**Desulfovibrio profundus** sp. nov., a Novel Barophilic Sulfate-Reducing Bacterium from Deep Sediment Layers in the Japan Sea

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Several strains of a strictly anaerobic, vibrio-shaped or sigmoid, sulfate-reducing bacterium were isolated from deep marine sediments (depth, 80 and 500 m) obtained from the Japan Sea (Ocean Drilling Program Leg 128, site 798B). This bacterium was identified as a member of the genus *Desulfovibrio* on the basis of the presence of desulfovibridin and characteristic phospholipid fatty acids (iso 17:1ω7 and iso 15:0), the small number of growth substrates utilized (lactate, pyruvate, and hydrogen), and 16S rRNA gene sequence analysis data. Based on data for 16S rRNA sequences (1,369 bp), all of the Japan Sea strains were identical to each other and were most closely related to *Desulfovibrio salexigens* and less closely related to *Desulfovibrio desulfuricans* (levels of similarity, 91 and 89.6%, respectively). There were, however, considerable phenotypic differences (in temperatures, pressures, and salinities tolerated, growth substrates, and electron donors) between the Japan Sea isolates and the type strains of previously described desulfovibriads, as well as important differences among the Japan Sea isolates. The Japan Sea isolates were active (with sulfide production) over a wide temperature range (15 to 65°C) and a wide sodium chloride concentration range (0.2 to 10%) (moderate halophile), and they were barophiles that were active at pressures up to about 40 MPa (400 atm). The optimum pressures for activity corresponded to the calculated pressures at the depths from which the organisms were isolated (for isolates obtained at depths of 80 and 500 m the optimum activities occurred at 10 and 15 MPa, respectively [100 and 150 atm, respectively]). This confirms that the organisms came from deep sediments and indicates that they are well-adapted for deep sediment conditions, which is consistent with other characteristics (utilization of hydrogen, fermentation, and utilization of ferric iron and organic sulfonates as electron acceptors). We propose that Japan Sea isolate 500-1 is the type strain of a new species, *Desulfovibrio profundus*.

**MATERIALS AND METHODS**

**Sample collection.** Sediment samples were collected from Ocean Drilling Program Site 798B in the Japan Sea (water depth, 900 m). The deepest sediment obtained was sediment from 518 m below the seafloor. Whole round core samples were cut from intact cores under sterile anaerobic conditions (9) and were transported anaerobically (8) at 4°C back to a laboratory for analysis a few weeks later. Full details concerning the subsequent handling of the samples have been published elsewhere (9, 31). Briefly, 5-ml subcores were removed under sterile anaerobic conditions from the centers of whole round cores and serially diluted prior to injection into most-probable-number (MPN) vials containing modified Postgate's sulfate-reducing bacterial medium supplemented with either acetate or lactate and adjusted to in situ levels of salinity. The medium contained (per liter) 0.5 g of KH$_2$PO$_4$, 1.0 g of NH$_4$Cl, 1.0 g of CaSO$_4$, 5.0 g of MgSO$_4$·7H$_2$O, 3.5 g of sodium lactate (70% [vol/vol] solution) or 1.8 g of sodium acetate (anhydrous). 1.0 g of yeast extract, 0.1 g of thiglycolic acid, 0.5 g of FeSO$_4$·7H$_2$O, 32.0 g of NaCl, 5.0 g of MgCl$_2$·6H$_2$O, and 1.0 ml of resazurin (0.1%). The pH was adjusted to 7.2, and the medium was autoclaved at 121°C and then cooled under 90% N$_2$-10% CO$_2$. When the medium was cool, 30 ml of a 1 M bicarbonate solution was added, the pH was adjusted to 7.5 with Na$_2$CO$_3$·HCl, and the medium was dispensed under N$_2$-CO$_2$ into sterile, evacuated, 7-ml crimp top vials with butyl septa. The vials were incubated at 6°C, the mean temperature of the core from the sediment surface to a depth of 518 m.

