***Dethiosulfovibrio russensis* sp. nov.,
Dethiosulfovibrio marinus sp. nov. and
Dethiosulfovibrio acidaminovorans sp. nov.,
novel anaerobic, thiosulfate- and sulfur-
reducing bacteria isolated from 'Thiodendron'
sulfur mats in different saline environments**

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Four strains of strictly anaerobic, sulfur- and thiosulfate-reducing bacteria, SR12^T, SR13, SR15^T and WS100^T, were isolated from 'Thiodendron' sulfur mats obtained from different saline environments. All isolates were motile, Gram-negative, non-spore-forming curved rods with pointed or rounded ends. The sizes of cells varied from 0.9 × 3–5 µm for strains SR12^T, SR13 and SR15^T to 0.9 × 4–8 µm for strain WS100^T. All strains could form long spiral filamentous cells up to 70–110 µm during the early stage of growth. All strains were motile by a tumbling movement and possessed lateral flagella arranged at the concave side of cells. Incomplete cross-septa were distinctive features of all strains. Growth occurred at temperatures of 10–40 °C with an optimum at 28 °C. The pH limits for growth were 5.5 to 8.0, with optimal growth at pH 6.5–7.0. All isolates were obligately anaerobic and slightly halophilic and grew in media containing 0.5–5 % NaCl with an optimum at 2 % NaCl. All strains were chemoorganoheterotrophic, having a fermentative type of metabolism and utilized proteins, peptides, amino acids and some organic acids, but not sugars, fatty acids or alcohols. Some organic substrates (isoleucine, valine, alanine, glutamate) were utilized only by strain SR12^T in the presence of sulfur or thiosulfate. Fermentation of citrate yielded mainly acetate, CO₂ and H₂. Sulfur and thiosulfate were reduced to hydrogen sulfide during the fermentation of organic substances, which increased cell yields and growth rates. Sulfate, sulfite, fumarate, nitrate, Fe₂O₃, MnO₂, DMSO and elemental selenium were not used as electron acceptors by these strains. The G+C contents of the DNA were 51 mol % for strains SR12^T, SR13 and SR15^T and 52 mol % for strain WS100^T. Based on morphological, physiological and phylogenetic similarities, all four isolates could be assigned to three new species of the genus *Dethiosulfovibrio*, named *Dethiosulfovibrio russensis* (type strain DSM 12538^T), *Dethiosulfovibrio marinus* (type strain DSM 12537^T) and *Dethiosulfovibrio acidaminovorans* (type strain DSM 12590^T).

Keywords: 'Thiodendron', *Dethiosulfovibrio*, fermentation, phylogeny, sulfidogenesis

The GenBank accession numbers for the 16S rRNA gene sequences of *D. russensis* strains SR12^T and SR13, *D. marinus* WS100^T and *D. acidaminovorans* SR15^T are AF234542, AF234543, AF234544 and AY005466, respectively.

INTRODUCTION

The subphylum of the Gram-positive bacteria with G + C contents of less than 55 mol % contains several saccharolytic and asaccharolytic micro-organisms that can be combined in different clusters (Baena *et al.*, 1999b). The newly established genera *Thermoanaerovibrio*, *Aminomonas*, *Aminobacterium*, *Dethiosulfovibrio* and *Anaerobaculum* form an independent branch of descent adjacent to cluster V (Baena *et al.*, 1998, 1999a, b; Magot *et al.*, 1997; Rees *et al.*, 1997). The genus *Dethiosulfovibrio* was established by Magot *et al.* (1997) with *Dethiosulfovibrio peptidovorans* as the type and only species of the genus. Like its closest relatives, *D. peptidovorans* showed a fermentative type of metabolism, but was unable to use carbohydrates or to grow at temperatures above 45 °C, unlike the closely related genera *Thermoanaerovibrio* and *Anaerobaculum* (Rees *et al.*, 1997; Baena *et al.*, 1999a). In contrast to *Aminomonas paucivorans* and *Aminobacterium columbiense*, which degrade amino acids in syntrophic association with methanogens, *D. peptidovorans* could circumvent the thermodynamically unfavourable production of hydrogen as a result of substrate fermentation by reducing thiosulfate and sulfur to sulfide, like many other members of the domains *Bacteria* and *Archaea* (Magot *et al.*, 1997; Baena *et al.*, 1998, 1999b). This process results in higher growth yields and even allows the fermentation of amino acids, which would otherwise not be used as growth substrates (Magot *et al.*, 1997).

The genus '*Thiodendron*' (type and only species, '*Thiodendron latens*') was established by Perfiliev (1969), who first isolated cells from the surface of sediment in mineral springs and saline lakes of the Staraja Russa health resort (Novgorod region, Russia), in small lakes near Solzi (Leningrad region, Russia) and on the shore of Lake Chokraskoye (Crimea, Ukraine) (Perfiliev, 1969; Perfiliev & Gabe, 1961). Later, '*Thiodendron latens*' was found as the dominant member of sulfur-bacterial mats in highly productive shallow water regions of marine ecosystems, in the littoral zone and in regions of underwater hydrothermal and volcanic activity of the White Sea and Japan Sea (Bight Kraternaya, Kuril Isles, Matupi Harbour, Papua New Guinea, Pacific Ocean) (Dubinina *et al.*, 1993a, b).

