Desulfosporosinus hippei sp. nov., a mesophilic sulfate-reducing bacterium isolated from permafrost

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The sulfate-reducing strain 343^T was isolated from ancient permafrost deposits in Siberia, Russia. Based on 16S rRNA gene sequence analyses, this strain was closely related to *Desulfosporosinus* species, showing 97.9% 16S rRNA gene sequence similarity to *Desulfosporosinus meridiei* DSM 13257^T, 97.6% similarity to *Desulfosporosinus auripigmenti* DSM 13351^T, 97.2% similarity to *Desulfosporosinus lacus* DSM 15449^T and 96.2% similarity to *Desulfosporosinus orientis* DSM 765^T. The strain was found to contain *b*-type cytochromes and to reduce only sulfate and thiosulfate using lactate as an electron donor but not sulfite, elemental sulfur, fumarate, nitrate or Fe(III). These data, considered in conjunction with DNA–DNA hybridization data, cell-wall chemotaxonomy and data on physiology, support recognition of strain 343^T as representing a distinct and novel species within the genus *Desulfosporosinus*, namely *Desulfosporosinus hippei* sp. nov., with the type strain 343^T (=DSM 8344^T =VKM B-2003^T).

The genus *Desulfosporosinus* was proposed in 1997 to accommodate the species *Desulfotomaculum orientis* (Stackebrandt *et al.*, 1997). The genus currently contains four species with validly published names, *Desulfosporosinus orientis* (Stackebrandt *et al.*, 1997), *Desulfosporosinus auripigmenti* (Stackebrandt *et al.*, 2003), *Desulfosporosinus meridiei* (Robertson *et al.*, 2001) and *Desulfosporosinus lacus* (Ramamoorthy *et al.*, 2006). *Desulfosporosinus*-like organisms are widespread in different habitats. They exist in environments such as pristine aquifers, municipal drinking water, rice plant roots and permafrost. Members of the genus also inhabit industrially impacted soil and sediments, coal-mining-impacted lakes and radionuclide-contaminated sediments (Vainshtein *et al.*, 2007).

Vainshtein *et al.* (1994, 1995) isolated the mesophilic, spore-forming, sulfate-reducing strain 343 from ancient permafrost in Russia. At that time, strain 343 was identified as a member of *Desulfotomaculum orientis* and placed in

the German Collection of Microorganisms and Cell Cultures under the number DSM 8344 and in the All-Russian Collection of Microorganisms under the number VKM B-2003. The strain number DSM 8344 is used in this article because all comparative taxonomic studies published so far have referred to this strain as DSM 8344. During the reclassification of *Desulfotomaculum orientis* as *Desulfosporosinus orientis* (Stackebrandt *et al.*, 1997), strains *Desulfosporosinus* (= *Desulfotomaculum*) *orientis* DSM 765^T and DSM 8344 were compared. They were shown to have a value of only 39% DNA–DNA reassociation, which clearly indicated the presence of two genospecies (Stackebrandt *et al.*, 1997). At that time, the lack of distinguishing phenotypic properties prevented the authors from describing a novel species for strain DSM 8344 (Stackebrandt *et al.*, 1997). We describe genetic and phenotypic attributes of DSM 8344 investigated in parallel with the four *Desulfosporosinus* species that support the recognition of DSM 8344 as representing a distinct and novel species within the genus *Desulfosporosinus*.

Sampling and isolation procedures were described in detail previously (Vainshtein *et al.*, 1995). In brief, strain DSM 8344 was isolated from 20 to 30 000-year-old permafrost deposits at a depth of 4.2–4.5 m; the temperature *in situ* was –5 °C. The strain was isolated from the highest positive

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DSM 8344^T is Y11571.

