***Desulfotomaculum alkaliphilum* sp. nov., a new alkaliphilic, moderately thermophilic, sulfate-reducing bacterium**

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A new moderately thermophilic, alkaliphilic, sulfate-reducing, chemolithoheterotrophic bacterium, strain S1^T, was isolated from a mixed cow/pig manure with neutral pH. The bacterium is an obligately anaerobic, non-motile, Gram-positive, spore-forming curved rod growing within a pH range of 8.0–9.15 (optimal growth at pH 8.6–8.7) and temperature range of 30–58 °C (optimal growth at 50–55 °C). The optimum NaCl concentration for growth is 0.1 %. Strain S1^T is an obligately carbonate-dependent alkaliphile. The G+C content of the DNA is 40.9 mol %. A limited number of compounds are utilized as electron donors, including H₂+acetate, formate, ethanol, lactate and pyruvate. Sulfate, sulfite and thiosulfate, but not sulfur or nitrate, can be used as electron acceptors. Strain S1^T is able to utilize acetate or yeast extract as sources of carbon. Analysis of the 16S rDNA sequence allowed strain S1^T (= DSM 12257^T) to be classified as a representative of a new species of the genus *Desulfotomaculum*, *Desulfotomaculum alkaliphilum* sp. nov.

Keywords: *Desulfotomaculum alkaliphilum* sp. nov., alkaliphile, thermophile, sulfate-reducing bacterium

INTRODUCTION

At present, the biodiversity of sulfate-reducing bacteria (SRB) comprises 29 genera and more than 100 species, differing in their metabolism of substrates and distribution in various ecosystems. Among them, only two species belonging to the domain *Archaea* can grow at acidic pH (from 4.5 to 6.5), whereas the majority of SRB are neutrophiles, which grow optimally at pH values close to 7.0. However, two novel mesophilic SRB isolated recently from soda lakes of Africa and Central Asia, *Desulfonatronovibrio hydrogenovorans* and *Desulfonatronum lacustre*, can grow at alkaline pH (from 8 to 10.2) (Zhilina *et al.*, 1997; Pikuta *et al.*, 1998). The alkaliphily of these bacteria is not surprising, since they were isolated from high-carbonate waters, of high salinity and high pH. Nevertheless, alkaliphiles (mainly representatives of the genera

Bacillus and *Clostridium*) can be isolated from neutral soils (Horikoshi & Akiba, 1982).

The incubation of cow/pig manure, of neutral pH, in a high-carbonate medium at moderately high temperature (55 °C) led to an intense sulfidogenesis, which indicated the presence of thermophilic, alkaliphilic sulfate-reducers. Since such SRB have not been reported previously, we attempted to isolate this bacterium.

METHODS

Source of the isolate. Mixed cow/pig manure samples were collected at several farms in the Moscow region. The pH of samples ranged from 6.0 to 7.0. The samples were stored at 4 °C.

Media and cultivation. Enrichment, isolation and cultivation of SRB was performed in a modified medium (Zhilina *et al.*, 1997) under an atmosphere of 100 % nitrogen. The basal medium contained (g l⁻¹): KH₂PO₄, 0.2; MgCl₂·6H₂O, 0.1; KCl, 0.2; NH₄Cl, 1.0; Na₂CO₃, 0.5; NaHCO₃, 8.0; Na₂SO₄, 5.0; NaCl, 5.0; Na₂S·9H₂O, 0.5; yeast extract, 0.01; and sodium formate, 5.0. The medium was supplemented with 10 ml vitamin solution (Wolin *et al.*, 1963) and

Abbreviations: MTS, maximum topological similarity; SRB, sulfate-reducing bacteria.

The GenBank accession number for the 16S rDNA sequence of strain S1^T is AF097024.

1 ml trace element solution containing (mg 200 ml⁻¹ water): MnCl₂·4H₂O, 750; Fe(NH₄)(SO₄)₂·12H₂O, 400; FeSO₄·7H₂O, 200; CoCl₂·6H₂O, 200; ZnSO₄·7H₂O, 200; NiCl₂, 100; CuSO₄·5H₂O, 20; AlK(SO₄)₂·12H₂O, 20; H₃BO₃, 20; Na₂MoO₄·2H₂O, 20; and 5 ml concentrated HCl. After autoclaving, the pH of the medium at 50 °C was 8.7–9.0.