Dubinina *et al.* (1993a, b) showed that '*Thiodendron latens*', previously described as a single bacterium with a complicated life cycle (Perfiliev, 1969), is really a symbiotic association of two different bacteria. Anaerobic, aerotolerant spirochaetes that accumulate intracellular elemental sulfur were the main functional and structural component of '*Thiodendron*' macrocolonies or films and large vibrioid-shaped sulfidogenes showing an identical morphology to the motile stage of '*Thiodendron latens*' were the second component (Dubinina *et al.*, 1993a). Therefore, the taxonomic status of the genus '*Thiodendron*' was abolished. Here, we report on the characterization of three

sulfidogenic strains isolated from different '*Thiodendron*' sulfur mats. These strains represent three new species of the genus *Dethiosulfovibrio*, and the names *Dethiosulfovibrio russensis* sp. nov., *Dethiosulfovibrio marinus* sp. nov. and *Dethiosulfovibrio acidaminovorans* sp. nov. are proposed.

METHODS

Bacterial strains. Strains SR12^T (= DSM 12538^T), SR13 (= DSM 12577) and SR15^T (= DSM 12590^T) were isolated from sulfur '*Thiodendron*' mats in mineral springs at the Staraja Russa health resort (Novgorod region, Russia). Strain WS100^T (= DSM 12537^T) was isolated from the littoral zone at Kandalaksha Bay (White Sea, Murmansk region, Russia). *D. peptidovorans* DSM 11002^T was purchased from the DSMZ, whereas *Escherichia coli* VKM-12 and '*Mycobacterium rubrum*' VKM B-874 were obtained from the Russian Collection of Microorganisms.

Isolation and growth conditions. All strains were isolated from the last positive tube of a serial dilution series on agar medium for sulfur-reducing bacteria, inoculated with material obtained from '*Thiodendron*' mats. The composition of the medium for isolation (g l⁻¹) was: NH₄Cl (0.3), CaCl₂·2H₂O (0.3), NaCl (20), MgCl₂·6H₂O (3), KH₂PO₄ (1), sodium acetate (1), yeast extract (0.5 or 1), ferrous ammonium citrate (0.1), agar (10) and elemental sulfur (in excess; final concentration 30 g l⁻¹) (Pfennig & Biebl, 1976). For cultivating isolated strains, anaerobic citrate-containing medium (ACC) was used. Medium ACC contained (g l⁻¹): NaCl (20), MgCl₂·6H₂O (3), KH₂PO₄ (1), yeast extract (5), peptone (2), sodium citrate (10 mM) and resazurin (0.001). The medium was autoclaved at 120 °C and then cooled to room temperature under a stream of O₂-free N₂ gas. The cooled medium was supplied with 2 ml CaCl₂·2H₂O (from a 10% sterile anoxic stock solution), 1 ml trace element solution (Pfennig & Lippert, 1966), 1 ml thiamin solution (0.05 g l⁻¹), 1 ml biotin solution (0.02 g l⁻¹), 1 ml vitamin B₁₂ solution (0.05 g l⁻¹) and 2 ml Na₂S (from a 0.5 M sterile anaerobic stock solution). When required, 10 ml thiosulfate (from a 1 M sterile anoxic stock solution) or sulfur (as a powder; final concentration 30 g l⁻¹) was added to the medium. Anoxic stock solutions were prepared with deoxygenated water and stored under a N₂ gas phase. For organic substrate utilization experiments, peptone and citrate were omitted and the concentration of yeast extract was reduced to 0.5 g l⁻¹. The pH of the medium was adjusted to 6.7–6.8. The medium was distributed in 10 ml portions into Hungate tubes under a stream of N₂ gas.

Physiological and biochemical tests. All growth studies were performed with medium ACC. For determination of the pH optimum, prereduced medium (as indicated above) was dispensed into Hungate tubes and strongly buffered with carbonate (for pH 8.5–10.0) or citrate (for pH 4.5–7.5) buffers. The buffer solutions were added from 1 M sterile anaerobic stock solutions. Growth of all strains was tested at temperatures ranging from 0 to 50 °C. To determine the salt requirement for growth, sodium chloride was either weighed directly into tubes or injected from a 10% sterile stock solution to obtain the desired concentration. All growth experiments were initiated with an inoculum of 2% (v/v).

Differential cytochrome spectra of whole cells after reduction with dithionite and oxidation by air were made at room temperature using the SF-56 spectrophotometer attached to a computer for data processing.

Growth was measured by monitoring optical density at 650 nm by inserting tubes directly into a Shimadzu UV-1201V spectrophotometer. The growth yield was determined as dry weight of cells formed per mol substrate consumed from cultures grown in 500 ml bottles. A calibration curve was used to estimate the growth yield from optical density values. Sulfide was determined photometrically by the methylene blue reaction (Cline, 1969). Fermentation products (acetate, lactate and succinate) were measured by HPLC as described previously (Rabus *et al.*, 1996); gas products (H_2 and CO_2) were measured by GC as described previously (Harder, 1997).