Whole-cell fatty acid profiles of strain DSM 8344^T and related type strains are available as supplementary material with the online version of this paper.

tubes of a most-probable number (MPN) series. The culture medium used was Postgate B medium (Postgate, 1984) containing 1.0 g lactate l^{-1} . Prior to inoculation, the medium was reduced with 10 mg sodium dithionite l^{-1} , added from a freshly prepared anoxic stock solution (3 % w/v). Anaerobic techniques were used in all medium preparation and culture manipulations. Unless otherwise indicated, all physiological experiments were performed in triplicate at 30 °C, lasting until cultures reached stationary phase. Uninoculated media served as negative controls. Physiological attributes of strain DSM 8344 were evaluated and compared with those of *D. meridiei* DSM 13257^T (Robertson *et al.*, 2001), *D. orientis* DSM 765^T (Campbell & Postgate, 1965), *D. auripigmenti* DSM 13351^T (Newman *et al.*, 1997; Stackebrandt *et al.*, 2003) and *D. lacus* DSM 15449^T (Ramamoorthy *et al.*, 2006).

Cell morphology and spore formation were assessed by phase-contrast light microscopy and electron microscopy. Strain DSM 8344 was deemed to be positive for spore formation because cell incubation at 85 °C for 30 min was followed by active growth in fresh nutrient medium after reinoculation. Growth was confirmed by both hydrogen sulfide and biomass production.

Temperature and pH optima were estimated by culturing cells in basal medium amended with sodium lactate and sodium sulfate and monitoring the change in cell number and sulfide production. Cells were counted directly by the method of Yagodka (1975). Direct counting of bacterial cells under the microscope was previously limited in its application to liquid media by the chamber size; the modification of Yagodka (1975) uses direct counting in a drop under a conventional coverglass. The volume is calculated as the product of the area of the counted region (the area of one microscope field of vision multiplied by the number of fields counted) and the depth (calculated with calibration of the microscope focusing system). Sulfide (as H_2S) concentration was estimated by spectrophotometric assay of methylene blue as described by Cline (1969). To determine the temperature optimum, cells were grown at pH 7.0 at 5, 10, 20, 24, 28, 30, 37 and 40 °C. To define the pH optimum, 20 mM (each) HEPES, PIPES, MES and Tris base buffers were used and the initial medium pH was set using 1 M HCl or 1 M NaOH. Cell growth at pH 4.0, 5.0, 6.0, 6.5, 7.0, 7.5 and 8.0 was examined at 28 °C. NaCl concentrations up to 30 % were tested to determine the salinity optimum for cell growth at 28 °C and pH 7.0.

Strain DSM 8344 was evaluated for its ability to utilize terminal acceptors and to grow using electron donors as indicated in Table 1. To obtain information on chemotaxonomic properties, basal Postgate B medium was used with lactate or sulfate replaced by the appropriate compounds. Anaerobic stock solutions were prepared for each electron acceptor and electron donor and aliquots were added to medium up to 1.0 g acceptor l^{-1} . Each acceptor was tested using sodium lactate (10 mM) as the carbon source. Each donor was also tested using sulfate

(5 mM) as the terminal electron acceptor. The ability to use electron donors or acceptors was estimated by changes in cell number and sulfide production as described above. The organism was considered positive for utilization of an electron donor or acceptor if two to three doublings occurred after the third passage, with simultaneous production of sulfide. Uninoculated media served as negative controls. Fermentative growth of strain DSM 8344 on pyruvate or lactate (each at 1 g l^{-1}) was evaluated and compared with other members of the genus *Desulfosporosinus*. Direct cell counting was used to confirm fermentative growth.

Cell-wall preparations were obtained by boiling cells in 20 % (w/v) aqueous trichloroacetic acid solution for 20 min in accordance with well-known methods. Diaminopimelic acid isomers were detected in cell-wall hydrolysates by TLC on cellulose sheets (Merck) using the solvent system of Rhuland *et al.* (1955). These methods as well as that used for determination of cellular fatty acid patterns were the same as used for other *Desulfosporosinus* species (Ramamoorthy *et al.*, 2006).

