analyses with termini filter. The following are type species: *Desulfonatronovibrio hydrogenovorans*, *Desulfotomaculum retbaense*, *Desulfonauticus submarinus* and *Desulfonatronum lacustre*. Bar, 10% estimated sequence divergence.

thiodismutans, which were both able to oxidize ethanol (Table 1). The coupling of sulfidogenesis to lactate utilization has been demonstrated for the haloalkaliphilic community of the Tuva soda lakes in the past (Zavarzin *et al.*, 1996). As a result, the lactate-oxidizing, alkaliphilic, sulfate-reducer *Desulfonatronum lacustre* strain Z-7955 has been isolated (Zhilina, 2005).

Thus, strain Z-7999^T could be characterized as a hydrogenotrophic, alkaliphilic, halotolerant species of the genus *Desulfonatronum* with a broad pH range for growth that is able to oxidize lactate, but not ethanol. On the basis of physiological properties and phylogenetic analysis, it is proposed that strain Z-7999^T represents a novel species in

the genus *Desulfonatronum*, namely *Desulfonatronum cooperativum* sp. nov.

Description of *Desulfonatronum cooperativum* sp. nov.

Desulfonatronum cooperativum (co.op.er.at.i'vum. L. neut. adj. *cooperativum* cooperative).

Vibrioid cells, 0.4–0.5 μ m in diameter and 1.0–2.5 μ m in length, occurring singly or in chains. Cells exhibit rapid motility by means of one polar flagellum. Non-spore-forming. The cell wall has Gram-negative structure. Anaerobe. Alkaliphile with broad pH range for growth.

Table 1. Characteristics that differentiate strain Z-7999^T from the type strains of other species of the genus *Desulfonatronum*

Taxa: 1, *D. lacustre* (Pikuta *et al.*, 1998; Zhilina, 2005); 2, *D. thiodismutans* (Pikuta *et al.*, 2003); 3, strain Z-7999^T.

Characteristic	1	2	3
Cell size (µm)	0.7–0.9 × 2.5–3.0	0.6–0.7 × 1.2–2.7	0.4–0.5 × 1.0–2.5
Growth temperature (°C):			
Range	22–45	15–48	15–40
Optimum	40	37	35–38
Growth pH:			
Range	8.0–10.1	8.0–10.0	6.7–10.3
Optimum	9.3–9.5	9.5	8.0–9.0
Growth in NaCl (g l ⁻¹):			
Range	0–100	> 10–70	> 10–80
Optimum	0	30	5–15
DNA G+C content (mol%)	57.3 ± 1	63.1 ± 1	56.5 ± 1
Electron donors with sulfate:			
H ₂	+	+	+
Formate	+	+	+
Lactate	–†	–	+
Ethanol	+	+	–

*Autotrophic growth.

†Although the type strain of *D. lacustre* did not utilize lactate, *D. lacustre* strain Z-7955 was able to grow with lactate (see Zhilina, 2005).

Growth occurs at pH 6.7–10.3 and 15–40 °C, with optimum growth at pH 8.0–9.0 and 35–38 °C. Cl[–] is not required for growth. Growth occurs with sulfate as an electron acceptor and molecular hydrogen, formate and lactate as electron donors with production of H₂S. No growth occurs on malonate, pyruvate, oxalate, malate, aspartate, succinate, glutamate, fumarate, acetate, butyrate, propionate, choline, betaine, methanol, ethanol, Casamino acids or yeast extract. Unable to grow by fermentation of sugars, peptone, yeast extract or pyruvate. As well as sulfate, sulfite and thiosulfate are reduced to sulfide, but dithionite, elemental sulfur, nitrate, nitrite and fumarate are not used as electron acceptors with formate as electron donor. Grows lithotrophically with sulfate as electron acceptor and molecular hydrogen as electron donor in the presence of acetate as carbon source.

The type strain is Z-7999^T (=DSM 16749^T=VKM B-2329^T). The DNA G+C content of the type strain is 56.5 ± 1 mol%. Isolated from syntrophic acetate-decomposing culture enriched from anaerobic deposits of soda lake Khadin, Tuva, Russia.

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