Substrate utilization. Substrate utilization tests were performed with modified ACC medium containing 0.5 g l^{-1} yeast extract. All organic substrates were added from filter-sterilized (pore size, $0.2\text{ }\mu\text{m}$) 1 M stock solutions or as saturated stock solutions into prereduced sterile anaerobic medium (as indicated above) by sterile syringes. The usual substrate concentration was 10 mM for carbohydrates, organic acids and alcohols. Peptides and protein extracts were used at a final concentration of 2 g l^{-1} . The gelatin melting test was carried out with 10 % gelatin in the medium. Amino acids at a final substrate concentration of 10 mM as well as peptides and protein extracts were tested in either the presence or the absence of thiosulfate and sulfur (in excess).

Cell characterization. To determine the Gram reaction we stained cells (Sigma Diagnostics, kit HT90-A) and used the KOH lysis method (Buck, 1982). Cultures of *E. coli* VKM-12 and '*M. rubrum*' VKM B-874 were used as controls for the Gram reaction analysis.

Light microscopy was performed with a phase-contrast microscope Zeiss NU-2. For electron microscopy, exponentially grown cells were put on colloidal film previously placed on copper wire mesh. Precipitated cells were fixed for a few minutes in 2.5 % (w/v) glutaraldehyde solution prepared in 0.05 M phosphate buffer (pH 7.0). The fixed samples were contrasted for 15 s with 2 % NH_4MoO_4 , pH 7.0, and dried. Material for ultrathin sections was prepared according the method of Ryter & Kellenberger (1958). Electron photomicrographs were taken with a JEM-100C electron microscope at an accelerating voltage of 60 kV.

DNA base composition. Two grams of cells (wet weight) was washed with buffer containing 0.15 M NaCl and 0.1 M EDTA (pH 8.0) and treated with lysis buffer (0.6 M sucrose, 0.015 M Tris/HCl and 0.015 M NaCl with 0.01 M EDTA). Lysis was achieved by lysozyme treatment (Serva) for 1 h at 37°C including 1 % SDS. The DNA was purified with 2-propanol before fractionation and treated with Pronase ($50\text{ }\mu\text{g ml}^{-1}$) (Marmur, 1961). The G+C content of the DNA was determined by comparison of temperature denaturation curves using a Pye Unicam SP1800 spectrophotometer with a heating speed of $0.5^\circ\text{C min}^{-1}$ (Mesbah *et al.*, 1989). The melting procedure was carried out in $0.1\times\text{SSC}$ (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). The G+C content of the DNA was calculated according to Owen *et al.* (1969).

DNA-DNA hybridization analysis. DNA-DNA hybridization was measured by the method of optical reassociation (De Ley *et al.*, 1970).

Comparative analysis of the 16S rRNA sequences. Culture samples were destroyed by frequent, rapid freeze-thawing to release the DNA from cells. The 16S rRNA gene sequences were determined as described previously (Muyzer *et al.*, 1995). Sequences that were not included in the 16S rRNA

sequence database of the Technical University Munich using the program package ARB (Strunk *et al.*, 1999) were added from databases. The tool ARB_ALIGN was used for sequence alignment. The alignment was checked by eye and corrected manually. Tree topologies were evaluated by performing maximum-parsimony, neighbour-joining and maximum-likelihood analysis. Only sequences that were at least 90 % complete were used for treeing. Alignment positions at which fewer than 50 % of sequences of the entire data set shared the same residues were excluded from the calculations.

RESULTS

Enrichment and isolation

Samples from bacterial sulfur mats of 'Thiodendron'-like structure were collected from bottom sediments of mineral sulfur springs at the Staraja Russa health resort (Novgorod region, Russia) (strains SR12^T, SR13 and SR15^T) and the littoral zone of the White Sea (Kandalaksha Bay, Murmansk region, Russia) (strain WS100^T) and used as inocula for dilution series.

Pure cultures were obtained from enrichments by using black FeS-producing colonies for repeated serial agar dilution. These colonies contained large vibrioid cells of identical morphology to the cells observed in the sulfur mat samples. The medium was then optimized for further cultivation.

Morphology and ultrastructure

The single cells of all strains were slightly curved, non-spore-forming rods with pointed or rounded ends. The cells often occurred in pairs (Figs 1a and 2). Gram staining and Gram reaction with KOH were negative. The size of cells varied depending on the age of the cultures. Cells from the early exponential phase of growth resembled helical filaments, 30–32 μm in length. Very long filaments, up to 70–110 μm , were

Fig. 1. Phase-contrast photomicrograph of *D. russensis* SR12^T (= DSM 12538^T) grown on citrate-containing medium, at the middle of exponential phase of growth (50 h). Bar, 5 μm .

Fig. 2. Electron micrograph of motile cells of *D. marinus* WS100^T (= DSM 12537^T) with flagella located on the concave side of cells. Cells were stained with 2% ammonium molybdate. Bar, 1 μ m.

formed by strains SR12^T and WS100^T when cultures were freshly inoculated from 4- to 6-month-old cultures. The cell size was 3–5 $\times$ 0.9 μ m for strains SR12^T and SR13 and 4–8 $\times$ 0.9 μ m for strain WS100^T during the exponential phase of growth. During that stage of growth, cells occurred singly, in pairs and rarely as long helical filaments. Young cells were motile by an active tumbling movement, which gave them the appearance of an X shape. Up to six flagella were arranged linearly along the concave side of the cells (Fig. 2).

