Note

Desulfovibrio hydrothermalis sp. nov., a novel sulfate-reducing bacterium isolated from hydrothermal vents

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Mesophilic, hydrogenotrophic, sulfate-reducing bacteria were isolated from a deep-sea hydrothermal chimney sample collected at 13° N on the East-Pacific Rise at a depth of 2600 m. Two strains (BL5 and H9) were found to be phylogenetically similar to *Desulfovibrio profundus* (similarity >99 %), whereas two other strains (H1 and AM13^T) were found to be phylogenetically distinct (similarity 96.4 %) from *Desulfovibrio zosteræ*, their closest relative. Strain AM13^T was characterized further. It was a barophilic, Gram-negative, non-sporulating, motile, vibrio-shaped or sigmoid bacterium possessing desulfovibrin. It grew at temperatures ranging from 20 to 40 °C, with an optimum at 35 °C in the presence of 2.5 % NaCl. The pH range for growth was 6.7–8.2 with an optimum around 7.8. Strain AM13^T utilized H₂/CO₂, lactate, formate, ethanol, choline and glycerol as electron donors. Electron acceptors were sulfate, sulfite and thiosulfate, but not elemental sulfur or nitrate. The G + C content of DNA was 47 mol%. Strain AM13^T (=DSM 14728^T =CIP107303^T) differed from *D. zosteræ* not only phylogenetically, but also genomically (DNA–DNA reassociation value between the two bacteria was 23.8 %) and phenotypically. This isolate is therefore proposed as the type strain of a novel species of the genus *Desulfovibrio*, *Desulfovibrio hydrothermalis* sp. nov.

Deep-sea hydrothermal vents are among the most productive ecosystems on Earth, where unusual animal and microbial communities are able to survive the harsh combinations of toxic chemicals, high pressures, high temperatures and total darkness. This high biomass productivity is based largely on the activity of ecto- and endosymbiotic associations of chemolithotrophic micro-organisms and vent fauna (Jeanthon, 2000; Polz & Cavanaugh, 1995). Microbial hydrothermal vent communities also include free-living bacteria associated with the discharged vent fluids, free-living microbial mats and micro-organisms within the deep-sea hydrothermal vent plume (Karl, 1995). Although increasing attention has been paid to the thermophilic and hyperthermophilic micro-organisms that inhabit deep-sea hydrothermal vents, because of their high biotechnological potential, mesophilic and psychophilic micro-organisms are known to be significant components of the microbial community along the physico-chemical gradient of the vent ecosystem

(Jeanthon, 2000). The occurrence of mesophilic sulfate-reducing bacteria (SRB) was made evident by (i) significant sulfate-reduction activities in vent sediments (Elsgaard *et al.*, 1994; Jørgensen *et al.*, 1992) and (ii) their isolation from various samples collected at different hydrothermal sites (Elsgaard *et al.*, 1995). However, none of them has yet been characterized.

Here, we report the isolation from deep-sea hydrothermal chimneys of SRB that are phylogenetically similar to *Desulfovibrio profundus* (Bale *et al.*, 1997) and the characterization of a novel mesophilic, barophilic species (strain AM13^T) of the genus *Desulfovibrio*, *Desulfovibrio hydrothermalis* sp. nov.

Strains BL5, H9, H1 and AM13^T were isolated from a deep-sea hydrothermal chimney sample stored in sea water at 4 °C until processing. The samples were collected by the deep-submergence vehicle *Nautil* in June 1999 from the Grandbonum vent site at latitude 13° N along the East-Pacific Rise during the AMISTAD cruise at a depth of 2600 m. *Desulfovibrio zosteræ* DSM 11974^T and *D. profundus* DSM 11384^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen

Abbreviation: SRB, sulfate-reducing bacteria.

The GenBank accession number for the 16S rDNA sequence of strain AM13^T is AF458778.