16S rRNA gene sequence analysis of strain DSM 8344 (GenBank accession no. Y11571) was reported previously (Stackebrandt *et al.*, 1997). The almost-complete 16S rRNA gene sequence of DSM 8344 was aligned with sequences included in the ARB database and those obtained from EMBL (<http://www.ebi.ac.uk>) using tools implemented in the ARB package (Ludwig *et al.*, 2004). The resulting alignment was inspected visually and potential errors were corrected manually. Evolutionary distances were calculated based on the algorithms of Jukes & Cantor (1969), using neighbour-joining and parsimony methods of tree reconstruction as implemented in the ARB program package. The databases of 16S rRNA gene sequences used in this study, as well as the phylogenetic programs, are available at <http://www.arb-home.de>.

Genomic DNA for DNA–DNA hybridization studies was isolated using a French pressure cell and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out according to the method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter.

Morphology and physiology

Cells of strain DSM 8344 were motile, curved rods, 4.5–5.5 µm long and 1.0–1.2 µm wide, and formed terminal, round endospores after 2–3 days incubation (Vainshtein *et al.*, 1995). Cells of strain DSM 8344^T stained Gram-negative. Flagellation was variable; subpolar flagella were occasionally observed.

Strain DSM 8344 coupled oxidation of lactate to sulfate reduction, conserving energy for growth, and belongs to physiological group I of the sulfate-reducing bacteria,

Table 1. Distinguishing traits of strain DSM 8344^T and related *Desulfosporosinus* type strains

Data for reference strains are from Ramamoorthy *et al.* (2006) and Spring & Rosenzweig (2006). Cells of all strains are curved rods. All strains are positive for the following traits: utilization of sulfate and thiosulfate as electron acceptors and lactate as electron donor and fermentative growth with pyruvate. All strains failed to grow with nitrate as electron acceptor. LL-Diaminopimelic acid is the diagnostic diamino acid in the cell-wall murein of all strains. ND, No data available.

Trait	<i>D. hippei</i> DSM 8344 ^T	<i>D. lacus</i> DSM 15449 ^T	<i>D. orientis</i> DSM 765 ^T	<i>D. meridiei</i> DSM 13257 ^T	<i>D. auripigmenti</i> DSM 13351 ^T
Gram stain	Negative	Negative	Negative	Negative	Positive
Morphology	Pairs	Pairs	Pairs	Short chains	Long chains
Cell diameter (µm)	1.0–1.2	0.5–0.7	0.7–1.0	0.7–1.1	0.4
Endospores	Round, terminal	Ellipsoidal, subterminal	Ellipsoidal, subterminal	Ellipsoidal, subterminal	Ellipsoidal, subterminal to terminal
Motility	+	+	+	+	–
Flagellation type	(Sub)polar	Peritrichous	(Sub)polar or lateral	Lateral	ND
Temperature for growth (°C)					
Optimum	28	30	30–37	28	25–30
Range	5–37	4–32	<42	10–37	ND
pH for growth					
Optimum	7	ND	7	7	6.4–7.0
Range	6.5–7.5	6.5–7.5	ND	ND	ND
NaCl range (%)	<2.0	ND	<5.0	<4.0	ND
Electron acceptors in the presence of lactate					
Sulfite	–	+	+	+	+
Sulfur	–	–	+	+	+
Fe(III)	–	+	–	–	–
Fumarate	–	–	+	–	+
Electron donors in the presence of sulfate					
Formate	–	+	+	+	+
Fumarate	–	–	+	–	–
Methanol	–	+	+	+	–
Glycerol	–	+	–	+	+
Oxidation of H ₂ (with CO ₂)	–	+	+	+	+
Fermentative growth on lactate	–	+	+	+	ND
Cytochromes	<i>b</i> *	<i>c</i> , traces of <i>b</i>	<i>b</i>	ND	ND
DNA G + C content (mol%)	42 (HPLC)*	43 (HPLC)	46 (<i>T</i> _m)	47 (<i>T</i> _m)	42 (HPLC)

