Desulfovibrio piezophilus sp. nov., a piezophilic, sulfate-reducing bacterium isolated from wood falls in the Mediterranean Sea

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A novel sulfate-reducing bacterium, designated C1TLV30T, was isolated from wood falls at a depth of 1693 m in the Mediterranean Sea. Cells were motile vibrios (2–4 μm). Strain C1TLV30T grew at temperatures between 15 and 45 °C (optimum 30 °C) and at pH 5.4–8.6 (optimum 7.3). It required NaCl for growth (optimum at 25 g NaCl l−1) and tolerated up to 80 g NaCl l−1. Strain C1TLV30T used as energy sources: lactate, fumarate, formate, malate, pyruvate and ethanol. The end products from lactate oxidation were acetate, H2S and CO2 in the presence of sulfate as terminal electron acceptor. Besides sulfate, thiosulfate and sulfite were also used as terminal electron acceptors, but not elemental sulfur, fumarate, nitrate or nitrite. Strain C1TLV30T possessed desulfoviridin and was piezophilic, growing optimally at 10 MPa (range 0–30 MPa). The membrane lipid composition of this strain was examined to reveal an increase in fatty acid chain lengths at high hydrostatic pressures. The G+C content of the genomic DNA was 49.6 % and the genome size was estimated at 3.5 ± 0.5 Mb. Phylogenetic analysis of the SSU rRNA gene sequence indicated that strain C1TLV30T was affiliated to the genus Desulfovibrio with Desulfovibrio profundus being its closest phylogenetic relative (similarity of 96.4 %). On the basis of SSU rRNA gene sequence comparisons and physiological characteristics, strain C1TLV30T (DSM 21447T = JCM 15483) is proposed to be assigned to a novel species of the genus Desulfovibrio, Desulfovibrio piezophilus sp. nov.

Abbreviations: FA, fatty acid; SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain C1TLV30T is HM852532.

Two supplementary figures are available with the online version of this paper.
Desulfovibrio piezophilus sp. nov.

Desulfovibrio profundus was the first SRB ascribed as growing at up to 40 MPa, with optimum activities occurring between 10 and 15 MPa. Thereafter, Desulfovibrio hydrothermalis was shown to grow faster at 26 MPa (the pressure in the environment from where it was extracted) than at atmospheric pressure (0.1 MPa), thus demonstrating its piezophilic characteristic.

In this study, we report on the isolation of strain C1TLV30T from wood falls at a depth of 1693 m in the Mediterranean Sea, which grows optimally at 10 MPa and shows unique changes in membrane lipid composition. The phylogenetic and physiological characteristics of this strain suggest it as representing a novel species, for which we propose the name Desulfovibrio piezophilus sp. nov.

Strain C1TLV30T was isolated from wood falls consisting of natural Douglas fir wood cubes (2 x 2 x 2 cm) placed for one year on deep-sea sediments in a colonization device named CHEMECOLI (Gaudron et al., 2010). One device could harbour roughly 100 cubes. The CHEMECOLI (M70/2b_833_TRAC-13) was deployed at the cold seep site ‘Central Zone 2A’ in the Pockmark area in the Nile Deep-Sea Fan in the eastern Mediterranean (32°31’39” N 30°21’18” E, 1693 m deep, in situ temperature: 14°C) on 18 November 2006 during the BIONIL cruise (RV Meteor M70/2b) by the ROV (Remotely Operated Vehicle) Quest 4000 (Marum, Bremen, Germany) and recovered by the ROV Victor 6000 (Ifremer, Toulouse, France) on 10 November 2007 during the MEDECO-2 cruise (RV Pourquoi Pas ?). All metadata are stored in the PANGAEA database (http://www.pangaea.de) and PANGAEA event labels for the experiments are cited accordingly. For deployment and recovery, a hermetic box was used with separate compartments for each device, to avoid washing and mixing. On board, some randomly selected wood cubes issued from a CHEMECOLI were stored anaerobically and frozen at -20°C in 10% glycerol until isolation of SRB.