Unless otherwise noted, enrichment and pure cultures were grown in 10 ml medium in 15 ml Hungate tubes under an atmosphere of N₂ (100%). All transfers and samplings of cultures were performed with syringes. The medium was sterilized at 120 °C for 30 min. All incubations were carried out at 50–55 °C. Upon cultivation on 3% (w/v) Difco agar in Hungate tubes, carbonate solution was added to 4 ml previously sterilized, carbonate-free medium.

Growth measurements. Bacterial growth was measured by direct cell counting under an MBI-3 phase-contrast microscope, by measuring sulfide produced from sulfate in the growth medium (Trüper & Schlegel, 1964) or by measuring the increase in OD₆₀₀ (Spekol 11).

Light microscopy. The morphology of living cultures was examined by using an MBI-3 phase-contrast microscope.

Electron microscopy

(i) **Ultrathin sections.** Bacterial cells were pre-fixed with 1.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 5 °C for 1 h, washed three times with the buffer and fixed with 1% OsO₄ in the same buffer at 20 °C for 4 h. After dehydration, the preparations were embedded in epoxy resin Epon 812 (Fluka), and ultrathin sections were mounted on copper grids and contrasted with 3% uranyl acetate in ethanol and then with lead citrate (Reynolds, 1963).

(ii) **Electron-microscopic cryofractography.** Bacterial cells were frozen in liquid propane cooled by a surrounding vessel of liquid nitrogen using attachments to a JEE-4X set-up for vapour-phase deposition and fractured when a vacuum of 3 × 10⁻⁴ Pa and a temperature of -100 °C were reached (Fikhte *et al.*, 1973). The surface of the fracture was contrasted with a platinum/carbon mixture at an angle of 45 ° and fixed by deposition of a carbon layer at an angle of 90 °. Ultrathin sections and replicas were examined in a JEM-100 B electron microscope at an accelerating voltage of 60 kV.

Effects of pH, temperature and NaCl concentration on growth. The effect of pH on growth was determined at 50 °C. The pH of the basal medium was adjusted to defined values with sterile 6 M solutions of HCl or NaOH at 50 °C under a stream of N₂ and measured using a pH meter calibrated at 50 °C. The temperature range for growth was determined in basal medium at pH 8.8. The effect of NaCl on growth was determined in basal medium containing 0.0, 0.5, 1.0, 2.0, 3.0, 5.0 and 7.0% (w/v) NaCl. NaCl requirements were studied by using a modified medium in which Na₂CO₃ and NaHCO₃ were replaced with 5 g K₂CO₃ l⁻¹ and Na₂S was replaced with K₂S.

Substrate utilization. The ability of the bacterium to utilize various substrates was tested by using basal medium supplemented with autoclaved or filter-sterilized substrates to a final concentration of 5 g l⁻¹. The cultures were incubated for 2 weeks and growth due to substrate utilization was checked by microscopic examination.

Catalase activity. Catalase was assayed by adding 3% H₂O₂ to the culture liquid (Gerhardt *et al.*, 1981).

Electron acceptors. The utilization of various electron acceptors was studied in basal medium containing formate (5 g l⁻¹) as an electron donor. Electron acceptors were added in the form of autoclaved or filter-sterilized stock solutions. The final concentrations (in mM unless stated) of electron acceptors were: Na₂SO₄, 20; Na₂SO₃, 5; Na₂S₂O₃, 10; NaNO₃, 10; benzenesulfonate, 5; fumarate, 10; and elemental sulfur (S⁰), 2 g l⁻¹.

Antibiotic susceptibility. Sensitivity to antibiotics was determined by transferring an exponentially growing culture into the basal medium containing filter-sterilized antibiotics at a concentration of 100 µg ml⁻¹ (bacitracin and chloramphenicol) or 250 µg ml⁻¹ (penicillin, ampicillin, streptomycin and vancomycin). Antibiotic-containing cultures were pre-incubated for 12 h at 37 °C and subsequently incubated for 2 weeks at 50–55 °C.

Analytical procedures. The volatile products of pyruvate fermentation were quantified by using a Pye Unicam 304 gas chromatograph equipped with a flame-ionization detector and a Chromosorb 101 column (0.9 m × 3.3 mm) kept at 160 °C. The carrier gas was CO₂.