*Data from Vainshtein *et al.* (1995).

members of which oxidize lactate incompletely to acetate. The strain was able to reduce sulfate over the range of 5–40 °C with an optimum at 28 °C. All temperature experiments were conducted at pH 7.0. Although strain DSM 8344 was isolated from a permafrost habitat, the strain grows optimally under mesophilic conditions. Sulfidogenic growth of DSM 8344 was confined to the range pH 6.5–7.5. NaCl concentrations of 0–0.1 % were optimal for growth of strain DSM 8344. No cell growth was detected with NaCl concentrations of more than 2 %.

Heat-treated cultures of DSM 8344 incubated in fresh medium increased in cell density and accumulated sulfide. This observation can be attributed to the ability of the cells to form high-temperature-resistant endospores.

Electron acceptor and donor preferences for all *Desulfosporosinus* type strains are summarized in Table 1. All the type strains utilized oxidized inorganic sulfur

compounds. However, unlike other *Desulfosporosinus* strains, DSM 8344 was unable to utilize sulfite as an electron acceptor. Furthermore, DSM 8344 was incapable of using elemental sulfur, fumarate, nitrate or Fe (III) as electron acceptors. Strain DSM 8344 and *D. orientis* DSM 765^T had different electron donor utilization profiles; DSM 8344 was distinct in that it was unable to couple growth to sulfate respiration using formate, fumarate or glycerol as both electron donors and carbon sources. Ethanol and propanol supported poor growth of cultures of DSM 8344. Strain DSM 8344 did not grow autotrophically with hydrogen and either CO₂ or acetate as carbon source. No cell growth or sulfide accumulation was observed when cells were cultured on acetate, benzoate, butanol, propionate, malate, succinate, maltose, lactose, sucrose, hexadecane, 2,4-hydrobenzoate, methanol, catechol or choline. Strain DSM 8344 grew fermentatively on pyruvate but not on lactate in the absence of sulfate.

Chemotaxonomy

The cellular fatty acid profile for strain DSM 8344 (Supplementary Table S1, available in IJSEM Online) showed that its qualitative composition was similar to those of other representatives of the genus *Desulfosporosinus*. However, there are quantitative differences, especially in the abundance of 16:0, 16:0 dimethylacetal (dma) and 18:1*cis*11. Fatty acids 16:1*cis*9 (34.2%), 16:0 (9.4%) and 18:1*cis*11 (13.9%) were predominant in strain DSM 8344. The diagnostic diamino acid of the peptidoglycan of strain DSM 8344 as well of all other type strains of *Desulfosporosinus* species was LL-diaminopimelic acid (Table 1). Cells of strain DSM 8344 contained *b*-type cytochromes, which is a characteristic of most endospore-forming, sulfate-reducing bacteria studied so far; *c*-type cytochromes were not observed in this strain.

Phylogeny and comparative genomics

A parsimony tree was constructed (Fig. 1) based on 16S rRNA gene sequences; the tree demonstrates that strain DSM 8344 and other members of the genus *Desulfosporosinus* group together to form a cluster with 97% bootstrap support. Sequence similarities based on the comparison of almost-complete 16S rRNA gene sequences indicate that strain DSM 8344 is most closely related to *D. meridiei* DSM 13257^T (97.9% 16S rRNA gene similarity), whereas the similarity value to *D. orientis* DSM 765^T is only 96.2%. Trees were also reconstructed using the neighbour-joining method, but displayed no significant differences in branching order from the parsimony tree.