Basal medium (BM) contained (per litre of distilled water): 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 1 g NH₄Cl, 25 g NaCl, 4.0 g sodium sulfate, 0.1 g KCl, 0.1 g CaCl₂, 2H₂O, 3 g MgCl₂, 6H₂O, 0.5 g cysteine hydrochloride, 0.1 g yeast extract (Difco), 10 ml trace element solution (Widdel & Pfennig, 1984) and 1 ml 0.1% resazurin. The pH was adjusted to 7.0 with 10 M KOH solution at atmospheric pressure and the medium was prepared anaerobically as previously described (Fardeau et al., 1997). Prior to culture inoculation with pieces of wood falls (around 1 g), 0.1 ml 10% (w/v) NaHCO₃ and 0.1 ml 2% (w/v) Na₂S·9H₂O and 20 mM lactate as energy source were injected from sterile stock solutions into the tubes for enrichment and isolation. The Hungate technique (Hungate, 1969) was used throughout this study and pure cultures were obtained as reported by Alazard et al. (2003).

Morphological characteristics and purity were observed with an Optiphot (Nikon) phase-contrast microscope. For transmission electron microscopy studies, cell preparations were negatively stained with sodium phosphotungstate, as previously described (Fardeau et al., 1997). Growth experiments were performed at atmospheric pressure in duplicate, using Hungate tubes containing basal medium and different electron donors at 20 mM (lactate, fumarate, formate, malate, pyruvate, ethanol, acetate, propionate, butyrate, succinate, fructose, Casamino-acids, benzoate, phenol) and hydrogen at 2 bars. Elemental sulfur (1%, w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM), fumarate (20 mM), nitrate (10 mM) and nitrite (2 mM) were tested as terminal electron acceptors. The pH, temperature and NaCl concentration ranges for growth were determined using basal medium supplemented with 20 mM lactate as electron donor at atmospheric pressure. Temperatures for growth were tested between 10 and 50°C. H₂S production was determined using the method of Cord-Ruwisch (1985). The presence of spores was checked as described by Fardeau et al. (1997). End products of metabolism and bacterial growth were measured as described by the same authors (Fardeau et al., 1997).

For growth experiments under hydrostatic pressure, completely filled Hungate tubes (18 ml) containing BM were inoculated in triplicate and incubated at 30°C under anaerobic conditions. Incubations under high-pressure were performed using pressure vessels. The screwed top end-cap was connected, via a stainless steel tube, to the piloted pressure generator (Tamburini et al., 2009) allowing a linear increase of the hydrostatic pressure (0.5 MPa sec⁻¹) by the programmable computer-driven system of the piloted pressure generator. The software Metrolog (Metro-Mesures, Mennecey, France) allows an increase (or decrease) in the range of hydrostatic pressure with precise regulation (0.2%); it also allows constant or variable flow injection so that the volume injected can be measured precisely. The working pressure was up to 40 MPa. Hungate tubes were placed three per pressure vessel. Optical density (OD₅₈₀) was measured regularly over 4 days.

To study cellular fatty acid composition, strain C1TLV30T was grown as preculture to an optical density of 0.2 at 600 nm under atmospheric pressure (0.1 MPa). Then, 1/100 of the preculture was inoculated in fresh medium and grown under atmospheric pressure or under high hydrostatic pressure (10 MPa or 25 MPa) for 40 h at 30°C under anoxic conditions. A second round of growth was performed under the same conditions, from 1/100 of the first round cultures. Cells obtained from the first and second rounds were used for comparative lipid composition analysis. Cultures of 40 ml from both conditions (atmospheric and 10 MPa- or 25 MPa-exposed cultures) were harvested by centrifugation at 6000 g for 20 min at 4°C and then pellets were stored at -20°C. Fatty acid composition from these pellets was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, in accordance with the procedure of Vainshtein et al. (1992). Results are means of three independent experiments.

For pulsed-field gel electrophoresis, preparation of DNA plugs, digestions, electrophoresis conditions and pattern
analyses were performed according to Lefèvre et al. (2009). Restriction enzymes (PacI, NotI, I-CeuI, XhoI, PstI and DraI) were used according to the manufacturer’s instructions (New England Biolabs). Molecular masses of all fragments produced by PacI restriction endonuclease digestion of genomic DNA were calculated as the mean from three pulsed-field gels. Plasmids were prepared by the alkaline lysis method described by Kado & Liu (1981).

The G + C content of DNA was determined at the DSMZ. The DNA was isolated and purified by chromatography on hydroxyapatite and the G + C content was determined by HPLC as described by Mesbah et al. (1989).