**Enrichment and isolation of pure cultures.** To increase growth rates, the incubation temperature for subcultures obtained from positive MPN vials was increased to 25°C. Once stable enrichment cultures had been established from samples obtained from 2, 10, 80, and 500 m below the seafloor, subsequent enrichment cultures were grown in both Postgate's medium and the better-defined Widdel's medium (46) (with NaCl and MgCl$_2$·6H$_2$O concentrations adjusted as described above). These cultures were inoculated into agar shake dilution series containing different growth substrates (31), and when individual black colonies developed, they were inoculated into liquid Widdel's medium containing the same substrate. This process was repeated until pure cultures were obtained. Purity was assessed by observing a uniform cell type, Gram staining, a lack of aerobic growth, and a lack of growth on a range of complex substrates under anaerobic conditions and by analyzing 16S rRNA gene libraries from putative pure cultures.

**Physiological characterization.** The characteristics of Japan Sea isolates were compared with the characteristics of the type strains of *Desulfovibrio desulfuri- cursus* (DSM 642) and *Desulfovibrio salexigens* (DSM 2638) obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig.

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Germany. The type strains were grown in the medium recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen. All cultures were routinely incubated in Widdel's medium at 25°C and 0.1 MPa (1 atm). As growth in this medium was accompanied by production of an iron sulfide precipitate and a visible turbidity was detectable (e.g., when lignosulfonate and dimethyl sulfoxide were used). The presence of bisulfite reductase (desulfoviridin) was determined by adding NaOH to a resuspended pellet of cells and viewing the preparation under UV light as described by Postgate (35).

**Sodium chloride and pH ranges for growth.** Media containing NaCl at concentrations ranging from 0 to 14% were prepared by adding different volumes of a sterile stock solution of NaCl (35%) to NaCl-free medium. Media with pH values ranging from 3.5 to 9 were prepared as described by Widdel and Bark (46).

**Bile tolerance.** Resistance to bile, a known characteristic of *D. autotrophicus* (40), was determined by incubating cultures in standard growth medium containing bile at concentrations ranging from 0.01 to 1 g/liter.

**Substrate removal and fermentation products.** Volatile fatty acid concentrations in culture media were determined by ion exclusion chromatography (Di-ones, Sunnyvale, Calif.) as described by Wellsbury et al. (45). Samples were removed anaerobically from growing cultures and were filtered through a 0.1-μm-pore-size Anotop filter (Whatman, Maidstone, United Kingdom) prior to analysis.

**Analysis.** The presence of bisulfite reductase (desulfoviridin) was determined by adding NaOH to a resuspended pellet of cells and viewing the preparation under UV light as described by Postgate (35).

**DNA base composition.** DNA was purified and digested by the method of Ausubel et al. (1). The G+C content was determined by the method of Tamaoka and Komagata (42). DNA was treated with P1 nuclease and alkaline phosphatase, and the nucleotides were separated by high-performance liquid chromatography by using a model System Gold separations (Beckman) equipped with an UltraspHERE C18, 3 μm column. DNA from the following three bacteria were used as standards: *Clostridium pasteurianum*, *E. coli* and *B. subtilis*.

**Whole-cell protein profiles.** Total cellular proteins were extracted from 10 ml portions of pure cultures grown in Widdel's medium. A pellet of each pure culture was resuspended in 200 μl of sterile H2O, 225 μl of sample buffer (0.125 M Tris.HCl [pH 6.8], 20% glycerol, 4% sodium dodecyl sulfate, 0.05% bromophenol blue) was added, and then 25 μl of 14.3 M mercaptoethanol was added. The preparations were incubated at 100°C for 5 min, centrifuged at 15,000 × g for 5 min, and stored at −20°C until they were needed. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis essentially as described by Laemmli (24). Electrophoresis was performed with a stacking gel containing 4% polyacrylamide and a resolving gel containing 12.5% polyacrylamide. Each gel was silver stained by using a modified method of Morrissey (28).

