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Desulfonauticus submarinus gen. nov., sp. nov., a novel sulfate-reducing bacterium isolated from a deep-sea hydrothermal vent

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A novel moderately thermophilic, hydrogenotrophic, sulfate-reducing bacterium, strain $6N^T$ ($=DSM\ 15269^T=CIP\ 107713^T$), was isolated from matrixes of *Alvinella* and *Riftia* originating from deep-sea hydrothermal-vent samples collected on the 13°N East-Pacific Rise at a depth of approximately 2600 m. It was a Gram-negative, non-sporulating, curved rod, motile with one polar flagellum, that did not possess desulfoviridin. It grew at temperatures ranging from 30 to 60 °C, with an optimum at 45 °C, in the presence of 0–5 % NaCl (optimum 2 %). Strain $6N^T$ utilized only H_2/CO_2 and formate as electron donors with acetate as carbon source. Sulfate, sulfite, thiosulfate and elemental sulfur were used as terminal electron acceptors during hydrogen oxidation. The G + C content of DNA was 34·4 mol%. Strain $6N^T$ grouped with members of the family *Desulfohalobiaceae* in the δ-subclass of the *Proteobacteria*. Its closest phylogenetic relative was *Desulfonatronovibrio hydrogenovorans*, with only 90 % similarity between the sequences of the genes encoding 16S rRNA. Because of significant phylogenetic differences from all sulfate-reducing bacteria described so far in the domain *Bacteria*, this novel thermophile is proposed to be assigned to a new genus and species, *Desulfonauticus submarinus* gen. nov., sp. nov.

Bacterial sulfate-reducers are widespread in marine, terrestrial and subterranean environments, where they contribute significantly with methanogens to the overall degradation of organic matter. In the last two decades, much attention has been paid by scientists to microbial communities living in deep environments, with particular emphasis on deep-sea hydrothermal vents (Chevaldonné *et al.*, 1992; Jeanthon, 2000). Such environments are known to be inhabited by a wide range of mesophilic to hyperthermophilic microorganisms involved in the oxidation and/or reduction of sulfur compounds including sulfide, thiosulfate, sulfite and elemental sulfur (Jeanthon, 2000). Amongst these microorganisms, particularly aerobic sulfide-oxidizers, but also anaerobic sulfur-reducers, were recognized to be of ecological significance. Molecular ecological and microbiological studies of hydrothermal chimneys or sediments and of growth chambers deployed in hydrothermal vents (Burggraf *et al.*, 1990; Jeanthon, 2000; Jeanthon *et al.*, 2002; Reysenbach *et al.*, 2000) have demonstrated the prevalence of thermophilic microbes that use (i) hydrogen as an electron donor and (ii) sulfur compounds (e.g., S^0, sulfate, thiosulfate) as terminal electron acceptors and their probable importance in hydrogen and sulfur metabolism *in situ*.

In contrast to the so-called 'moderately to hyperthermophilic sulfur-reducers', the community of sulfate-reducing bacteria, some of which are also able to reduce thiosulfate, sulfite and S^0, has been poorly studied. However, the dominance of sulfate-reducing bacteria in hydrothermal communities has been confirmed by measurements of sulfate-reduction activity in sediments (Elsgaard *et al.*, 1994; Jorgensen *et al.*, 1992) and by their repeated isolation from hydrothermal samples (Alazard *et al.*, 2003; Elsgaard *et al.*, 1995). Thermophilic sulfate- and/or sulfite-reducing members of the domain *Archaea* were found to be representative of these deep microbial communities. They include *Archaeoglobus profundus* (Burggraf *et al.*, 1990), isolated from hydrothermal vents at Guaymas Basin, and *Archaeoglobus veneficus* (Huber *et al.*, 1997), isolated from several chimney samples collected at the Mid-Atlantic Ridge (23°N) and East-Pacific Rise (9°N). Thermophilic and mesophilic representatives of the domain *Bacteria* include *Thermodesulfobacterium hydrogeniphilum* (Jeanthon *et al.*, 2002), isolated from a deep-sea hydrothermal vent at Guaymas Basin, '*Desulfothermus naphthae*' (Kuever *et al.*, 2003; Rueter *et al.*, 1994), also isolated from Guaymas Basin sediments, and *Desulfovibrio hydrothermalis* and

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA and *dsr* gene sequences of strain $6N^T$ are AF524933 (16S rDNA), AY187623 (*dsrA*) and AY187624 (*dsrB*).