Methods for purification of the DNA, PCR amplification and sequencing of the 16S rRNA gene were as described by Cashion et al. (1977) and Hernandez-Eugenio et al. (2000). The partial sequences generated were assembled using BioEdit v. 5.0.9. (Hall, 1999) and the consensus sequence of 1539 nt was corrected manually for errors. The most closely related sequences in GenBank (version 178) (Benson, 2009), and the Ribosomal Database Project (release 10), identified using BLAST (Altschul, 2007), and the Sequence Match program (Cole et al., 2009), were extracted and aligned. The consensus sequence was then manually adjusted to conform to the 16S rRNA secondary structure model (Winker & Woese, 1991). Nucleotide ambiguities were omitted and evolutionary distances were calculated using the Jukes and Cantor option (Jukes & Cantor, 1969). Dendrograms were reconstructed with the TREECON program using the neighbour-joining method (Saitou & Nei, 1987). Tree topology was re-examined by the bootstrap method (1000 replications) of resampling (Felsenstein, 1985). The topology was also supported using the maximum-parsimony and maximum-likelihood algorithms.

Enrichments in the medium containing lactate as electron donor and sulfate as electron acceptor and inoculated with pieces of wood falls (around 1 g) were positive after one week of incubation at 30 °C. Thereafter, colonies of SRB developed in roll-tubes after 15 days of incubation at the same temperature. They were picked separately and the process of serial dilution was repeated until the isolates were deemed to be axenic. Several strains similar in morphology (vibrio-like micro-organisms) and phylogeny (sequence similarities ranging from 99 to 100%) and incompletely oxidizing lactate to acetate were isolated. One of these strains, designated C1TLV30T, was selected and used for further characterization.

Cells stained Gram-negative. They were motile, non-spore-forming vibrios (2 to 4 μm in length and 0.5 μm in diameter), and generally occurred singly or in pairs (data not shown). Strain C1TLV30T was mesophilic and grew at temperatures ranging from 15 to 45 °C, with an optimum at 30 °C. The isolate required NaCl for growth (optimum 25 g l⁻¹), and tolerated up to 80 g NaCl l⁻¹. The optimum pH for growth was 7.3 (range between pH 5.4 and 8.6). Strain C1TLV30T grew optimally at 10 MPa (range 0–30 MPa) (Supplementary Fig. S1, available in IJSEM Online). Under optimal growth conditions, doubling time was estimated to be around 10 h. Sulfate, thiosulfate and sulfite were utilized as electron acceptors, but not elemental sulfur, fumarate, nitrate or nitrite. When using sulfate as terminal electron acceptor, strain C1TLV30T grew on lactate, fumarate, formate, malate, pyruvate, ethanol and hydrogen. Hydrogen was only used in the presence of acetate as carbon source. Pyruvate and fumarate were fermented. No growth was observed on the following substrates: acetate, propionate, butyrate, succinate, fructose, Casamino-acids, benzoate or phenol. The end products from lactate oxidation were acetate, CO₂ and H₂S in the presence of sulfate as terminal electron acceptor.

SRB are widely distributed in nature with a peculiar emphasis in marine ecosystems (Fauque & Ollivier, 2004). They are known to inhabit extreme environments submitted to several hundred bars of pressure such as deep-sea hydrothermal vents, oil reservoirs and other subterrestrial ecosystems (Ollivier et al., 2007; Parke & Sass, 2007), thus suggesting that they have to cope with high hydrostatic pressure within these habitats. Only a few reports deal with the growth responses of SRB to high hydrostatic pressures. They include two species of the genus Desulfovibrio, Desulfovibrio hydrothermalis and Desulfovibrio profundus, which have been isolated from hydrothermal vents in the Pacific Ocean (Alazard et al., 2003) and deep sediment layers in the Japan Sea (Bale et al., 1997), respectively. Here we report the isolation of a novel SRB (strain C1TLV30T) from wood falls located at a depth of 1693 m in the Mediterranean Sea which has Desulfovibrio profundus as its closest phylogenetic relative (96.4% similarity) (Fig. 1). It grew optimally at 10 MPa and tolerated hydrostatic pressures up to 25–30 MPa, thus demonstrating its piezophilic nature (Supplementary Fig. S1). However, in contrast to Desulfovibrio profundus, strain C1TLV30T was not active at hydrostatic pressures up to about 40 MPa, with the upper limit of pressure for growth being 30 MPa. In the case of Desulfovibrio hydrothermalis, the optimum pressure for growth was not reported, but this bacterium grew faster at 26 Mpa, corresponding to the pressure at the depth from which it was isolated (2600 m), than at 0.1 MPa. This is also true for strain C1TLV30T (better growth at 17 MPa than at 0.1 MPa), thus demonstrating that these species of the genus Desulfovibrio together with Desulfovibrio profundus are well adapted to deep-sea environments.