Lipid analysis. Lipids were extracted from cell biomass that was dried in a stream of helium and then under a vacuum. To 30 mg of dry biomass, 200 µl of a 5.4 M solution of anhydrous HCl in methanol was added, and the mixture was heated at 70 °C for 2 h. The methyl esters of fatty acids and aldehyde derivatives obtained were extracted twice with 100 µl hexane. The extract was dried and silylated in 20 µl *N,O*-bis(trimethylsilyl)trifluoroacetamide for 15 min at 65 °C. A 1 µl portion of the reaction mixture was analysed with a model HP-5985B GC-MS system (Hewlett Packard) equipped with a capillary column (25 × 0.25 mm) consisting of fused quartz containing an Ultra-1 nonpolar methyl-silicone phase. The temperature program was run from 150 °C (2 min isotherm) to 250 °C at 5 °C min⁻¹ and then from 250 to 300 °C at 10 °C min⁻¹. Data processing was carried out with an HP-1000 computer by using the standard programs of the GC-MS system (Hewlett Packard).

DNA analysis. The G + C content of DNA was determined by the thermal denaturation method using the DNA of *Escherichia coli* K-12 as a standard (Owen *et al.*, 1969). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970). The genome size was determined from renaturation rates (Gillis *et al.*, 1970).

16S rRNA sequencing. For amplification and sequencing of 16S rRNA genes, DNA was obtained by standard phenol/chloroform extraction. The 16S rRNA genes were selectively amplified by PCR with the following primers: 5'-AGAG-TTGATCCTGGCTCAG-3' (forward) and 5'-TACGGT-TACCTTGTTACGACTT-3' (reverse). PCR was carried out in 50 µl standard ammonium sulfate buffer [17 mM (NH₄)₂SO₄, 67 mM Tris/HCl, 200 µg BSA ml⁻¹, 1 mM DTT, 2 mM MgCl₂] containing 1 mg DNA template, 200 mM of each primer, 200 mM of each dNTP and 5 U Tet-z polymerase (BioMaster).

Temperature cycling was performed in 30 cycles with the following profile: 0.5 min at 94 °C, 0.5 min at 42 °C and 2 min at 72 °C. The final incubation was at 72 °C for 7 min. PCR products were purified using the PCR-prep kit (Promega) according to the manufacturer's instructions. 16S rRNA was sequenced in both directions (Lane, 1991). DNA was sequenced using the Promega Silver Sequencing kit (Promega) according to the manufacturer's instructions with minor modifications.

16S rDNA sequence analysis. The sequence of strain S1^T was aligned manually with the equivalent 16S rDNA and rRNA sequences of all closely related strains found in GenBank. The initial sets of pre-aligned eubacterial sequences were obtained from the Ribosomal Database Project (Larsen *et al.*, 1993). Regions that were either not sequenced in one or more reference organisms or that exhibited alignment uncertainty, i.e. positions 1–108, 201–218, 453–479, 999–1035 and 1416–3' terminus (*E. coli* nomenclature), were omitted from the analysis. In total, 1120 nucleotides were used in the analysis. Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed by use of the correction of Jukes & Cantor (1969). The tentative unrooted phylogenetic tree was constructed using the 'maximum topological similarity' (MTS) algorithm (Chumakov & Yushmanov, 1988) and the TREE program from the GENESEE software package (Brodsky *et al.*, 1991). A phylogenetic tree rerooted by an outgroup (*E. coli*) was constructed by the neighbour-joining method (Saitou & Nei, 1987) with bootstrap analysis of 100 trees by the TREECON program (Van de Peer & De Wachter, 1994).

Nucleotide sequence accession numbers. The reference accession numbers were as follows: *Desulfotomaculum haloophilum* SEBR 3139^T, U88891; *Desulfotomaculum nigrificans* NCIMB 8395, X62176; *Desulfotomaculum ruminis* DSM 2154, Y11572; *Desulfotomaculum aeronauticum* DSM 10349^T, X98407; '*Desulfotomaculum reducens*' MI-1, U95951; *Desulfotomaculum putei* SMCC W459^T, AF053929; *Desulfotomaculum acetoxidans* DSM 771^T, Y11566; *Desulfotomaculum kuznetsovii* DSM 6115, AF009646; *Desulfotomaculum luciae* SLT^T, AF069293; *Desulfotomaculum australicum* AB33^T, M96665; *Desulfotomaculum thermocisternum* ST90^T, U33455; *Desulfotomaculum thermobenzoicum* DSM 6193^T, L15628; *Desulfotomaculum thermoacetoxidans* DSM 5813^T, Y11573; *Desulfotomaculum geothermicum* DSM 3669, Y11567; *Desulfotomaculum thermosapovorans* DSM 6562^T, Y11575; *Desulfosporosinus orientis* DSM 765^T, Y11570; '*Desulfotomaculum auripigmentum*' OReX, U85624; *Desulfotobacterium dehalogenans* JW/IU-DC1^T, U40078; *Dethiosulfovibrio peptidovorans* SEBR 4207^T, U52817; *Thermoanaerobacter thermocopriae* JT-3, L09167; *Moorella thermoacetica* LJD, X58352; *Syntrophospora bryantii* DSM 3014B^T, M26491; *Sporomusa paucivorans* X, M59117; *Peptococcus niger* DSM 20475, X55797; *Heliobacterium chlorum* ATCC 35205^T, M11212; *Thermoanaerobacterium saccharolyticum* B6A-RI^T, L09169; *Desulfonatronovibrio hydrogenovorans* Z-7935^T, X99234; and *Desulfonatronum lacustre* Z-7951^T, Y14594.