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Desulfovibrio profundus-like micro-organisms (Alazard *et al.*, 2003), isolated from a deep-sea hydrothermal chimney sample collected on the 13°N East-Pacific Rise.

Here, we report the isolation of a novel hydrogenotrophic, thermophilic sulfate-reducer isolated from the same deep marine hydrothermal site from which *Desulfovibrio hydrothermalis* was isolated. Because of significant phylogenetic, phenotypic and genomic differences from sulfate-reducing bacteria within the subclass *δ-Proteobacteria*, we propose to assign this isolate to a new genus and species within the family *Desulfohalobiaceae*, *Desulfonauticus submarinus* gen. nov., sp. nov.

Nine strains were isolated from various matrixes of *Alvinella* stored in glycerol/sea water (80/20, v/v) at −80 °C and of *Riftia* stored in sea water at 4 °C until processing. The samples were collected during the AMISTAD cruise by the deep-submergence vehicle *Nautile* in June 1999 from the Grandbonum vent site at 13°N, 103°56′W along the East-Pacific Rise at 2600 m depth. Enrichments were performed using sulfate-reducing bacteria (SRB) growth medium containing (l^{-1} distilled water): 1 g NH_4Cl, 0·3 g K_2HPO_4, 0·3 g KH_2PO_4, 1·8 g $MgCl_2.6H_2O$, 0·4 g $CaCl_2.2H_2O$, 23 g NaCl, 0·1 g KCl, 3 g Na_2SO_4, 1 g sodium acetate dihydrate, 0·5 g cysteine hydrochloride, 2 g yeast extract (Difco), 2 g bio-Trypticase (bioMérieux), 10 ml trace-element solution (Widdel & Pfennig, 1981; Imhoff-Stuckle & Pfennig, 1983) and 1 mg resazurin. The pH was adjusted to 7·0 with 10 M KOH and the medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Aliquots were then dispensed into Hungate tubes (5 ml) or serum bottles (20 ml) under a stream of N_2/CO_2 (80 : 20, v/v) and the vessels were autoclaved for 45 min at 110 °C. Prior to inoculation, $Na_2S.9H_2O$ and $NaHCO_3$ were injected from sterile stock solutions to obtain respective final concentrations of 0·04 and 0·2 % (w/v). The matrixes of *Riftia* and *Alvinella* were inoculated in 20 ml SRB medium and incubated at 45 and 55 °C with agitation under an atmosphere of H_2/CO_2 (80 : 20, 2 bar) to initiate enrichment cultures. Isolation was performed in modified SRB medium [$MgCl_2$, $CaCl_2$, KCl and NaCl were replaced by sea salts (Sigma; 30 g l^{-1}), the concentrations of yeast extract and bio-Trypticase were respectively lowered to 0·3 and 0·2 g l^{-1} and 0·2 ml of the vitamin solutions of Balch *et al.* (1979) and Widdel & Pfennig (1981) were added per litre medium]. The culture was purified by repeated use of the roll-tube method (Hungate, 1969) with medium solidified with 2 % (w/v) Noble agar (Difco). Several colonies obtained were picked and cultured in the culture medium. The process of isolation was repeated several times until the isolates were deemed to be axenic.