**Ribotyping and hybridization.** Genomic DNA were isolated from strains 80-55 and 509-1T (T = type strain) by the method of Ausubel et al. (1) and were digested with restriction endonucleases EcoRI and PstI as recommended by the manufacturer (Boehringer Mannheim). DNA fragments were separated by electrophoresis on 0.7% agarose gels with Tris-borate-EDTA buffer at 10 V/cm. The gels were exposed to Kodak X-OMAT X-ray photographic film and analyzed by the method of Southern (39), and 16S rRNA gene sequences were amplified by the PCR as described previously (37, 38). The PCR primers used for 16S rRNA gene amplification were based on the primers described by Lane (25), with slight modifications; the primers used were primers 27F (5′-GTG CTG CAG AGA TTT TGA TCC TGG-3′) (the 3′ end of the primer annealed), followed by a 45°C annealing temperature and a 15 s extension time, and a PCR cycle number of 26. The PCR products were ligated into vector pCRII and used to transform *E. coli* INVaF with a TA cloning kit (Invitrogen) as recommended by the manufacturer. Cloned 16S rRNA genes were sequenced by using a restriction fragment length polymorphism analysis of the whole genome insert of 16S rRNA gene as previously described by the DNAsIS suite of programs (Hitachi). Sequences were aligned for the phylogenetic analysis with the Clustal V program (18). Additional analyses were
performed with the PHYLIP suite of programs (15) by using data and services provided by the Ribosomal Database Project (University of Illinois, Urbana-Champaign) (26) and the Antwerp Database (29). A phylogenetic tree was constructed by using TREECON software (44).

Phospholipid fatty acid (PLFA) analysis. Freeze-dried bacterial cells were extracted by using a modified Bligh-Dyer monophasic solvent system with phosphate buffer (2, 17). Phospholipids were isolated from the total lipid extracts by column chromatography (aminopropyl-phase bonded silica cartridges) and were released as the methyl esters after mild alkaline methanolysis (17) prior to identification by gas chromatography and gas chromatography-mass spectrometry. Positions of unsaturation were determined by the formation of dimethyl disulfide adducts (47).

Nucleotide sequence accession number. The 16S rRNA gene sequence of Desulfovibrio profundus sp. nov. has been deposited in the GenBank database under accession number U90726.

RESULTS

Enrichment and isolation. Enrichment in Postgate's medium required long incubation times (in some cases more than 9 months) in order to obtain stable, sulfide-producing cultures. An unavoidable 18-month delay between inoculation of the initial MPN vials and subsequent processing may have contributed to this. Cultures were obtained from the 2-, 10-, 80-, and 500-m samples, and these cultures grew on a range of substrates in agar shake preparations (31), although long incubation times (3 to 4 months) were required for some substrates (particularly palmitate, fructose, and benzoate; there was also variation in incubation times with sample depth). Initially, however, growth of colonies from agar shake preparations was obtained only from the 80-m sample grown on liquid medium with lactate; five similar isolates were obtained, and two of these, strains 80-55 and 80-94, were studied further. An isolate from the 500-m sample, strain 500-1T, was finally obtained on an acetate agar plate, although this organism did not grow in liquid acetate medium and growth in liquid culture was continued with lactate. While initial enrichment cultures required at least 6 weeks of incubation before growth was detectable, later enrichment cultures, and eventually pure cultures, produced significant amounts of sulfide within 2 weeks after inoculation into lactate medium.

Cell morphology. The Japan Sea isolates were motile, gram-negative, vibrio-shaped or sigmoid cells (0.5 to 1 by 1 to 2 μm) (Fig. 1) with some pleomorphism.

Ranges and optimum conditions for activity. Based on sulfide production, Japan Sea strains 80-55 and 500-1T were active at temperatures between 15 and 65°C, and optimum activity occurred at temperatures around 25°C (Fig. 2). The activity temperature profile of these organisms was unusual, with considerable reproducible activity at the extremes of the temperature range tested (60% of the sulfide production at the optimum temperature). This was significantly different ($P <$
were moderate halophiles; they were active in the presence of D. salexigens. Were active at pressures up to ca. 5 and 15 MPa, respectively (50 and 150 atm, respectively). The maximum sulfide production was ca. 40 MPa (500 atm), and the maximum pressure for activity was ca. 40 MPa (1 atm), and the maximum pressure for activity was ca. 0.6 to about 10% NaCl (Fig. 3), in contrast to the slightly branched fatty acids characteristic of desulfovibrios (iso 17:0 7 and iso 15:0) (14, 43). The protein profiles of strains 80-55 and 500-1T were identical, but distinct from the protein profiles of D. salexigens and D. desulfuricans (data not shown).