The genome of strain C1TLV30T was characterized by PFGE analysis. The overall DNA G + C content of strain C1TLV30T was 49.6 mol%. We used several restriction endonucleases to cleave strain C1TLV30T genomic DNA into a reasonable number of fragments. As shown in Supplementary Figure S2, PacI generated a manageable number of fragments that were relatively easily resolvable by PFGE. Analysis of the digestion pattern using QUANTITY ONE quantification software (Bio-Rad) indicated that the genome size of strain C1TLV30T was about 3.5 + 0.5 Mb. The density of the band around 800 bp was
clearer than those of bands with lower molecular sizes, suggesting a randomly linearized fragment. Hence it was not considered here. No plasmid was revealed using the Kado and Liu method for plasmid extraction (data not shown; Kado & Liu, 1981).

A major characteristic of piezophilic micro-organisms seems to reside in membrane fatty acid (FA) composition. For example, increases in the levels of lower-melting-temperature branched or unsaturated FAs in piezophilic bacteria have been reported to increase membrane fluidity (Kates, 1986; Yano et al., 1998). The FA composition of total lipids from strain C1TLV30T, grown at 30 °C and 0.1, 10 or 25 MPa, indicated that the major FAs of the strain were C15:0, C16:0, C16:1, C17:0, C18:0 and C18:1. Relative changes in the proportions of these seven major types of FA after exposure to hydrostatic pressure-dependent growth conditions are shown in Table 1. Total branched FAs were between 59 and 68%, whatever the growth conditions. Total mono-unsaturated FAs were 24% after growth during 35 h under atmospheric pressure (0.1 MPa) but were 42 and 35% when growth was performed at 10 or 25 MPa, respectively. After two rounds of growth at 0.1, 10 or 25 MPa, mono-unsaturated FAs were 30, 31.5 and 40%, respectively. Thus, no evident correlation was made between the levels of branched or unsaturated FAs and hydrostatic pressure for strain C1TLV30T. Interestingly, regarding chain length, the proportion of C15 and C16 FAs was 62% at 0.1 MPa, and 46–48% at 10 and 25 MPa after the first round of growth. It was 58, 52.5 and 38.5%, respectively, after the second round. Thus, Desulfovibrio piezophilus sp. nov. could exhibit a novel mechanism in which an overall increase in the level of FA chain length, both saturated and mono-unsaturated, branched or not, would occur during hydrostatic pressure increase.

Although increases in only unsaturated FAs were reported to be involved in hydrostatic pressure adaptation in microorganisms such as species of the genus Shewanella and

<table>
<thead>
<tr>
<th>Fatty acid type</th>
<th>Total fatty acids (%) at indicated pressure</th>
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<tbody>
<tr>
<td></td>
<td>Round 1</td>
</tr>
<tr>
<td></td>
<td>25 MPa</td>
</tr>
<tr>
<td>15:0b</td>
<td>22</td>
</tr>
<tr>
<td>16:0</td>
<td>12</td>
</tr>
<tr>
<td>16:1b</td>
<td>3</td>
</tr>
<tr>
<td>17:0</td>
<td>9</td>
</tr>
<tr>
<td>17:1b</td>
<td>14</td>
</tr>
<tr>
<td>18:0</td>
<td>17</td>
</tr>
<tr>
<td>18:0b</td>
<td>1</td>
</tr>
<tr>
<td>18:1</td>
<td>10</td>
</tr>
<tr>
<td>18:1b</td>
<td>2</td>
</tr>
<tr>
<td>Total branched FAs</td>
<td>59</td>
</tr>
<tr>
<td>Total mono-unsaturated FAs</td>
<td>35</td>
</tr>
<tr>
<td>Total C15+C16 FAs</td>
<td>46</td>
</tr>
</tbody>
</table>
Photobacterium profundum (Allen et al., 1999; Bartlett, 2002; Simonato et al., 2006), it is the combined ratio of different FAs that determines the viscosity of the membranes. In strain C1TLV30T, increase in FA chain length may be the major bacterial response in the establishment of optimal membrane fluidity. Thus, strain C1TLV30T could present new particularities in FA metabolism linked to hydrostatic pressure changes that have not been reported for piezophilic species of the genus Desulfovibrio so far. In this respect, this strain provides a particularly good model for understanding adaptation of members of the genus Desulfovibrio to the deep ocean.