pH, temperature and NaCl ranges for growth were determined as described previously (Hernandez-Eugenio *et al.*, 2000) using SRB medium with the addition of vitamin solutions (Balch *et al.*, 1979; Pfennig & Widdel, 1981). Substrates were tested at a final concentration of 20 mM in SRB medium. To test for electron acceptors, sodium thiosulfate, sodium sulfate, sodium sulfite, sodium nitrate and elemental sulfur were added to the medium at respective final concentrations of 20, 20, 2 and 10 mM and 2 % (w/v). Phase-contrast microscopy (model Eclipse E600; Nikon) was used for routine examination of the cultures and to obtain photomicrographs. For electron microscopy, thin sections were prepared as described by Fardeau *et al.* (1997). Photomicrographs were taken with a Hitachi model H600 electron microscope at 75 kV. Unless otherwise indicated, duplicate culture tubes were used throughout these studies. Growth was measured by inserting tubes directly into a model Cary 50 Scan spectrophotometer (Varian) and measuring the OD_{580}. Sulfide was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). Fermentation products were determined as described by Fardeau *et al.* (1993). Desulfoviridin was determined as described by Postgate (1959).

The G + C content of DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, using HPLC as described previously (Hernandez-Eugenio *et al.*, 2000). Genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega), according to the manufacturer's protocol. DNA extracts were stored at −20 °C in Tris/HCl (10 mM, pH 8·0). The 16S rRNA gene was amplified with the primers Fd1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and Rd1 (5′-AAGGAGGTGATCCAGCC-3′) and the following reaction conditions: 1 min at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 55 °C and 2 min at 72 °C and a final extension of 10 min at 72 °C. The genes encoding DSR (α- and β-subunits) were amplified with the primers DSR1F (5′-ACSCACTGGAAGCACG-3′) and DSR4R (5′-GTGTAGCAGTTACCGCA-3′) (Wagner *et al.*, 1998) and the following reaction conditions: 1 min at 94 °C, 45 cycles of 30 s at 94 °C, 1 min at 45·7 °C and 3 min at 72 °C and a final extension of 10 min at 72 °C. PCR products were purified with the Nucleo Spin extract kit (Macherey Nagel) and cloned using the pGEM-T-easy cloning kit (Promega), according to the manufacturers' protocols. The clone libraries were screened by direct PCR amplification from a colony using the vector-specific primers SP6 (5′-ATTTAGGTGACACTATAGAA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) and the following reaction conditions: 2 min at 96 °C, 40 cycles of 30 s at 94 °C, 1 min at 55 °C and 3 min at 72 °C and a final extension of 10 min at 72 °C. Plasmids containing inserts of the correct length were isolated using the Wizard Plus SV Minipreps DNA purification system (Promega) according to the manufacturer's protocol. Purified plasmids were sent for sequencing to Genome Express. The nucleotide sequence of the 16S rRNA gene and the amino acid sequence deduced from the nucleotide sequence of the DSR genes were aligned manually with reference sequences of various members of the genus *Desulfovibrio* using the sequence alignment editor BioEdit (Hall, 1999). Reference sequences were obtained from the Ribosomal Database Project II (Maidak *et al.*, 2001), EMBL and GenBank databases (Benson *et al.*, 1999).

Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on 1111 unambiguous nucleotides (16S rRNA gene) and on 428 unambiguous amino acids (DSR genes) were computed by using respectively the Jukes & Cantor (1969) method and the Kimura (1980) method. Dendrograms were constructed by using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined by bootstrap analysis using 100 resamplings of the sequences (Felsenstein, 1985).

The enrichment medium used in the absence of sulfate in this study was first designed for the isolation of methanogens. Because of significant sulfide production in some of these enrichments, we decided to investigate the presence of novel thermophilic sulfate-reducing bacteria. Sulfate-reducing enrichment cultures were obtained after 3 weeks incubation at 45 and 55 °C. Microscopic observations revealed the presence of long, motile, curved rods. The enrichment was subcultured in Hungate roll tubes. Single, brown, discus-shaped colonies (1 mm diameter) that developed after 45 days incubation at 45 and 55 °C were picked and serially diluted in roll tubes before the culture was considered pure. Five strains (7V, 7B, $6N^T$, 8V, 8B) were isolated at 45 °C and four strains (21G, 32G, 41G, 52G) were isolated at 55 °C. The purity of these strains was confirmed by morphological homogeneity observed under a phase-contrast microscope and by the absence of growth in liquid sulfate-free SRB medium supplemented with 20 mM glucose under aerobic or anaerobic conditions. The nine strains were found to be phylogenetically very similar. Strain $6N^T$ was characterized further.