The G+C content of strain 500-1T was estimated to be 53 mol%; this compares with previously reported values of 49 and 59 mol% G+C for D. salexigens and D. desulfuricans, respectively (13). When genomic DNAs from strains 80-55 and 500-1T were separately digested with EcoRI and PstI and hybridized with eubacterium-specific 16S ribosomal DNA probe EUB338, both organisms produced the same band patterns, indicating that they had identical ribotypes.

Growth substrates and electron acceptors. Growth substrate and electron acceptor data for the Japan Sea strains, D. salexigens, and D. desulfuricans are shown in Table 1. Compared with other desulfovibrios, the Japan Sea strains used a relatively limited number of growth substrates during sulfate reduction (46) (Table 1); both lactate and pyruvate were incompletely oxidized to acetate, and hydrogen was utilized. Lactate and pyruvate were also fermented by the strains, but not by D. salexigens and D. desulfuricans. In contrast to both D. salexigens and D. desulfuricans, both Japan Sea strains were capable of weak growth on hydrogen in the absence of acetate as a carbon source. Sulfate, sulfite, thiosulfate (only strain 500-1T), iron oxyhydroxide, nitrate, dimethyl sulfoxide (only strain 80-55), and lignosulfonate were used as electron acceptors. Overall, the metabolic capabilities of the two Japan Sea strains were similar but not identical and were substantially different from the metabolic capabilities of both D. salexigens and D. desulfuricans.

Molecular analysis of pure cultures obtained from Japan Sea sediment. DNAs were extracted from all six Japan Sea isolates, and small 16S rRNA gene libraries were prepared. Selected clones (5 to 12 clones from each library) were compared and were shown to produce identical 16S ribosomal DNA restriction fragment length polymorphism profiles with restriction endonucleases EcoRI, AvaI, and TaqI. The results of a partial sequence analysis of a subset of the clones obtained from the sulfate-reducing bacterial cultures were consistent with the purity established previously by microscopy and growth checks and also suggested that the isolates in the different cultures were members of the same species.

Phylogenetic analysis of sulfate-reducing bacterial strains isolated from Japan Sea sediments based on 16S rRNA gene sequences. 16S rRNA genes from sulfate-reducing bacterial strains 80-55 and 500-1T were sequenced over almost their entire length (1,369 bp) and were found to be identical. The sequences obtained was compared with the sequences of other sulfate-reducing bacteria, and this comparison revealed that strains 80-55 and 500-1T were most closely related to D. salexigens and less closely related to D. desulfuricans (levels of sequence similarity for the 1,369 bp, 91.0 and 89.6%, respectively). Figure 5 shows a dendrogram based on a phylogenetic
there has been no physiological evidence that bacteria obtained from deep sediments were adapted to deep conditions to support a deep sediment origin for these organisms.

In the Japan Sea sediments the presence of deep bacterial populations and activity was confirmed by a range of independent methods (9, 30), and viable counts of sulfate-reducing bacteria correlated with sulfate reduction rates, changes in pore water sulfate content, and concentrations of reduced sulfur species. Sulfate-reducing bacterial strains 80-55 and 500-1.7 were isolated from the original MPN vials inoculated with sediment obtained from 80 and 500 m below the seafloor, respectively. These isolates are gram-negative, desulfoviridin-positive, sulfate-reducing bacteria which incompletely oxidize lactate and pyruvate. This, together with characteristic of desulfovibrios (14), places them in the Desulfovibrio group. This was confirmed by 16S rRNA gene sequence analysis data, which also indicate that these bacteria are closely related, but not identical, to Desulfaxigens and Desulfuricans (levels of sequence similarity, 91.0 and 89.6%, respectively) (Fig. 5).