Examination of cell ultrastructure for strains SR12^T and WS100^T by electron microscopy revealed a multi-layered cell wall typical of Gram-negative bacteria. The cell wall was composed of at least three layers (Fig. 3a, b). The most distinctive ultrastructural features of strains SR12^T and WS100^T were numerous incomplete cross-septa within each cell at various stages of growth (Fig. 3a, b). The mean number of these developing cross-septa was 10–20 per cell unit. Cross-septa were formed by regular ingrowth of the cytoplasmic membrane and the thin peptidoglycan layer of the cell wall. The external wall layer did not participate in septum formation, but took part in binary cell division. The cross sections of strains SR12^T and WS100^T were similar to that of trochomes of the large, Gram-positive bacterium *Caryophanon latum* (Peshkov & Marek, 1973; Trentini & Gilleland, 1974). Very similar cross-septum formation was found for *D. peptidovorans* cells during stationary growth.

Growth characteristics

All strains grew over a temperature range of 15–40 °C, with an optimum at 28 °C. No growth was observed at 50 °C. A NaCl concentration of at least 0.5% (w/v) was required for growth and up to 7% (w/v) NaCl was

tolerated. The optimum NaCl concentration for growth was 2% (w/v). No growth occurred at 10% (w/v) or below 0.5% (w/v) NaCl. The pH range for growth was 5.5–8.0, with an optimum at 6.5–7.0. The maximum specific growth rate ($\mu_{\max}$) on ACC medium was 0.16 h⁻¹ (doubling time 4.3–4.4 h) for both strain SR12^T and strain WS100^T.

Substrate utilization and physiological characteristics

All strains showed a fermentative type of metabolism. The substrate preferences for all strains were similar (Table 1). Typical substrates were polypeptides (gelatin, peptone, tryptone), amino acids mixtures (meat extract, casein amino acids), individual amino acids and a limited number of organic acids such as citrate, pyruvate and 2-oxoglutarate. Strains SR12^T and SR15^T also used malate. With respect to the utilization of amino acids, all four strains and *D. peptidovorans* showed remarkable variations (Table 1). In contrast to the type strain of the genus, all four strains were able to depolymerize gelatin and to ferment citrate. A concentration of 0.5–1 g yeast extract l⁻¹ was required for the utilization of organic acids by all strains. Growth of all strains in medium containing yeast extract as the only organic substrate was extremely poor (Fig. 4).

All strains reduced elemental sulfur and thiosulfate, but not sulfate or sulfite, to sulfide during the fermentation of organic substrates. Growth did not occur with H₂ as electron donor and carbon dioxide or acetate as carbon sources in the presence of thiosulfate or elemental sulfur as electron acceptor. The addition of either elemental sulfur (30 g l⁻¹) or thiosulfate (10 mM) gave up to 7 mM sulfide at the end of growth and increased the cell yield and growth rate significantly during fermentative growth on peptides, organic acids and some amino acids. No cytochromes were observed in the cells of strain SR12^T, SR15^T or WS100^T grown on ACC medium in the presence of sulfur or thiosulfate.

Analysis (HPLC and GC) of the end products of citrate fermentation indicated that strains SR12^T and WS100^T produced acetate, CO₂ and H₂.

DNA base composition

The G + C contents were 50.8 mol% for strain SR12^T, 51.2 mol% for strain SR13, 50.6 mol% for strain SR15^T and 51.8 mol% for strain WS100^T, as determined by thermal denaturation (Table 1).

Comparative analysis of 16S rRNA sequences

Phylogenetic analysis revealed that strains SR12^T, SR13, SR15^T and WS100^T were closely related to *D. peptidovorans* (DSM 11002^T), which is a member of the subdivision containing Gram-positive bacteria with G + C contents of less than 55 mol% (Magot *et al.*, 1997; Saiki *et al.*, 1985). Our tree was calculated on

Fig. 3. Electron micrograph of longitudinal (a) and transverse (b) cell cross-sections of *D. russensis* SR12^T (= DSM 12538^T) grown on citrate-containing medium for 3 d, showing multitude septum formations by regular, deep, cytoplasmic membrane invaginations. (a) Overview; bar, 0.25 μm; inset; bar, 0.125 μm. Inset indicates ingrowth of inner dense thin cell wall layer (peptidoglycan) participating in the formation of septa. (b) Bar, 0.2 μm.

almost full-length sequences only (1205 bases) and was corrected by taking into consideration the different results of various tree-construction algorithms. Bifurcations indicate branching that appeared stable and well separated from neighbouring branches in all cases. Multifurcations indicate tree topologies that could not be resolved significantly on the basis of the available dataset. The new strains form a stable cluster together

with *Aminobacterium columbiense*, *Anaerobaculum thermoterrenum*, *Synergistes jonesii*, *Thermoanaerovibrio acidaminovorans* and *Aminomonas paucivorans* (Fig. 5). The 16S rRNA sequence of strain SR12^T was identical to those of strains SR13, SR15^T and WS100^T and showed a similarity of 98.5% to that of *D. peptidovorans*. DNA–DNA hybridization was therefore used for differentiation at the species level.