(DSMZ), Braunschweig, Germany. Enrichment and isolation were performed using SRB growth medium containing the following (l^{-1} distilled water): NH_4Cl , 1.0 g; K_2HPO_4 , 0.3 g; KH_2PO_4 , 0.3 g; $MgCl_2 \cdot 6H_2O$, 3.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $NaCl$, 25 g; KCl , 0.2 g; Na_2SO_4 , 3.16 g; sodium acetate, 0.5 g; sodium lactate, 2.24 g; cysteine hydrochloride, 0.5 g; yeast extract (Difco Laboratories), 0.5 g; bio-Trypticase (bioMérieux), 0.5 g; trace mineral element solution (Balch *et al.*, 1979), 10 ml; resazurin, 1.0 mg. 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 of 5 or 20 ml were then dispensed respectively into Hungate tubes or serum bottles, under a stream of N_2/CO_2 gas (80:20, v/v), and the vessels were autoclaved for 45 min at 110 °C. Prior to inoculation, $Na_2S \cdot 9H_2O$ and $NaHCO_3$ were injected from sterile stock solutions to obtain respective final concentrations of 0.04% (w/v) and 0.2% (w/v). A chimney sample was inoculated in 20 ml SRB medium and incubated at 35 °C without agitation to initiate an enrichment culture. The culture was purified by repeated use of the roll-tube method (Hungate, 1969) with medium solidified with 2% (w/v) agar (Difco). Several colonies that developed 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 using SRB medium according to Hernandez-Eugenio *et al.* (2000). Substrates were tested at a final concentration of 20 mM in SRB medium. To test for electron acceptors, sodium thiosulfate, sodium sulfate, sodium sulfite, elemental sulfur and nitrate were respectively added to the medium at final concentrations of 20 mM, 20 mM, 2 mM, 2% (w/v) and 10 mM. For disproportionation studies, SRB medium without lactate but containing 20 mM thiosulfate and 2 mM acetate was used. Disproportionation was verified by the formation of sulfate (Madsen & Aamand, 1991) and sulfide (Cord-Ruwisch, 1985). Growth and product formation were analysed after 2 weeks of incubation at 35 °C. Cells of strain AM13^T from the early stationary phase of growth were used for pressurization tests. The culture broth was diluted in 20 ml tubes containing sterile medium to obtain an initial OD_{600} of 0.05. Half of the tubes were incubated at 260 atm hydrostatic pressure at 37 °C and the other half were incubated at 1 atm at 37 °C. At the appropriate times, three tubes from each pressure condition were removed to monitor the OD_{600} of the two cultures. Phase-contrast microscopy (model Eclipse E600; Nikon) was used for routine examinations of the cultures and to obtain photomicrographs. Thin sections for electron microscopy were prepared as described by Fardeau *et al.* (1997). Electron micrographs 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 the method of Cord-Ruwisch (1985). Fermentation products were determined as described by Fardeau *et al.* (1993). Desulfovibridin was determined as described by Postgate (1959). The G+C content of DNA was determined at the DSMZ using HPLC as described previously (Hernandez-Eugenio *et al.*, 2000). DNA–DNA hybridization studies were also performed at the DSMZ (Chamkha *et al.*, 2001). The methods for purification and extraction of DNA and the amplification and sequencing of the 16S rRNA gene have been described previously (Andrews & Patel, 1996). The sequencing reaction was performed by PCR amplification in a final volume of 20 μ l using 100 ng PCR products (or 500 ng plasmid), 5 pmol primer and 8 μ l BigDye Terminator premix according to the Applied Biosystems protocol. After heating to 96 °C for 3 min, the reaction was subjected to 30 cycles of 30 s at 96 °C, 30 s at 55 °C and 4 min at 60 °C (Perkin Elmer 9700 thermal cycler). Removal of excess BigDye Terminators was performed by using exclusion columns. The samples were dried in a vacuum centrifuge and dissolved in 1.6 μ l deionized formamide/EDTA pH 8.0 (5:1). The samples were loaded onto an Applied Biosystems 373XL sequencer and run for 12 h on a 4.5% denaturing acrylamide gel. The 16S rRNA gene sequence was 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 1190 unambiguous nucleotides were computed by using the method of Jukes & Cantor (1969). Dendrograms were constructed by using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined by using bootstrapped trees (Felsenstein, 1985).

Sulfate-reducing enrichment cultures were obtained after 4 days incubation at 35 °C. Microscopic observations revealed the presence of motile, vibrio-shaped bacteria. The enrichment was subcultured in Hungate roll tubes. Single, brown, discus-shaped colonies (1–2 mm diameter) that developed after 7 days incubation at 35 °C were picked and serially diluted in roll tubes before the culture was considered pure. Four strains (BL5, H9, H1 and AM13^T) were isolated. The purity of these strains was confirmed by the morphological homogeneity of cells 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. Strains BL5 and H9 were found to be phylogenetically similar to *D. profundus*, whereas strains H1 and AM13^T were phylogenetically distinct (see details below) from *D. zosteriae*. Strain AM13^T was characterized further. Microscopic observations revealed that the cells of strain AM13^T were motile, Gram-negative, vibrio-shaped or sigmoid and

Fig. 1. (a) Phase-contrast photomicrograph of strain AM13^T grown with lactate as a carbon and energy source. Bar, 5 μ m. (b) Electron micrograph of an ultrathin section of a cell of strain AM13^T, showing the multilayered cell wall. Bar, 0.2 μ m.

occurred mainly singly (Fig. 1a). Cells were 3–5 μ m long and 0.5–1.2 μ m wide. Sporulation was never observed. Electron microscopy of ultrathin sections of cells indicated the presence of a multilayered Gram-negative type of cell envelope, as reported for members of the genus *Desulfovibrio* (Fig. 1b).