Microscopic observations revealed that cells of strain $6N^T$ were rod-shaped, 5–6 µm long and 0·35–0·50 µm wide and occurred mainly singly (Fig. 1a). They were motile with one polar flagellum (Fig. 1b). Sporulation was never observed. Electron microscopy of ultrathin sections of cells indicated the presence of a thin tripartite cell wall analogous to an outer membrane covering a clear periplasm (Fig. 1c). Strain $6N^T$ was strictly anaerobic, growing optimally in basal SRB medium containing H_2+CO_2 and sulfate at 45 °C (temperature growth range between 30 and 60 °C) and pH 7·0. It was slightly halophilic, growing optimally in the presence of 2 % (w/v) NaCl, the upper limit for growth being 5 % NaCl. Under optimal growth conditions, the mean doubling time was about 12 h. Strain $6N^T$ used only H_2+CO_2 in the presence of sulfate as electron acceptor and acetate as carbon source. Growth on formate and acetate (carbon source) was only obtained when NaCl, KCl, $MgCl_2$ and $CaCl_2$ were replaced by sea salts (30 g l^{-1}). No growth was observed on the following substrates using sulfate as electron acceptor: lactate, fumarate, malate, succinate, glycerol, acetate, propionate, butyrate, methanol, ethanol, fructose, glucose, mannose, rhamnose, sucrose, choline, Casamino acids, yeast extract and bio-Trypticase. Sulfate, thiosulfate, sulfite and elemental sulfur served as electron acceptors.

It is clear from ecological, molecular ecological and microbiological studies that sulfate-reducers, together with sulfur-reducers, play an important role in the hydrogen and/or sulfur metabolism within deep-sea hydrothermal-vent environments (Jeanthon, 2000). Although the biodiversity of sulfur-reducers has been widely studied in these deep ecosystems, there are few reports on the isolation and characterization of sulfate- and/or sulfite-reducers (Alazard *et al.*, 2003; Burggraf *et al.*, 1990; Elsgaard *et al.*, 1995; Huber *et al.*, 1997; Jeanthon *et al.*, 2002; Rueter *et al.*, 1994). Here, we describe a novel thermophilic sulfate-reducer (strain