RESULTS

Enrichment culture and isolation of pure culture

Samples of cow and pig manures mixed at a ratio of 1:1 were incubated for 4–5 d at 55 °C under anaerobic conditions in Hungate tubes containing medium with 5 g formate l⁻¹. The pH of the medium (9.2) was typical for many alkaliphiles. By the time of inoculation, the pH values of the samples of cow and pig manures were 6.8 and 6.0, respectively. The enrichment culture showed intense sulfidogenesis (15–20 mM) during five sequential transfers.

A pure culture was obtained by the method of serial dilution in medium containing 2 mM formate and 2 mM acetate (pH 9.0). The purity of the isolated

culture was confirmed by the absence of growth in glucose–peptone medium lacking sulfate but containing 1.5 g yeast extract l⁻¹.

One of the colonies (referred to as strain S1^T) obtained via roll tubes was chosen for further characterization. Colonies, which appeared on agar after 8–12 d growth, were white, matt, dentate, rough and 0.5–2.0 mm in diameter. Surface colonies were round and granular with a dense centre. Subsurface colonies were round and white with a dense yellowish centre and friable, irregular edges.

Cell morphology

Cells of strain S1^T are curved, oval-ended, non-motile rods, 0.6–0.7 × 3.0–3.5 µm, occurring singly, in pairs or as short, spiral chains. Endospores are located proximally or distally (Fig. 1). Cells reproduce by binary fission. After 4–5 d growth on ethanol or pyruvate, intense spore formation may be observed. The cell wall has a typical Gram-positive structure in the form of a thin electron-dense layer. The cell envelope is composed of two structurally ordered S layers. As follows from the freeze–fracture data, the outer S layer has tetragonal symmetry and was readily scaled off the cells, so that large numbers of fragments of this layer occurred in the medium. The fragmentary inner S layer, which was observable in both cross and longitudinal sections, is located beneath the mucopeptide layer of the cell wall. The symmetry of this layer remains unknown, since we were unable to reveal it on freeze–fracture replicas, presumably due to its location in a hydrophilic region of the cell envelope.

Effects of pH, temperature and NaCl concentration on growth and sulfidogenesis

The optimum pH for sulfidogenesis was 8.6–8.7. The strain grew within a pH range of 8.0–9.15 (Fig. 2a). No growth was observed at pH 7.5 or 9.5. When the initial pH of the medium was suboptimal, strain S1^T began to grow after the pH of the medium had increased gradually to optimal values.

The dependence of strain S1^T on CO₃²⁻ ions was tested by using growth medium buffered with glycine or serine (in both cases the pH of the medium was adjusted to 8.8) and supplemented with 3 g sulfate l⁻¹ and 5 g ethanol l⁻¹. Experiments showed that the carbonate-free media could not support growth of strain S1^T. The suggestion that glycine and serine could inhibit the growth of strain S1^T must be excluded, since it grew well in carbonate-containing medium supplemented with 5 g glycine or serine l⁻¹. Thus, strain S1^T is an obligately carbonate-dependent alkaliphile. The optimum growth temperature of this strain is 50–55 °C (Fig. 2b); no growth occurred below 25 °C or above 58 °C. Hence, strain S1^T is a moderate thermophile.

The requirement of strain S1^T for NaCl was not absolute. There was no growth at 0% NaCl. The

Fig. 1. Electron micrographs of cells of strain $S1^T$. (a) Cell in the initial phase of binary fission. (b) Freeze-fractured cells with spores (Sp). (c) Freeze-fractured cell with the tetragonal outer S-layer. (d) A fragment of the Gram-positive cell wall: S_{out} , outer S-layer; S_{in} , inner S-layer; PG, peptidoglycan layer; CM, cytoplasmic membrane. Bars, 0.5 μ m (a–c) and 0.1 μ m (d).

Fig. 2. Dependence of the doubling time of strain $S1^T$ on pH (a) and temperature (b).

optimum concentration of NaCl was 0.1–1 %. At 5 % NaCl, the length of the lag phase increased. At 7 % NaCl, there was no growth. Strain $S1^T$ was not Na^+ -dependent; the equimolar substitution of potassium salts for sodium salts did not affect growth.