Table 1 Comparison of strains SR12^T, SR13, WS100^T and SR15^T and *D. peptidovorans*

Abbreviations used are: +, used; –, not used; +¹, used only in the presence of thiosulfate or sulfur; +², growth without H₂S production; ND, not determined. Data for *D. peptidovorans* were taken from Magot *et al.* (1997). Cells of all taxa were motile. Peptone, tryptone, yeast extract and Casamino acids were utilized by all strains tested. The following sugars were tested and not utilized: arabinose, fructose, galactose, glucose, lactose, maltose, mannose, ribose, sucrose, trehalose and xylose. In addition, none of the organisms listed could use lactate, formate, acetate, propionate, butyrate, fumarate, succinate, ethanol, propanol, glycerol or mannitol. All taxa listed reduced sulfur and thiosulfate to sulfide and did not reduce sulfate to sulfide.

Character	SR12 ^T (= DSM 12538 ^T)	SR13 (= DSM 12577)	WS100 ^T (= DSM 12537 ^T)	SR15 ^T (= DSM 12590 ^T)	<i>D. peptidovorans</i>
Cell morphology	Curved rods or spirals	Curved rods or spirals	Curved rods or spirals	Curved rods or spirals	Vibrio
Cell size (µm)*	From 0.9 × 3–5 to 0.9 × 100–110	From 0.9 × 3–5 to 0.9 × 100–110	From 0.9 × 4–8 to 0.9 × 70	0.9 × 3–5	1 × 3–5
Intracellular cross-investigations†	+	ND	+	ND	+
DNA G+C content (mol%) (<i>T_m</i> method)	50.8	51.2	51.8	50.6	56‡
Temperature range for growth (°C) (optimum)	4–37 (28)	4–37 (28)	4–37 (28)	4–40 (25–30)	20–45 (42)
Range of NaCl for growth (% v/v) (optimum)	0.5–5 (2)	0.5–5 (2)	0.5–5 (2)	0.5–7 (2)	(3)
pH range for growth (optimum)	5.5–8.0 (6.5–7.0)	ND	5.5–8.0 (6.5–7.0)	5.5–8.0 (6.5–7.0)	5.5–8.8 (7.0)
Utilization of:					
Citrate	+	+	+	+	–
Malate	+	+	–	+	ND
2-Oxoglutarate	+	–	+ ²	+ ²	ND
Pyruvate	+ ²	–	+ ²	+	ND
Gelatin	+	+	+	+	–
Meat extract	+	+	+	+	ND
Alanine	+ ¹	+ ¹	–	+ ¹	+ ¹
Arginine	+ ¹	–	–	–	+ ¹
Asparagine	–	–	–	–	+ ¹
Aspartate	–	–	–	ND	ND
Cysteine	+ ¹	+	+	+	ND
Glycine	–	–	–	ND	–
Glutamate	+ ¹	+ ¹	+	+	+ ¹
Histidine	+	–	+	+	+
Isoleucine	+ ¹	–	–	–	+ ¹
Leucine	–	+ ²	–	–	+ ¹
Lysine	+	–	+ ¹	ND	–
Methionine	–	+ ¹	–	–	+ ¹
Proline	–	–	–	ND	–
Serine	+	+	+ ²	+	+
Threonine	+ ²	–	+	+ ²	–
Valine	+ ¹	+ ¹	–	–	+ ¹
Reduction of sulfite to sulfide	–	–	–	–	ND

* Measured in the middle of exponential phase.

† Distinctly visible at stationary growth phase.

‡ Determined by HPLC.

DNA–DNA hybridization analysis

Total DNA obtained from all isolated strains was compared with the total DNA of *D. peptidovorans* DSM 11002^T (Magot *et al.*, 1997), which appeared to be closely related to the new isolates. The DNA–DNA similarity of the isolates to *D. peptidovorans* varied from 21 to 27%, which separates them taxonomically from *D. peptidovorans*. The high level of DNA homology, 74%, between strains SR12^T and strain SR13 leaves both as one species, whereas the low levels for WS100^T (22–59%) and SR15^T (29–40%) to all other strains and to each other (Table 2) identify them as two separate species.

DISCUSSION

In this paper, we describe four new strains of bacteria isolated from ‘*Thiodendron*’ sulfur mats from different

Fig. 4. Growth of strain SR12^T on several organic substrates in the presence of thiosulfate (10 mM). Substrates tested were: meat extract (2 g l⁻¹) and yeast extract (0.5 g l⁻¹) (○), histidine (10 mM) and yeast extract (0.5 g l⁻¹) (□), 2-oxoglutarate (10 mM) and yeast extract (0.5 g l⁻¹) (■) and yeast extract (0.5 g l⁻¹) alone (●).

Fig. 5. Maximum-likelihood tree based on 1205 positions of almost full-length 16S rRNA sequences from 124 bacterial strains. Selected sequences from Gram-positive organisms with high DNA G+C contents were taken to root the tree. Trees constructed with other tree-reconstruction algorithms (neighbour-joining and maximum-parsimony) generally resulted in the same overall tree topology. In bootstrap analysis using parsimony criteria, the branching of the genus *Dethiosulfovibrio* was supported by values of 100%. Bar, 10% sequence divergence.