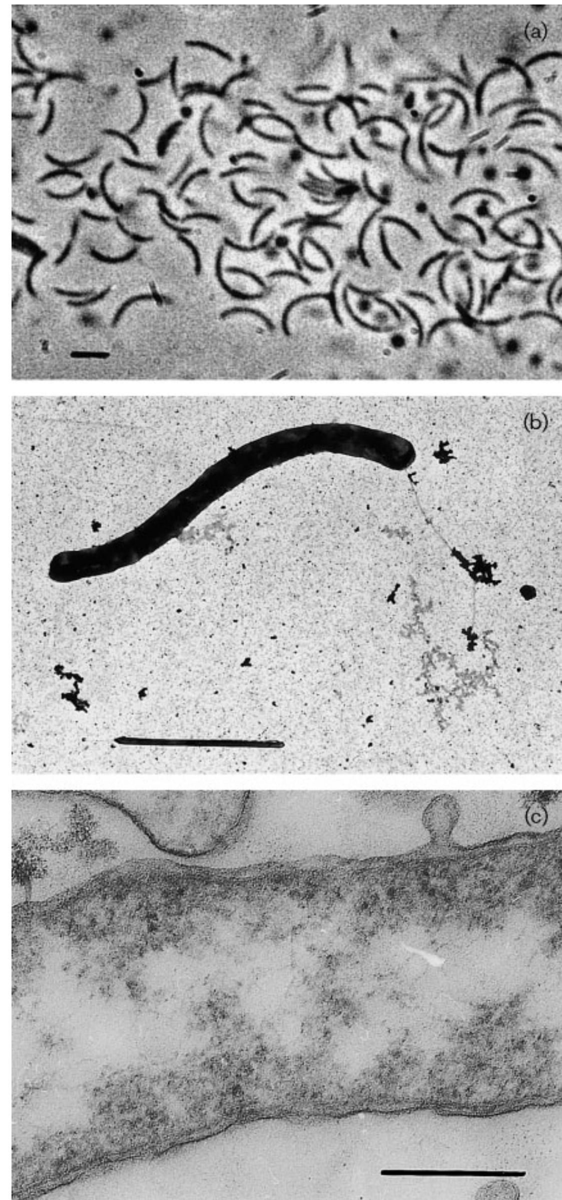

Fig. 1. (a)–(b) Phase-contrast photomicrograph (a) and electron transmission micrograph (b) of strain $6N^T$ grown with hydrogen as an energy source. Bars, 2 µm. (c) Electron micrograph of an ultrathin section of a cell of strain $6N^T$ showing the multilayered cell wall. Bar, 0·2 µm.

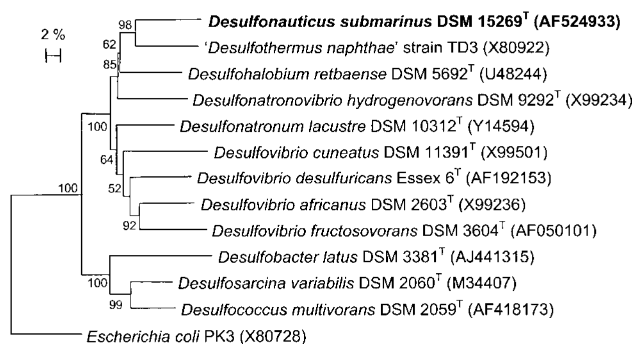

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparison indicating the position of strain 6N^T amongst the closest members of the family *Desulfohalobiaceae* and genera *Desulfomicrobium* and *Desulfovibrio*. Sequence accession numbers are given in parentheses. Bootstrap values, expressed as percentages of 100 replications, are shown at branching points. The 16S rRNA gene of *Escherichia coli* PK3 was used as the outgroup. Bar, 2 substitutions per 100 nucleotides.

6N^T), isolated from various matrixes of *Alvinella* and *Riftia*. Analysis of the almost complete sequence (1111 bp) of the 16S rRNA gene of strain 6N^T revealed that this novel isolate groups with members of the family *Desulfohalobiaceae* (Kuever *et al.*, 2003) in the δ-subclass of the *Proteobacteria* and is related to *Desulfonatronovibrio hydrogenovorans* (similarity 86·5 %) (Fig. 2). The G+C content of DNA of strain 6N^T was 34·4 mol%. The phylogeny derived from 428 amino acids of DSR was congruent with the phylogeny derived from the 16S rRNA gene and supported the phylogenetic position of strain 6N^T within the family *Desulfohalobiaceae*, its closest relative being *Desulfonatronovibrio hydrogenovorans* (Fig. 3). This family comprises the genera *Desulfohalobium* (*Desulfohalobium retbaense*) (Ollivier *et al.*, 1991), *Desulfonatronovibrio* (*Desulfonatronovibrio hydrogenovorans*) (Zhilina *et al.*, 1997) and '*Desulfothermus*' ('*Desulfothermus naphthae*') (Kuever *et al.*, 2003; Rueter *et al.*, 1994). In contrast to strain 6N^T, '*Desulfothermus naphthae*', the only thermophilic member of the family *Desulfohalobiaceae*, is unable to use hydrogen and performs the complete oxidation of organic substrates (hydrocarbons). *Desulfohalobium retbaense* differed markedly from strain 6N^T by (i) using not only hydrogen but also lactate and ethanol and (ii) growing in a moderate range of salinity. Finally, *Desulfonatronovibrio hydrogenovorans* is a non-halophilic, mesophilic, sulfate-reducing bacterium that grows optimally in alkaline conditions. In this respect, there are numerous phenotypic, genomic and phylogenetic differences (Table 1) between strain 6N^T and members of family *Desulfohalobiaceae* that warrant its assignment to a new genus of the family.