Taking into account that strain $S1^T$ was isolated from manure, its requirement for NH_4^+ was tested. The strain grew well at NH_4Cl concentrations from 0.1 to 1.5 %. At 5 % NH_4Cl , an extended lag phase was observed.

Substrate utilization

In the presence of sulfate, strain $S1^T$ grew on H_2 (2 atmospheres) plus acetate (2 mM), formate, ethanol, lactate and pyruvate. When strain $S1^T$ grew on ethanol, lactate or pyruvate, in addition to acetic acid as an end product, trace amounts of other volatile fatty acids (propionic, valeric and isovaleric) were present in the cultivation medium. CO_2 measurement was intricate

Table 1. Comparison of the fatty acids of *Desulfotomaculum alkaliphilum*, *Desulfotomaculum nigrificans*, *Desulfotomaculum acetoxidans* and *Ruminococcus palustris*

Percentages of total fatty acids are shown. Data were taken from this study (*Desulfotomaculum alkaliphilum* S1^T), Dowling *et al.* (1986) (*Desulfotomaculum acetoxidans* 5575), Ueki & Suto (1979) (*Desulfotomaculum nigrificans* NCIB 8395) and Zhilina *et al.* (1995) (*Ruminococcus palustris* Z-7189^T).

Fatty acid	<i>Desulfotomaculum alkaliphilum</i> S1 ^T	<i>Ruminococcus palustris</i> Z-7189 ^T	<i>Desulfotomaculum nigrificans</i> NCIB 8395	<i>Desulfotomaculum acetoxidans</i> 5575
C _{12:0}	—	—	—	—
Iso-C _{14:0}	—	—	—	—
C _{14:0}	2.3	—	6.1	1.3
Iso-C _{15:0}	—	—	21.1	0.5
Anteiso-C _{15:0}	—	—	11.0	—
C _{15:0}	1.9	—	1.6	0.3
Iso-C _{16:0}	—	—	3.4	—
C _{16:1}	21.5	21.0	5.0	24.4
C _{16:0}	31.7	20.5	5.8	34.0
C _{16:1α}	1.9	5.6	—	—
C _{16:0α}	6.2	14.5	—	—
C _{10Me16:0}	—	—	—	—
Iso-C _{17:1}	—	—	2.0	—
Iso-C _{17:0}	2.4	—	4.1	—
Anteiso-C _{17:0}	0.6	—	3.0	—
Hydroxyiso-C _{15:0}	—	—	—	—
C _{17:0}	2.0	0.7	0.5	—
C _{18:2}	—	—	—	—
C _{18:1δ9}	3.3	9.1	0.7	1.0
C _{18:1δ1}	13.3	8.6	0.7	37.0
C _{18:0}	5.6	1.5	1.5	1.2
Hydroxy-C _{16:0}	0.1	—	—	—
2-Hydroxy-C _{16:0}	0.3	—	—	—
C _{18:1α}	4.5	2.1	—	—
C _{18:0α}	2.7	2.0	—	—
C _{20:0}	—	—	—	—
Hydroxy-C _{18:0}	0.1	—	—	—

because of the high content of carbonates in the medium. In the absence of sulfate, pyruvate was poorly utilized. Strain S1^T utilized acetate and yeast extract as sources of carbon and required vitamins for growth.

The strain did not grow on acetate, propionate, butyrate, methanol, glycerol, yeast extract, peptone, methylamine, casein, cysteine, valine, sorbitol, dulcitol, mannitol, inositol, glucose, sucrose, starch, rhamnose, arabinose, lactose or xylose.

Electron acceptors

Strain S1^T utilized Na₂SO₄, Na₂SO₃ and Na₂S₂O₃, but not NaNO₃, S⁰, benzenesulfonate or fumarate, as electron acceptors. Tests for the dismutation reaction (Bak & Pfennig, 1987) were negative: neither sulfite or thiosulfate supported growth. It should be noted that mesophilic, alkaliphilic sulfate-reducers were able to bring about the dismutation reaction with sulfite and thiosulfate (Zhilina *et al.*, 1997; Pikuta *et al.*, 1998). Strain S1^T is an obligate anaerobe and catalase-negative.

Antibiotic susceptibility

Strain S1^T was resistant to chloramphenicol at 100 µg ml⁻¹ and to vancomycin and ampicillin at 250 µg ml⁻¹. The strain grew well in the presence of these antibiotics in three subsequent subcultures without noticeable morphological changes in cells. At the same time, penicillin, streptomycin and bacitracin inhibited growth of the strain at 100 or 250 µg ml⁻¹.