Table 2 Percentage DNA–DNA hybridization between *D. peptidovorans* and strains SR12^T, SR13, WS100^T and SR15^T

	<i>D. peptidovorans</i>	SR12 ^T	SR13	SR15 ^T
SR12 ^T	21	—		
SR13	27	74	—	
SR15 ^T	29	32	40	—
WS100 ^T	22	52	59	38

saline environments. It was demonstrated that all isolates were strictly anaerobic, obligately moderately halophilic, Gram-negative bacteria with a fermentative type of metabolism. Elemental sulfur and thiosulfate were reduced to sulfide during fermentation, but growth of the new isolates was not dependent upon sulfur reduction, as has been shown for some sulfur-respiring mesophilic and thermophilic *Bacteria* and *Archaea* (Widdel & Hansen, 1992; Schauder & Kröger, 1993; Kelly & Adams, 1994; Bonch-Osmolovskaya, 1994; Ma *et al.*, 1995; Stetter, 1996). As has been demonstrated already for members of the genera

Dethiosulfovibrio, *Thermoanaerobacter*, *Thermosipho* and *Haloanaerobium*, sulfidogenesis was accompanied by a decrease in the hydrogen concentration in the growth medium and by a shift of the fermentation products to more oxidized compounds, e.g. acetate (Fardeau *et al.*, 1993, 1997; Faudon *et al.*, 1995; Ravot *et al.*, 1996, 1997). Since sulfur and thiosulfate increased both the cell yield and the growth rate in the cultures, the organisms must derive some advantage from the reduction process. As has been found for some *Bacteria* (Janssen & Morgan, 1992; Childers *et al.*, 1992) and *Archaea* (Adams, 1990; Ma *et al.*, 1995; Stetter, 1996), there was a significant stimulation of growth by the removal of high concentrations of hydrogen (one of the final products of fermentation). A possible explanation would be that these compounds are used as alternatives to protons by an enzyme similar to sulfhydrogenase, as described for *Pyrococcus furiosus* (Kelly & Adams, 1994).

For other organisms, such as *Thermotoga maritima*, it has been shown that sulfur reduction is preferred over H₂ formation, although coupling with energy conservation was excluded because there was no increase of the growth yield (Schröder *et al.*, 1994). The

biochemical mechanism underlying the stimulating effect of sulfur compound reduction for the strains described here is still unknown. Fiala & Stetter (1986) could prevent the inhibitory effect of hydrogen on the growth of *Pyrococcus furiosus* by gassing with nitrogen.

In the absence of thiosulfate, growth by fermentation of single amino acids was restricted to particular amino acids. Alanine, glutamate, isoleucine, leucine, methionine and valine were only utilized in the presence of thiosulfate as electron acceptor. The same results were obtained for *Thermoanaerobacter* species (Faudon *et al.*, 1995; Fardeau *et al.*, 1997). This dependence on thiosulfate can be explained by the metabolic pathways used for degradation. The initial step in the degradation of these amino acids is a thermodynamically unfavourable oxidative deamination to the corresponding keto acid (Stams, 1994; Schink, 1997). To pull this reaction in the desired direction, a steady removal of reducing equivalents by an exergonic reaction is necessary, for example by H_2 -scavenging organisms or by the reduction of sulfur or thiosulfate (Stams, 1994; Schink, 1997).

The newly isolated strains were compared, using phenotypical and genotypical properties and phylogenetic analysis, with other anaerobic, fermentative, sulfur-reducing bacteria in order to determine their taxonomic and phylogenetic affiliations. A comparative 16S rRNA sequence analysis placed the three new strains within the domain *Bacteria* in the same region as the genera *Thermoanaerovibrio*, *Anaerobaculum*, *Aminomonas*, *Aminobacterium* and *Synergistes*, but with the closest relationship to the genus *Dethiosulfovibrio* (Fig. 5). Due to the limited number of sequences currently available, it can only be speculated whether these organisms really represent a new monophyletic deep-branching phylum or whether this is simply a temporal treeing artifact caused by long branch attraction. The exact placement within the 16S rRNA tree must await the availability of further sequences. The similarity value for the 16S rRNA gene sequence between the only known species of the genus, *D. peptidovorans*, and strains SR12^T, SR13, SR15^T and WS100^T was 98.5%. It is interesting that the metabolism of members of the genus *Dethiosulfovibrio* is similar to that of their closest phylogenetic relatives. Although some of them are thermophiles, many share the ability to use elemental sulfur or thiosulfate as a hydrogen (electron) sink and grow by fermentation of amino acids or other organic compounds to produce H_2S (Rees *et al.*, 1997; Baena *et al.*, 1999a). Others, such as *Synergistes jonesii*, isolated from the rumen of a goat, and *Aminomonas paucivorans*, live in habitats where hydrogen would be removed continuously by methanogens or other H_2 -scavenging organisms (Allison *et al.*, 1992; McSweeney *et al.*, 1993; Baena *et al.*, 1999b).