Studies on the diversity of DSR genes in the bacterial community associated with the back of *Alvinella pompejana* suggested a prominent role for anaerobic sulfate-reducing bacteria in the ecology of this worm (Cottrell & Cary, 1999). The DSR genes of *Desulfovibrio gigas*, *Desulfobacterium autotrophicum* and *Desulfobacter latus* were found to be the closest relatives of the clones analysed (Cottrell & Cary, 1999). DSR genes similar to that of strain 6N^T were not recovered from these molecular studies, suggesting that the isolate might inhabit only the matrix of *Alvinella*. Interestingly, strain 6N^T, isolated from various matrixes of *Alvinella* but also of *Riftia* on the 13°N East-Pacific Rise, uses mainly hydrogen as an energy source (formate was poorly used), thus suggesting that its metabolic role in hydrothermal vents is probably limited to the oxidation of this gaseous compound, which can be (i) produced by several deep mineral reactions (Devereux & Stahl, 1993; Stetter *et al.*, 1993) and (ii) emitted from smokers. Such hydrogenotrophic activity renders strain 6N^T an important protagonist within the biogeochemistry but also in detoxification processes within deep-sea hydrothermal vents. In contrast to the recently isolated *Thermodesulfobacterium hydrogeniphilum* (Jeanthon *et al.*, 2002),

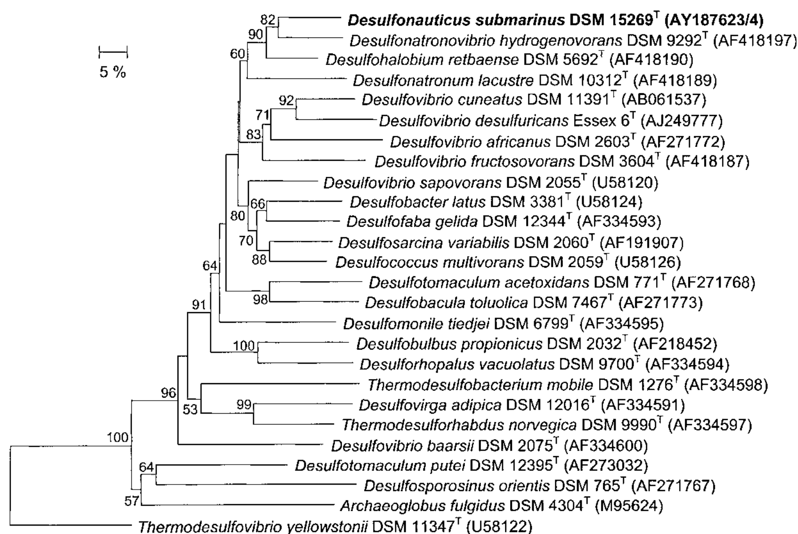

Fig. 3. Phylogenetic tree based on comparison of deduced DSR amino acid sequences indicating the position of strain 6N^T amongst the closest members of the family *Desulfohalobiaceae* and genera *Desulfomicrobium* and *Desulfovibrio*. Nucleotide sequence accession numbers from which the amino acid sequences were deduced are given in parentheses. Bootstrap values, expressed as percentages of 100 replications, are shown at branching points. The DSR amino acid sequence of *Thermodesulfovibrio yellowstonii* DSM 11347^T was used as the outgroup. Bar, 5 substitutions per 100 amino acids.