Membrane lipids

The fatty acid profile of cells of strain S1^T is presented in Table 1. Comparison with the profiles of 72 strains of SRB (Vainstein *et al.*, 1992) did not reveal any similarity between them. Strain S1^T does not contain iso-C_{17:1} (isoheptadecenic acid), a specific marker of the genus *Desulfovibrio*. Furthermore, it is far (beyond the genus) from *Desulfotomaculum acetoxidans* and *Desulfotomaculum nigrificans*. At the same time, the fatty acid profile of strain S1^T is similar to that of the phylogenetically dissimilar homoacetogenic bacterium *Ruminococcus palustris* (Zhilina *et al.*, 1995).

Fig. 3. Phylogenetic dendrogram showing the position of *Desulfotomaculum alkaliphilum* S1^T among representatives of the genus *Desulfotomaculum* and some genera of Gram-positive bacteria with low G+C content and Gram-negative, alkaliphilic, sulfate-reducing bacteria. Bootstrap values (greater than 95%) are shown at nodes. Scale bar corresponds to five substitutions per 100 nucleotides.

DNA analysis

The G + C content of the genomic DNA of strain S1^T was 41.5 mol %. The level of DNA relatedness between the new isolate and the type species of the genus *Desulfotomaculum*, *Desulfotomaculum nigrificans*, was very low (< 10% by hybridization analysis).

Phylogenetic analysis

The 16S rDNA sequence (1502 nucleotides) of strain S1^T corresponded to positions 8–1528 of the 16S rDNA of *E. coli*. The G + C content of this sequence was 55.1 mol %. The sequence was compared with all sequences of *Desulfotomaculum* species presently available in the GenBank database and with some reference sequences of strains from the neighbouring taxa of the clostridial subphylum.

The topologies of two phylogenetic trees constructed by using the neighbour-joining method (Fig. 3) and the MTS method (data not shown) were almost identical and correlated well with earlier data concerning *Desulfotomaculum* species (Nilsen *et al.*, 1996; Liu *et al.*, 1997; Stackebrandt *et al.*, 1997; Nazina *et al.*, 1999). Most previous studies were performed with only a few species of the genus *Desulfotomaculum*, which contributed to the instability of the phylogenetic

trees. It has been shown previously (Love *et al.*, 1993; Nazina *et al.*, 1999) that tree topologies for members of the genus *Desulfotomaculum* and its relatives are altered by selecting different reference sequences and by using different treeing algorithms.

With the increased number of *Desulfotomaculum* species used for our analysis, these factors had less effect on the branching pattern of the genus. According to the phylogenetic data, all *Desulfotomaculum* species formed a monophyletic cluster except for *Desulfotomaculum auripigmentum*, which appeared to be related to the recently renamed *Desulfosporosinus orientis* (Stackebrandt *et al.*, 1997). This cluster contains some subclusters (Stackebrandt *et al.*, 1997; Nazina *et al.*, 1999) whose similarity values ranged from 83.7 to 91.2% (Fig. 3). Within the multistrain subclusters, similarity values were greater than 94.0%.

In the phylogenetic tree, strain S1^T was located close to *Desulfotomaculum* species. The sequence similarity of strain S1^T to species of the genus *Desulfotomaculum* was relatively low, ranging from 88.4 to 92.7%. The highest degree of relatedness of this strain (92.7%) was observed with a recently described species, *Desulfotomaculum halophilum* (Tardy-Jacquenod *et al.*, 1998). In order to determine the affiliation of strain S1^T to individual subclusters, its 16S rDNA was analysed for the presence of subcluster-specific signature nucleotides (Stackebrandt *et al.*, 1997). Subclusters 1, 2, 3 and 4 can be defined on the basis of significant signature nucleotides amounting to no more than 18 nucleotides of a total of 33 common nucleotides. Strain S1^T has 28 common nucleotides with *Desulfotomaculum halophilum*, 22 common nucleotides with *Desulfotomaculum acetoxidans* (subcluster 4) and no more than 18 common nucleotides with the members of other subclusters. Thus, strain S1^T does not belong to any of the previously identified subclusters of the genus *Desulfotomaculum* and forms, together with *Desulfotomaculum halophilum*, a new subcluster, 5.

DISCUSSION

On the basis of phenotypic characteristics (i.e. dissimilatory sulfate reduction to sulfide, Gram-positive cell wall and formation of endospores), strain S1^T was identified as a member of the genus *Desulfotomaculum*.