Besides the significant differences between the 16S rRNA sequences, all three strains can be distinguished

from *D. peptidovorans* by the G+C content of the DNA, values of DNA–DNA homology, their fermentable substrate ranges, morphology and physiological characteristics (Table 1). The DNA–DNA hybridization results indicate that they are new species within this genus (Stackebrandt & Goebel, 1994). The levels of DNA homology between strains SR12^T, SR13, SR15^T and WS100^T (see Table 2) leave the four as three species. New species are therefore proposed with the names *D. russensis*, for strains SR12^T and SR13, *D. acidaminovorans* for strain SR15^T and *D. marinus* for strain WS100^T. All three type strains of the newly proposed species differ from *D. peptidovorans* by their ability to utilize citrate, threonine and lysine and inability to utilize leucine, methionine and asparagine and by biochemical and morphological features (Table 1).

Ecological role

As was mentioned above, the sulfur mat-forming bacterium described earlier as '*Thiodendron latens*' was really a symbiotic association of aerotolerant spirochaetes and anaerobic sulfidogenes (Dubinina *et al.*, 1993a). The spirochaete species are the main structural and functional component of these mats and they may accumulate elemental sulfur intracellularly. The sulfidogenes isolated previously from '*Thiodendron*' mats as pure cultures are morphologically identical to the motile stage of '*Thiodendron latens*' and they show the same tumbling movement (Dubinina *et al.*, 1993a). They are described in this paper as new species of the genus *Dethiosulfovibrio*. A stable co-culture between *Dethiosulfovibrio* species and a *Spirochaeta* species in a medium for spirochaete cultivation supplemented with glucose and elemental sulfur under microaerophilic conditions resulted in the formation of structures identical to natural '*Thiodendron*' sulfur mats (Dubinina *et al.*, 1993a).

Such an association of micro-organisms would be typical of sulfide-rich habitats, where the chemical oxidation of sulfide by oxygen, manganese or ferric iron or by the activity of sulfide-oxidizing bacteria results in the formation of thiosulfate or elemental sulfur (Cline & Richards, 1969; Zhang & Millero, 1993; Yao & Millero, 1996; Jørgensen, 1990). These partly oxidized sulfur compounds can then be either completely oxidized to sulfate by sulfur-oxidizing bacteria, if enough oxygen is present, or reduced to sulfide by sulfidogenic bacteria. Oxygen limitation would not be unusual in such places, as indicated by micro-profile measurements from such habitats (Jørgensen, 1990). The relationship between the aerotolerant *Spirochaeta* species and the *Dethiosulfovibrio* species (or other sulfidogenes) would represent a very effective shortcut in the sulfur cycle. In contrast to the well-known interaction between green-sulfur bacteria and sulfur-reducing bacteria, '*Thiodendron*'-like associations of colourless sulfur bacteria depend on the input of organic compounds other than acetate or

ethanol (Wolfe & Pfennig, 1977) and exist in highly productive marine ecosystems with high rates of H_2S production (Dubinina *et al.*, 1993b).

Description of *Dethiosulfovibrio russensis* sp. nov.

Dethiosulfovibrio russensis (rus.sen'sis. M.L. adj. *russensis* pertaining to Staraja Russa in Russia).

Cells are Gram-negative curved or vibrioid-like rods, $3\text{--}5 \times 0.9\text{ }\mu\text{m}$ with pointed or rounded ends. Cells are motile by tumbling movement with flagella located on the concave side of cells. Long helical filamentous cells up to $110\text{ }\mu\text{m}$ in length were observed at earlier stages of growth (22–36 h). Multiple cross-septa formed by regular, deep, cytoplasmic membrane invaginations are typical for cells of all growth phases. Growth occurred at temperatures between 15 and $40\text{ }^\circ\text{C}$ (optimum $28\text{ }^\circ\text{C}$) and at pH values between 5.5 and 8.0 (optimum 6.5–7.0). Obligately anaerobic and slightly halophilic. Requires NaCl at 0.5–5% (optimum 2%), but no growth occurs at 10% NaCl. Ferments proteins, peptides, amino acids and some organic acids. Able to ferment serine, histidine, lysine, arginine, cysteine and threonine. Can use alanine, glutamate, isoleucine, leucine and valine in the presence of elemental sulfur or thiosulfate only. Also ferments yeast and meat extracts, peptone, tryptone, casein hydrolysate, citrate, malate, pyruvate and 2-oxoglutarate in both the presence and absence of sulfur or thiosulfate. Yeast extract is required for growth. Elemental sulfur and thiosulfate, but not sulfate or sulfite, are reduced to sulfide in the presence of fermentable substrates. The presence of sulfur or thiosulfate increases the cell yield and growth rate. Cytochromes were not found. Unable to utilize carbohydrates (glucose, sucrose, trehalose, arabinose, ribose, xylose, maltose, lactose, fructose, galactose, mannose), some organic acids (acetate, succinate, lactate, formate, fumarate, propionate, butyrate) or alcohols (ethanol, mannitol, glycerol, propanol) in either the presence or absence of yeast extract and thiosulfate. Sulfate, sulfite, fumarate, nitrate, Fe_2O_3 , MnO_2 , DMSO and elemental selenium are not reduced. The G + C content of the DNA is 50.8 mol% (T_m).