Table 1. Physiological characteristics of strain 6N^T and its closest relatives in the family *Desulfohalobiaceae*

Taxa: 1, strain 6N^T; 2, *Desulfonatronovibrio hydrogenovorans* (data from Zhilina *et al.*, 1997); 3, *Desulfohalobium retbaense* (Ollivier *et al.*, 1991); 4, '*Desulfothermus naphthae*' (Kuever *et al.*, 2003). ND, Not determined.

Characteristic	1	2	3	4
Source	Hydrothermal vent	Alkaline lake	Saline lake	Marine sediment
Temperature for growth (°C):				
Range	30–60	15–43	25–45	50–69
Optimum	45	37	37–40	60–65
NaCl concentration for growth (%):				
Range	0–5	1–12	3–24	ND
Optimum	2	3	10	2
Reduction of S^0	+	−	+	ND
Substrates used	H_2+CO_2	H_2+CO_2, formate	Lactate, pyruvate, ethanol	Alkanes (C_6–C_{14}), fatty acids (C_4–C_{18})
G+C content (mol%)	34·4	48·6	57	37·4

strain 6N^T also shows its perfect adaptation to the physico-chemical conditions prevailing in deep-sea hydrothermal environments through its use of not only sulfate, but also thiosulfate, sulfite and S^0 as terminal electron acceptors, these sulfur compounds being commonly found in hydrothermal fluids (Jannasch & Mottl, 1985). As noted above, because of its significant phenotypic, genotypic and phylogenetic characteristics, we propose the assignment of strain 6N^T to a new genus and species, *Desulfonauticus submarinus* gen. nov., sp. nov.

Description of *Desulfonauticus* gen. nov.

Desulfonauticus (De.sul'fo.nau'ti.cus. L. pref. *de* from; L. n. *sulfur* sulfur; N.L. pref. *Desulfo-* desulfuricating, use to characterize a dissimilatory sulfate-reducing prokaryote; L. adj. *nauticus* nautical; N.L. masc. n. *Desulfonauticus* a marine sulfate-reducer).

Cells are curved rods, motile with one polar flagellum, Gram-negative. Moderate thermophile, neutrophile and slightly halotolerant. In the presence of sulfate, only hydrogen plus acetate (carbon source) serve as growth substrates. Sulfate, thiosulfate, sulfite and elemental sulfur are utilized as electron acceptors. The G+C content of DNA of the type strain of the type species is 34 mol% as determined by HPLC. The type species is *Desulfonauticus submarinus.*

Description of *Desulfonauticus submarinus* sp. nov.

Desulfonauticus submarinus (sub.ma'ri.nus. L. pref. *sub-* under; L. adj. *marinus* marine; N.L. adj. *submarinus* from a submarine area).

Cells are curved rods (5–6 × 0·35–0·50 μm), motile with one polar flagellum, Gram-negative. The temperature range for growth is 30–60 °C, the optimum being 45 °C. The optimum pH is 7·0. The optimum NaCl concentration for growth is 2 % (range 0–5 %). In the presence of sulfate, hydrogen plus acetate (carbon source) serve as growth substrate. Formate plus acetate (carbon source) used slowly when $MgCl_2$, $CaCl_2$, KCl and NaCl are replaced by sea salts (30 g l^{-1}). Lactate, fumarate, malate, succinate, glycerol, acetate, propionate, butyrate, methanol, ethanol, fructose, glucose, mannose, rhamnose, sucrose, choline, Casamino acids, yeast extract and bio-Trypticase are not used. Sulfate, thiosulfate, sulfite and elemental sulfur are utilized as electron acceptors. The G+C content of DNA of the type strain is 34·4 mol%, as determined by HPLC.

The type strain, 6N^T (=DSM 15269^T=CIP 107713^T), was isolated from matrixes of *Riftia* and *Alvinella* in the 13°N East-Pacific Rise at a depth of 2600 m.

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