Strain AM13^T was strictly anaerobic, growing optimally in basal SRB medium containing lactate and sulfate at 35 $^{\circ}$ C (temperature range for growth; 20–40 $^{\circ}$ C). The pH optimum was 7.8; strain AM13^T grew in a pH range between 6.2 and 8.4. It was slightly halophilic, growing optimally in the presence of 2.5% (w/v) NaCl; the upper NaCl concentration for growth was 4%. Under optimal conditions of growth, the mean doubling time was about 15 h. Cells grew faster at 260 atm than at 1 atm, suggesting that AM13^T was barophilic (Fig. 2). Strain AM13^T used hydrogen, formate, lactate, fumarate, malate, pyruvate, ethanol, glycerol and choline in the presence of sulfate as an electron acceptor. Lactate was oxidized incompletely to acetate, CO₂ and H₂S. H₂ and formate were used only in the

Fig. 2. Effect of hydrostatic pressure on growth of strain AM13^T. Growth was monitored as OD₆₀₀ at atmospheric pressure (●) and 260 atm (■).

presence of acetate as a carbon source. No growth was observed on the following substrates using sulfate as electron acceptor: acetate, succinate, propionate, butyrate, benzoate, methanol, 1-propanol, fructose, xylose, glucose and alanine. Pyruvate was fermented to acetate, hydrogen and CO₂ in sulfate-free medium. Sulfate, thiosulfate and sulfite, but not elemental sulfur or nitrate, served as electron acceptors in the presence of lactate as energy and carbon source.

Phylogenetic analysis revealed that strains BL5 and H9 were similar to *D. profundus* (similarity > 99%), whereas strains H1 and AM13^T were distinct from *D. zosteræ* (similarity < 97%) as their closest relative. Analysis of the almost complete sequence (1535 bp) of the 16S rRNA gene of strain AM13^T revealed that this novel isolate groups with the members of the genus *Desulfovibrio* in the δ -Proteobacteria and is related to *D. zosteræ* (similarity 96.4%) (Fig. 3). The G+C content of DNA of strain AM13^T was 47 mol%. DNA–DNA hybridization studies indicated that strain AM13^T and *D. zosteræ* have an overall relatedness value

of 23·8 %, whereas strain BL5 and *D. profundus* have an overall relatedness value of 68·7 %.

This is the first report of the characterization of a mesophilic sulfate reducer of the domain *Bacteria* that originates from deep-sea hydrothermal vents. Most microbial studies within such environments have focussed on thermophiles and particularly hyperthermophiles that reduce elemental sulfur and belong to the domain *Archaea* (Jeanthon, 2000). Within this domain, only one thermophilic sulfate-reducing archaeon, *Archaeoglobus profundus*, has been isolated at great depths (Burggraf *et al.*, 1990). Despite the great interest of scientists in studying thermophilic or hyperthermophilic micro-organisms, it is generally admitted that mesophiles, as well as moderate thermophiles, may represent the most abundant microbes in hydrothermal vents (Campbell *et al.*, 2001; Jeanthon, 2000). This is exemplified by the isolation of (i) metabolically diverse non-filamentous mesophilic bacteria from the dorsal integument of *Alvinella pompejana* (Prieur & Jeanthon, 1987; Prieur *et al.*, 1990; Raguénès *et al.*, 1996), (ii) several mesophilic sulfur-oxidizing bacteria (Durand *et al.*, 1993; Jannasch *et al.*, 1985; Ruby & Jannasch, 1982; Wirsén *et al.*, 1998) and (iii) moderate thermophiles belonging to the ϵ -*Proteobacteria* (Campbell *et al.*, 2001). Sulfate reduction activity was evident in sediments from Guaymas Basin (Elsgaard *et al.*, 1994; Jørgensen *et al.*, 1992) and successful isolation was achieved from various samples collected at different marine hydrothermal environments, thus suggesting the probable ecological significance of SRB in the biogeochemistry of such environments. Despite this ecological importance, only one thermophilic and no mesophilic SRB have been fully characterized (Burggraf *et al.*, 1990).