Isolated from 'Thiodendron' bacterial sulfur mats of mineral springs of the Staraja Russa health resort (Russia). The type strain is SR12^T, which has been deposited in the DSMZ as DSM 12538^T. The type strain can be distinguished from strains SR13, SR15^T and WS100^T by DNA–DNA homology levels.

Description of *Dethiosulfovibrio marinus* sp. nov.

Dethiosulfovibrio marinus (ma'ri.nus. L. adj. *marinus* marine).

Cells are Gram-negative curved or vibrioid-like rods, $4\text{--}8 \times 0.9\text{ }\mu\text{m}$ with pointed or rounded ends. Cells are motile by tumbling movement with flagella located on

the concave side of cells. Long helical filamentous cells up to $70\text{ }\mu\text{m}$ in length were observed at earlier stages of growth (22–36 h). Multiple cross-septa formed by regular, deep, cytoplasmic membrane invaginations are typical for cells of all growth phases. Growth occurred at temperatures between 15 and $40\text{ }^\circ\text{C}$ (optimum $28\text{ }^\circ\text{C}$) and at pH values between 5.5 and 8.0 (optimum 6.5–7.0). Obligately anaerobic and slightly halophilic. Requires NaCl at 0.5–5% (optimum 2%), but no growth occurs at 10% NaCl. Ferments proteins, peptides, amino acids and some organic acids. Able to ferment glutamate, histidine, cysteine and threonine. Can use lysine, serine and valine in the presence of elemental sulfur or thiosulfate only. Also ferments yeast and meat extracts, peptone, tryptone, casein hydrolysate, citrate, pyruvate and 2-oxoglutarate in both the presence and absence of sulfur or thiosulfate. Yeast extract is required for growth. Elemental sulfur and thiosulfate, but not sulfate or sulfite, are reduced to sulfide in the presence of fermentable substrates. The presence of sulfur or thiosulfate increases the cell yield and the growth rate. Cytochromes were not found. Unable to utilize carbohydrates (glucose, sucrose, trehalose, arabinose, ribose, xylose, maltose, lactose, fructose, galactose, mannose), some organic acids (acetate, succinate, lactate, formate, fumarate, propionate, butyrate) or alcohols (ethanol, mannitol, glycerol, propanol) in either the presence or absence of yeast extract and thiosulfate. Sulfate, sulfite, fumarate, nitrate, Fe_2O_3 , MnO_2 , DMSO and elemental selenium are not reduced. The G + C content of the DNA is 51.8 mol% (T_m).

Isolated from 'Thiodendron' bacterial sulfur mats in the littoral zone at Kandalaksha Bay (White Sea, Murmansk region, Russia). The type strain is WS100^T, which has been deposited in the DSMZ as strain DSM 12537^T. The type strain can be distinguished from strains SR12^T and SR15^T by DNA–DNA homology levels.

Description of *Dethiosulfovibrio acidaminovorans* sp. nov.

Dethiosulfovibrio acidaminovorans (a.cid.a.mi.no.vo'r-ans. M.L. n. *acidum aminum* amino acid, L. v. *vorare* to devour, swallow; M.L. part. adj. *acidaminovorans* devouring amino acids, the main substrate).

Cells are Gram-negative curved or vibrioid-like rods, $3\text{--}5 \times 1\text{ }\mu\text{m}$ with pointed or rounded ends. Cells are motile by tumbling movement with flagella located on the concave side of cells. Growth occurred at temperatures between 15 and $40\text{ }^\circ\text{C}$ (optimum $28\text{ }^\circ\text{C}$) and at pH values between 5.5 and 8.0 (optimum 6.5–7.0). Obligately anaerobic and slightly halophilic. Requires NaCl at 0.5–7% (optimum 2%), but no growth occurs at 10% NaCl. Ferments proteins, peptides, amino acids and some organic acids. Able to ferment serine, histidine, glutamate, cysteine and threonine. Can use alanine in the presence of elemental sulfur or thio-

sulfate only. Also ferments yeast and meat extracts, peptone, tryptone, casein hydrolysate, citrate, malate, pyruvate and 2-oxoglutarate in both the presence and absence of sulfur or thiosulfate. Yeast extract is required for growth. Elemental sulfur and thiosulfate, but not sulfate or sulfite, are reduced to sulfide in the presence of fermentable substrates. The presence of sulfur or thiosulfate increases the cell yield and growth rate. Cytochromes were not found. Unable to utilize carbohydrates (glucose, sucrose, trehalose, arabinose, ribose, xylose, maltose, lactose, fructose, galactose, mannose), some organic acids (acetate, succinate, lactate, formate, fumarate, propionate, butyrate) or alcohols (ethanol, mannitol, glycerol, propanol) in either the presence or absence of yeast extract and thiosulfate. Sulfate, sulfite, fumarate, nitrate, Fe_2O_3 , MnO_2 , DMSO and elemental selenium are not reduced. The G+C content of the DNA is 51 mol% (T_m).

Isolated from 'Thiodendron' bacterial sulfur mats of mineral springs of the Staraja Russa health resort (Russia). The type strain is SR15^T, which has been deposited in the DSMZ as strain DSM 12590^T. The type strain can be distinguished from strains SR12^T and WS100^T by DNA-DNA homology levels.

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