The growth substrates utilized by Japan Sea sulfate-reducing bacterial strains 80-55 and 500-1.7 were similar to the growth substrates utilized by other desulfovibrios, but even more restricted. For example, the Japan Sea isolates did not utilize ethanol, formate, or malate (Table 1). However, they did exhibit enhanced fermentation abilities and were able to ferment both lactate and pyruvate, unlike either Desulfaxigens or Desulfuricans. The utilization of inorganic electron acceptors, in addition to sulfate, iron oxyhydroxide, nitrate, sulfite, and thiosulfate (only strain 500-1.7) by the Japan Sea strains is similar to the utilization by other desulfovibrios. However, the ability of the Japan Sea strains to use lignosulfonate, although previously found in another sulfate-reducing bacterium (48), is in contrast to the characteristics of the Desulfovibrio type strains tested in this study (Table 1). Although not remarkable, the metabolic capabilities of the Japan Sea isolates, which exhibit enhanced fermentation, an ability to use complex organic sulfonates and ferric iron as electron acceptors, and an ability to grow on hydrogen, are characteristics that are consistent with the deep sulfate-limited environment from which they were isolated (9). Hydrogen can be produced by several deep mineral reactions (13a, 41a).

As the Japan Sea strains are the first bacteria from very deep sediment layers to be isolated and characterized, it was important that the ability of these organisms to be active under simulated in situ conditions be established and that the maximum temperature and pressure ranges for activity be determined. As considerable bacterial activity may occur in the environment without growth, a situation that is particularly true for anaerobic bacteria (45a), sulfide production rather than growth was used to determine activity ranges. As both the Japan Sea strains and type strains of previously described desulfovibrios were grown under the same conditions, comparisons between them were valid, but the results may not be identical to the results of experiments based on growth. Sulfide production, however, was correlated with bacterial concentration (P = 0.05), and the experiments were reproducible and statistically significant. The pressure characteristics of the Japan Sea strains, compared to either Desulfaxigens or Desulfuricans, are remarkable. Based on sulfide production, not only are the Japan Sea strains barophiles, but the optimum pressures for activity for 80-55 and 500-1.7 coincided with the pressures at the total depths (water column and sediment depths) from which they were isolated (10 and 15 MPa, respectively [100 and 150 atm, respectively]) (Fig. 4). This characteristic is similar to characteristics of other barophiles (46a) and pro-
vides strong evidence that the organisms were obtained from deep sediments. Furthermore, the Japan Sea isolates were active at pressures up to about 40 MPa (400 atm), in marked contrast to both *D. salexigens* and *D. desulfuricans*. Limited data for bacterial growth under elevated pressures confirmed that the Japan Sea strains could also grow better at elevated pressures than at 0.1 MPa (1 atm); however, overall, growth was reduced with increasing pressure more rapidly than activity.

The broad temperature profiles (15 to 65°C) (Fig. 2) of the Japan Sea isolates were also very different from the temperature profiles of other desulfuvibrios, which normally are not active at temperatures above 40°C (46). There was considerable sulfate reduction at the extreme temperatures and there was no sharp temperature optimum, characteristics that are markedly different from the characteristics of the type strains of the previously described desulfuvibrios. The thermal gradient in the Japan Sea was 11°C/Km (19), and hence the temperature characteristics of the Japan Sea isolates enable them to grow in sediments more than 500 m deep. Furthermore, the Japan Sea isolates were resistant to 1 g of ribitane per liter (34, 40); these characteristics of the type strains of *D. salexigens* (Fig. 3).

The ability to grow at high concentrations of NaCl is also a characteristic of *D. halophilus* (4a); however, this sulfate-reducing bacterium requires at least 3% NaCl for growth and can tolerate up to 18% NaCl, compared to 10% NaCl for the Japan Sea strains. *D. halophilus* is also different from the Japan Sea strains in terms of growth substrates since it is able to use formate and ethanol for sulfate reduction but cannot ferment pyruvate. *D. gabanensis*, a recently isolated moderately halophilic desulfuvibrio (42a), differs markedly from the Japan Sea isolates as it has a salinity range of 1 to 17%, can grow on a much wider range of substrates, and utilizes different electron acceptors. In contrast to *D. salexigens*, hibitane concentrations as low as 0.1 g/liter inhibited growth of the Japan Sea isolates. The inability of the Japan Sea isolates to respire or ferment choline distinguishes them from *D. desulfuricans*. Therefore, consistent with the 16S rDNA gene sequence analysis data (Fig. 5), the phenotypic characteristics of the Japan Sea isolates are similar to, but distinct from, the phenotypic characteristics of *D. salexigens* and other desulfuvibrios, and we propose a new species of the genus *Desulfovibrio*, *Desulfovibrio profundus*, for the Japan Sea isolates. *D. profundus* clusters with the moderately halophilic organism *D. halophilus* and the slightly halophilic organism *D. salexigens*, to which it is most closely related, on the basis of 16S rDNA gene analysis data.