In this paper, we report (i) the characterization of a novel species of the genus *Desulfovibrio*, strain AM13^T, and (ii) the isolation of sulfate reducers that are phylogenetically and genotypically similar to *D. profundus*, which originated from deep sediment layers in the Japan Sea (Bale *et al.*, 1997). The

isolation of *D. profundus*-like micro-organisms from deep-sea hydrothermal vents (chimney rocks) suggests that this species is ecologically significant within deep-sea environments, being distributed not only in deep marine sediments (Bale *et al.*, 1997), but also at the surface of these sediments (this report). Similarly to the *D. profundus*-like micro-organisms isolated during this work, the sulfate-reducing strain AM13^T was also isolated from a deep-sea hydrothermal chimney sample, but it differs greatly phylogenetically from *D. profundus* (similarity 87·8 %). Phenotypic and genotypic differences are also observed between *D. profundus* and strain AM13^T. They include the G+C content of the DNA and the range of temperature for growth (Table 1).

Phylogenetic studies confirm the assignment of strain AM13^T within the genus *Desulfovibrio*, *D. zosteræ* being its closest phylogenetic relative (similarity 96·4 %). Similarly to *D. zosteræ*, strain AM13^T grows optimally in the presence of NaCl, and this reflects the marine origin of both micro-organisms. However, in contrast to strain AM13^T, considered a slight halophile, *D. zosteræ* does not require NaCl for growth and is therefore halotolerant (Nielsen *et al.*, 1999). There are also other phenotypic differences between the two bacteria (Table 1); in particular, the inability of strain AM13^T to oxidize sugars. Genomic differences were also observed when performing DNA–DNA hybridization, since a low value of DNA relatedness (23·8 %) was found between strain AM13^T and *D. zosteræ*, thus confirming that strain AM13^T is a novel species of the genus *Desulfovibrio* (Wayne *et al.*, 1987). An important physiological feature of this novel species is that it is barophilic, having a higher growth rate at a hydrostatic pressure of 260 atm, which corresponds to the pressure from its extracted environment, than at atmospheric pressure. This provides evidence that the novel species, like *D. profundus*, originates from deep environments. Because of the significant phenotypic, genotypic and

Table 1. Discriminating characteristics of strain AM13^T, *D. zosteræ* and *D. profundus*

Data for reference species were taken from Nielsen *et al.* (1999) (*D. zosteræ*) and Bale *et al.* (1997) (*D. profundus*). ND, Not determined; +, growth; (+), utilization without growth; ±, poorly utilized.

Characteristic	Strain AM13 ^T	<i>D. zosteræ</i>	<i>D. profundus</i>
Temperature range for growth (°C)	20–40	5–34·5	15–65
Electron donors (using sulfate):			
Formate + acetate (2 mM)	+	(+)	ND
Glycerol	+	ND	ND
Fermentation of:			
Fructose	–	+	–
Fumarate	±	+	–
Electron acceptors:			
Elemental sulfur	–	+	–
Nitrate	–	–	+
DNA G+C content (mol%)	47	42·7	53

phylogenetic differences between *D. zosteriae* and strain AM13^T, the latter can be assigned to a novel species of the genus *Desulfovibrio*, for which the name *Desulfovibrio hydrothermalis* sp. nov. is proposed.

Description of *Desulfovibrio hydrothermalis* sp. nov.

Desulfovibrio hydrothermalis (hy.dro.ther.ma'lis. N.L. adj. *hydrothermalis* from a hydrothermal area).

Cells are motile, Gram-negative, vibrio-shaped or sigmoid (0.5–1 × 1–2 µm). The temperature range for growth is 20–40 °C, the optimum being 35 °C. The optimum pH for growth is 7.8. In the presence of sulfate, hydrogen plus acetate (carbon source), formate plus acetate (carbon source), lactate, fumarate, malate, pyruvate, ethanol, glycerol and choline serve as growth substrates. Fermentative growth occurs on pyruvate. Sulfate, thiosulfate and sulfite, but not elemental sulfur or nitrate, are utilized as electron acceptors. Oxidation of lactate with sulfate is incomplete, with acetate, CO₂ and H₂S as the end products of metabolism. The G+C content of the DNA is 47 ± 0.5 mol% as determined by HPLC. The GenBank accession number for the 16S rRNA gene sequence of strain AM13^T is AF458778. Isolated from a hydrothermal chimney at a depth of 2600 m at 13° N from the East-Pacific Rise. The type strain is strain AM13^T (=DSM 14728^T =CIP 107303^T).

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