To our knowledge, the greatest depth in a marine environment at which a sulfate reducer was isolated has so far not exceeded 3000 m, corresponding to in situ pressure of 30 MPa. It is therefore a challenge for microbiologists to cultivate SRB from depths around and below 3000 m in marine ecosystems to determine if they are of geomicrobiological relevance in oxidizing organic matter in abysses, due to their hydrogenotrophic metabolism in particular. This is probably the case for strain C1TLV30T, which should contribute to degradation of cellulose and hemicellulose contained in wood falls by oxidizing H2 delivered by fermentative micro-organisms.

However, at this stage, few microbiological studies regarding wood falls have been undertaken (Nilsson & Björdal, 2008; Palacios et al., 2009). Further experiments are needed to clarify the microbial community interactions existing within these deep-sea ecosystems.

Besides phylogenetic differences observed between Desulfovibrio profundus and strain C1TLV30T, several phenotypic and genetic characteristics allowed us to distinguish them. They include the temperature range for growth as Desulfovibrio profundus and strain C1TLV30T should be considered as thermophilic and mesophilic, respectively (Table 2). Moreover, unlike Desulfovibrio profundus, strain C1TLV30T used ethanol and formate as electron donors but not nitrate as terminal electron acceptor, and did not ferment lactate (Table 1). Desulfovibrio profundus also differed from strain C1TLV30T by having a higher G + C content of the DNA (Table 2). Therefore, based on phylogenetic, genetic, phenotypic and chemotaxonomic characteristics of strain C1TLV30T, we propose that this strain be assigned to a novel species of the genus Desulfovibrio, Desulfovibrio piezophilus sp. nov.

**Description of Desulfovibrio piezophilus sp. nov.**

Desulfovibrio piezophilus [pi.e.zo.phi’lus. Gr. v. piezo to press; N.L. adj. philus -a -um (from Gr. adj. philos -é -on) loving; N.L. masc. adj. piezophilus loving pressure].

Cells are strictly anaerobic, Gram-stain-negative, motile, non-spore-forming vibrios, approximately 2 to 4 µm in length and about 0.5 µm in diameter. They generally occur singly or in pairs. The temperature range for growth is 15 to 45 °C (optimum 30 °C). The optimum NaCl concentration for growth is 2.5 % (range 0.1–8 %). The optimum pH is 7.3.

**Table 2. Comparison of physiological and metabolic properties between strain C1TLV30T and Desulfovibrio profundus**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>Temperature range (°C)</td>
<td>15–45</td>
<td>15–65</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>pH range</td>
<td>5.4–8.6</td>
<td>4.5–9</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7.3</td>
<td>7</td>
</tr>
<tr>
<td>NaCl range (%)</td>
<td>0.1–8.0</td>
<td>0.2–10</td>
</tr>
<tr>
<td>Optimum NaCl (%)</td>
<td>2.5</td>
<td>0.6–10</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>49.6</td>
<td>53.0</td>
</tr>
<tr>
<td>Substrate utilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Malate</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Formate</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Lactate fermentation</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate as electron acceptor</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

Grows optimally at 10 MPa. Upper limit of hydrostatic pressure for growth is 25–30 MPa. Uses lactate, fumarate, formate, malate, pyruvate, ethanol, and hydrogen. Hydrogen is only used in the presence of acetate as carbon source. Ferments pyruvate and fumarate. Substrates not used are: acetate, propionate, butyrate, succinate, fructose, Casamino acids, benzooate or phenol. Incompletely oxidizes its substrates to acetate, CO2 and H2S in the presence of sulfate. Besides sulfate, thiosulfate and sulfite are also used as terminal electron acceptors, but not elemental sulfur, fumarate, nitrate or nitrite. At 10 MPa, the strain appears to exhibit an increase in FA chain lengths. The G+C content of DNA of the type strain is 49.6 mol% and the genome size is 3.5 ± 0.5 Mb.

The type strain is C1TLV30T (=DSM 21447T =JCM 15486T) isolated from wood falls at a depth of 1693 m in the Mediterranean Sea.

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**References**

**Desulfovibrio piezophilus** sp. nov.