The 16S rRNA gene sequences of strains 80-55 and 500-1T were identical despite clear phenotypic differences between these organisms, including differences in optimum pressure for activity (Fig. 4) and some metabolic activities (Table 1). Hence, bacteria can be very closely related as determined by 16S rRNA analysis but still have phenotypic differences that are environmentally important. The close relationship between the Japan Sea isolates and near-surface desulfuvibrios (*D. salexigens* and *D. desulfuricans*), combined with the high optimum pressures of genetically identical strains 80-55 and 500-1T, which were isolated from different sediment depths (80 and 500 m below the seafloor, respectively), suggests that adaptation during burial is a possible mechanism for the origin of specialized deep-sediment bacteria. When bacterial populations originally in surface sediments become buried, they have to adapt to increased temperatures and pressures and the geochemical changes that occur during burial to survive. In addition, the results of an analysis of 16S rRNA genes amplified from high-molecular-weight bacterial DNA from deep Japan Sea sediment indicated that there are other novel bacterial types present (37). Pressure tolerance and other characteristics of the Japan Sea isolates demonstrate that these bacteria are well-adapted to their deep sediment environment and hence that they are not dormant or just surviving in situ, but are an active and important component of this environment. *D. profundus* is the first sulfate-reducing bacteria from extreme depths in marine sediments that has been characterized. Bacterial populations deep within marine sediments represent a unique new source of bacterial diversity with potential significance for biotechnological application (for example, microbiologically enhanced oil recovery) (1a).

**Description of Desulfovibrio profundus sp. nov.** *Desulfovibrio profundus* (pro.fun’ dus. L. adj. profundus, deep). Motile, gram-negative, desulfoviridin-positive, vibrio-shaped or sigmoid cells (0.5 to 1 by 1 to 2 μm). The following growth ranges are based on sulfide production: temperature range for growth, 15 to 65°C (optimum growth occurs around 25°C); pH range for growth, 4.5 to 9.0 (optimum pH, around 7); salinity range for growth, 0.2 to 10% NaCl (optimum NaCl concentration, 0.6 to 8%); pressure range, 0.1 to 40 MPa (1 to 400 atm) (optimum pressure, 10 to 15 MPa [100 to 150 atm], depending on the strain). Strict anaerobe. Reduces sulfate, sulfite, and thiosulfate (depending on the strain) to sulfide. Nitrate, ferric iron, dimethyl sulfide (depending on the strain), and lignosulfonate are used as alternative electron acceptors. Substrates used for dissimilatory sulfate reduction are lactate and pyruvate, which are incompletely oxidized to acetate, and hydrogen. Lactate and pyruvate are fermented in the absence of sulfate. Desulfoviridin is present. The G+C content of the DNA is 53 mol% (as determined by high-performance liquid chromatography). Isolated from deep marine sediment. The type strain is strain 500-1 (DSM 11384).

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**References**


**Abbreviations**

BLAST, Basic Local Alignment Search Tool; CLS, complete-length sequence; DNA, deoxyribonucleic acid; EST, expressed sequence tag; FAME, fatty acid methyl ester; GC, guanine plus cytosine; G + C, guanine plus cytosine; HPLC, high-performance liquid chromatography; 16S rRNA, 16S ribosomal RNA; PLFA, phospholipid fatty acid; ssr, sulphate-sulfur reducing; TFA, trifluoroacetic acid; TMAO, trimethylamine N-oxide; TMA, trimethylamine.