Among the spore-forming SRB of the genus *Desulfotomaculum*, S1^T is the only alkaliphilic organism; all other 19 species of this genus are neutrophiles growing optimally at pH 7.0–7.6. It is interesting that the obligately alkaliphilic strain S1^T was isolated from a manure sample of neutral pH. The same manure sample was used for isolation of a number of anaerobic, moderately thermophilic, alkaliphilic bacteria, including a saccharolytic strain, K1, a proteolytic strain, N1, and a cellulolytic strain, K2. Like S1^T, all these bacteria are spore-forming and, hence, can survive unfavourable conditions. Since strain S1^T is able to utilize hydrogen, one can suggest that it can

Table 2. Comparison of *Desulfotomaculum alkaliphilum* S1^T with some other sulfate-reducing bacteria

Data were taken from this study (*Desulfotomaculum alkaliphilum* S1^T), Hagenauer *et al.* (1997) and Hippe *et al.* (1997) (*Desulfotomaculum aeronauticum* strain 9^T), Campbell & Postgate (1965) (*Desulfotomaculum nigrificans* NCIB 8395), Tardy-Jacquenod *et al.* (1998) (*Desulfotomaculum halophilum* SEBR 3139^T), Zhilina *et al.* (1997) (*Desulfonatovibrio hydrogenovorans* Z-7935^T) and Pikuta *et al.* (1998) (*Desulfonatovibrio lacustre* Z-7951^T).

Character	<i>Desulfotomaculum alkaliphilum</i> S1 ^T	<i>Desulfotomaculum aeronauticum</i> 9 ^T	<i>Desulfotomaculum nigrificans</i> NCIB 8395	<i>Desulfotomaculum halophilum</i> SEBR 3139 ^T	<i>Desulfonatovibrio hydrogenovorans</i> Z-7935 ^T	<i>Desulfonatovibrio lacustre</i> Z-7951 ^T
Cell morphology:						
Shape	Vibrio	Rod	Rod	Rod	Vibrio	Vibrio
Motility	—	+	—	+	+	+
Spores	+	+	+	+	—	—
Size (µm)	0.6–0.7 × 3.0–3.5	0.5–0.8 × 2.2–5.5	0.3–0.5 × 3.0–6.0	0.5 × 3–6	0.5 × 1.8–2.0	0.6 × 2.0–3.0
pH range (optimum)	7.8–9.2 (8.7)	(7.0)	(7.3)	6.9–8.0 (7.3)	8.0–10.2 (9.5–9.7)	8.0–10.1 (9.5)
Temperature range (optimum) (°C)	37–58 (53)	(37)	40–62 (55)	30–40 (35)	22–45 (37)	22–45 (37)
NaCl requirement (%)	0.1	—	±	4.0–6.0	+	—
NaHCO ₃ requirement	+	—	—	—	+	+
G + C content (mol %)	40.9	43.8	44.5	56.3	48.6	57.3
Genome size (Da)	2.2 × 10 ⁹	ND	3.6 × 10 ⁹	ND	2.1 × 10 ⁹	3.4 × 10 ⁹
Electron donors (+ SO ₄ ²⁻):						
H ₂ + CO ₂	—	+	—	(+)	—	—
H ₂ + acetate	+	+	+	+	+	+
Formate	+	+	—	+	+	+
Acetate	—	—	—	—	—	—
Propionate	—	—	—	—	—	—
Pyruvate	+	+	+	+	—	—
Lactate	+	+	+	+	—	—
Ethanol	+	—	+	+	—	+
Electron acceptors:						
Sulfate	+	+	+	+	+	+
Sulfite	+	+	+	+	+	+
Thiosulfate	+	+	+	+	+	+
Sulfur	—	—	—	—	—	—

ND, Not determined; +/—, substrate used/not used; (+), substrate used but growth is slow.

grow in a co-culture with the saccharolytic strain K1, which produces hydrogen. Generally, all of the aforementioned strains could be members of a community with close trophic relations. They can grow at 37 °C, albeit poorly, and, hence, can reside in the intestinal tract of warm-blooded animals. Investigations *in situ* are needed to confirm this assumption.