Currently DNA–DNA hybridization values between two strains of less than 70% are considered to warrant recognition of strains as members of separate species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Previously, DNA–DNA hybridization experiments have revealed that strain DSM 8344 shows only 39% reassociation with *D. orientis* DSM 765^T (Stackebrandt *et al.*, 1997), thereby excluding its assignment to this species as originally proposed by Vainshtein *et al.* (1995). In this

study, the ratio of DNA–DNA binding with the closest phylogenetic relative, *D. meridiei* DSM 13257^T, was determined and was found to be 57%.

Ribotype patterns determined previously also clearly indicate a distinct position of strain DSM 8344 separate from all other type strains of the genus *Desulfosporosinus* (Stackebrandt *et al.*, 2003; Spring & Rosenzweig, 2006).

The DNA G+C content of strain DSM 8344 was 42.1 mol%.

Strain DSM 8344 is also distinguished phenotypically from other members of the genus by its fatty acid profile and its inability to use sulfite or iron as an electron acceptor or to grow by oxidation of hydrogen (Table 1). This last trait is a novel feature amongst *Desulfosporosinus* species, meaning that an emendation of the genus description is necessary. Consequently, phylogenetic and chemotaxonomic data indicate that strain DSM 8344 represents a novel species of *Desulfosporosinus*, *Desulfosporosinus hippei* sp. nov.

Emended description of the genus *Desulfosporosinus* Stackebrandt *et al.* 1997 emend. Stackebrandt *et al.* 2003

The emended description of Stackebrandt *et al.* (2003) is further modified as follows. The genus also includes species that do not oxidize hydrogen with CO₂ or acetate as carbon source.

Description of *Desulfosporosinus hippei* sp. nov.

Desulfosporosinus hippei (hip'pe.i. N.L. gen. n. *hippei* of Hippe, named after Hans H. Hippe, of the DSMZ, for his numerous contributions to the cultivation and taxonomy of sulfate-reducing bacteria).

Cells are curved rods, stain Gram-negative and have subpolar flagella. Individual cells are 4.5–5.5 µm long and 1.0–1.2 µm wide. They produce heat-resistant, terminal, round endospores. Utilizes sulfate and thiosulfate as terminal electron acceptors in the presence of lactate.

Fig. 1. Phylogenetic tree based on an alignment of almost-complete 16S rRNA gene sequences showing the affiliation of strain DSM 8344^T to the genus *Desulfosporosinus*. The tree was reconstructed using the program DNAPARS implemented in the PHYLIP package of the ARB software (version 03_08_22). Only bootstrap values above 80% (as percentages of 1000 resamplings) are shown. The root is defined using sequences of unidentified strains of *Peptococcus niger* (X55797) and *Desulfotomaculum nigrificans* (AB026550) (not shown). Bar, 5% estimated sequence divergence.

Sulfite, elemental sulfur, fumarate, nitrate and Fe(III) are not used as electron acceptors. In the presence of sulfate, ethanol and propanol are utilized slightly as electron donors and carbon sources. Cannot use acetate, benzoate, butanol, propionate, malate, succinate, maltose, lactose, sucrose, hexadecane, 2,4-hydrobenzoate, methanol or choline as electron donors or carbon sources. Cells are capable of fermentative growth using pyruvate but not lactate as an energy source in the absence of any electron acceptor. The temperature and pH ranges for growth are 5–37 °C and pH 6.5–7.5. Cells grow well at NaCl concentrations up to 1 g l⁻¹. NaCl inhibits growth at concentrations above 20 g l⁻¹. Possesses *b*-type cytochromes. The diagnostic diamino acid of the peptidoglycan is LL-diaminopimelic acid. Major fatty acids (>5 %) are (as percentages of the total) 16:1*cis*9 (34.2 %), 16:0 (9.4 %), 18:1*cis*11 (13.9 %) and 18:1*cis*9 (9.7 %). The G+C content of the DNA of the type strain is 42.1 mol%.

The type strain is strain 343^T (=DSM 8344^T =VKM B-2003^T), isolated from ancient (20–30 thousand years old) permafrost deposits in Kolyma region, Siberia, Russia.

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