In the course of cytological studies, we performed a comparative analysis of the S layers of strain S1^T and some strains of *Desulfotomaculum nigrificans* possessing similar surface structures. Generally, structurally ordered surface layers (S layers) can be formed by a variety of bacteria, including Gram-positive and Gram-negative bacteria and archaea. These layers play an important role in the adhesion of substrates and exoenzymes and in cell-surface recognition, determine the antigenic properties of bacteria, serve as molecular traps and ion sieves and confer resistance of bacterial cells to phages and predatory bacteria (by means of structural variation of S layers). The layers, which are composed mainly of structurally ordered subunits with hexagonal or tetragonal symmetry (Sleytr, 1997), can occur not only on the outer face of the cell wall, but also within the cell wall. Comparative analysis of the structural organization of the cell wall of strain S1^T and *Desulfotomaculum nigrificans* 435 (Nazina & Pivovarov, 1979) showed that the outer layer of the cell wall of strain S1^T does not contain a

layer of bowl-shaped subunits typical of *Desulfotomaculum nigrificans* 435. The greatest similarity between the structural organization of the outer S layer and the symmetry of subunits was observed between strain S1^T and *Desulfotomaculum nigrificans* NCIMB 8395 (Sleytr *et al.*, 1969): in both cases the symmetry of subunits was tetragonal and the inner S layer occurred beneath the peptidoglycan layer.

Strain S1^T is similar to the type strain of *Desulfotomaculum nigrificans* (Table 2) in growth temperature and the range of donors (except formate) and acceptors of electrons utilized. On the other hand, these strains differ in morphology, cell size, optimum and ultimate pH, responses to carbonate, genome size, the G + C content of DNA and the level of DNA–DNA homology.

Desulfotomaculum aeronauticum DSM 10349^T and strain S1^T differ in their motility, cell shape, optimum pH and growth temperature, responses to carbonate and NaCl and their ability to utilize ethanol and lactate as electron donors. Strain S1^T differs from *Desulfotomaculum halophilum* SEBR 3139^T in morphology, optimum and ultimate values of pH and temperature, G + C content and requirements for NaCl and CO₃²⁻ ions.

Table 2 also summarizes data concerning the alkali-philic SRB *Desulfonatovibrio hydrogenovorans* Z-

7935^T and *Desulfonatromum lacustre* Z-7951^T. Strain S1^T differs from these representatives of relatively novel genera in morphology and physiology. In particular, S1^T has lower optimum and ultimate values of pH and temperature and a wider range of utilizable substrates.

In the phylogenetic trees derived from 16S rRNA sequences (Fig. 3), strain S1^T consistently branched together with members of the genus *Desulfotomaculum*. 16S rDNA analysis revealed only slight relatedness of strain S1^T to most species of the genus *Desulfotomaculum* (83.7–91.2% sequence similarity). This agrees with the results of phenotypic and genotypic analysis. In 16S rDNA similarity, strain S1^T is closest to *Desulfotomaculum halophilum* (sequence similarity 92.7%); however, this level of 16S rDNA similarity alone is insufficient to ascribe S1^T to the latter species (Stackebrandt & Goebel, 1994). Thus, the phenotypic and genotypic data allow the classification of strain S1^T as a new species of the genus *Desulfotomaculum*, *Desulfotomaculum alkaliphilum* sp. nov.

Description of *Desulfotomaculum alkaliphilum* sp. nov.

Desulfotomaculum alkaliphilum (al.ka.li.phi'lum. M. L. fem. n. *alkali* alkali; Gr. adj. *philos* loving; M. L. adj. *alkaliphilum* alkali-loving).

Cells are vibrioid or curved rods, 0.6–0.7 × 3.0–3.5 µm, non-motile, which form round endospores. Cells stain Gram-positive. Strictly anaerobic, chemolithoheterotrophic, alkaliphilic and moderately thermophilic. The temperature range for growth is 30–60 °C; optimum growth at 50–55 °C. Obligate alkaliphile that cannot grow at pH 7.0. The pH range suitable for growth at 50 °C is 8.0–9.15; the optimum pH for growth and sulfidogenesis at 50 °C in sodium carbonate medium is 8.7. NaCl concentration optimal for growth is 0.1% (w/v); the maximum tolerable concentration of NaCl is 5% (w/v). Requires carbonate anion. Utilizes hydrogen, formate, pyruvate, lactate and ethanol as electron donors and sulfate, sulfite and thiosulfate as electron acceptors. Elemental sulfur is not reduced. Requires vitamins or yeast extract for growth.

The G+C content of DNA is 40.9 mol% (as determined by the thermal denaturation method). Habitat: cow and pig manure. The type strain, S1^T, was isolated from a cow/pig manure mixture of a neutral pH. This strain has been deposited at the DSMZ as DSM 12257^T, at the All-Russia Collection of Microorganisms as VKM B-2192^T and at the ATCC as ATCC 700784^T.

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