Isolation of sulfate-reducing bacteria from Tunisian marine sediments and description of *Desulfovibrio bizertensis* sp. nov.

Olfa Haouari, Marie-Laure Fardeau, Laurence Casalot, Jean-Luc Tholozan, Moktar Hamdi and Bernard Ollivier

1Laboratoire de microbiologie IRD, UMR 180, IFR-BAIM, Universités de Provence et de la Méditerranée, ESIL case 925, 163 Avenue de Luminy, F-13288 Marseille cedex 09, France
2UR-Procédures Microbiologiques et Alimentaires, INSAT, 1080 Tunis, Tunisia

Several strains of sulfate-reducing bacteria were isolated from marine sediments recovered near Tunis, Korbous and Bizerte, Tunisia. They all showed characteristics consistent with members of the genus *Desulfovibrio*. One of these strains, designated MB3T, was characterized further. Cells of strain MB3T were slender, curved, vibrio-shaped, motile, Gram-negative, non-spore-forming rods. They were positive for desulfoviridin as bisulfite reductase. Strain MB3T grew at temperatures of 15–45 °C (optimum 40 °C) and at pH 6–8.1 (optimum pH 7.0). NaCl was required for growth (optimum 20 g NaCl l⁻¹). Strain MB3T utilized H₂ in the presence of acetate with sulfate as electron acceptor. It also utilized lactate, ethanol, pyruvate, malate, fumarate, succinate, butanol and propanol as electron donors. Lactate was oxidized incompletely to acetate. Strain MB3T fermented pyruvate and fumarate (poorly). Electron acceptors utilized included sulfate, sulfite, thiosulfate, elemental sulfur and fumarate, but not nitrate or nitrite. The G+C content of the genomic DNA was 51 mol%. On the basis of genotypic, phenotypic and phylogenetic characteristics, strain MB3T (= DSM 18034T = NCIMB 14199T) is proposed as the type strain of a novel species, *Desulfovibrio bizertensis* sp. nov.

The Hungate technique (Hungate, 1969) was then used throughout for cultivation. The basal medium contained (per litre of distilled water): 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 1.0 g NH₄Cl, 23 g NaCl, 3 g Na₂SO₄, 0.1 g KCl, 0.1 g CaCl₂·2H₂O, 0.1 g yeast extract (Difco), 0.5 g cysteine hydrochloride, 1 ml trace mineral element solution (Widdel & Pfennig, 1984) and 1 ml 0.1% resazurin; pH was adjusted to 7.2 with 10 M KOH. The basal medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature and 5 ml aliquots were distributed in Hungate tubes under a stream of O₂-free N₂ gas. The N₂ gas phase was replaced with N₂/CO₂ (80 : 20, v/v) and the tubes were autoclaved for 45 min at 110 °C. Prior to inoculation, 0.1 ml 2% Na₂S·9H₂O, 0.1 ml 10% NaHCO₃ and 0.1 ml 15% MgCl₂·6H₂O were added.

Abbreviation: SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MB3T is DQ422859.
Enrichments were performed in Hungate tubes containing 5 ml medium and inoculated with sample diluted to 10%. H₂ + CO₂ [80 : 20 (v/v), 2 bar], peptone (10 g l⁻¹) or yeast extract (0·2 g l⁻¹) were used as substrates. Acetate (2 mM) was added as the carbon source in the presence of hydrogen as electron donor. The tubes were incubated at 30 °C for 3 days. Three enrichment series were performed. Cultures were purified by repeated use of the Hungate roll-tube method with medium solidified with 2·5 % (w/v) agar (Difco). Several colonies that developed were picked and cultured in the corresponding culture medium. The process of isolation was repeated several times until isolates were deemed to be axenic. Physiological optimal growth conditions (for strain MB3T only) were determined in duplicate experiments conducted in basal medium containing lactate (20 mM) and thiosulfate (20 mM) as described by Fardeau et al. (1993). Growth was measured by inserting tubes directly into a model Cary 50 Scan spectrophotometer (Varian) and measuring the OD₅₇₈. Sulfide was determined photometrically as colloidal CuS following the method of Cord-Ruwisch (1985).

Genomic DNA was extracted according to the protocol described for the Wizard Genomic DNA purification kit (Promega). 16S rRNA genes were amplified by using primers Fd1 (5'--AGAGTTTGATCCTGGCTCAG--3') and Rd1 (5'--AAGGAGGTGATCCAGCC--3') and by using the following reaction conditions: 1 min at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 50 °C and 2 min at 72 °C, and a final extension step of 10 min at 72 °C. PCR fragments were then cloned into pGEM-T-easy (Promega). Recombinant clones, with inserts of the correct length, were sequenced by cloned into pGEM-T-easy (Promega). Recombinant clones, with inserts of the correct length, were sequenced by Larker et al. (1995). The nucleotide sequences of the 16S rRNA genes were compared with reference sequences from the GenBank database (Benson et al., 1999). The 16S rRNA gene sequence of strain MB3T was aligned with reference sequences of various Desulfovibrio species using programs provided by the Ribosomal Database Project II (Maidak et al., 2001). Sequence alignment was verified manually using the program BIOEDIT (Hall, 1999). Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on an unambiguous stretch of 1274 bp were computed by using the Jukes & Cantor (1969) method. The dendrogram was constructed by using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined by bootstrap analysis based on 100 resamplings (Felsenstein, 1985).

Several SRB were isolated from marine sediments recovered near Tunis, Korbous and Bizerte in the presence of hydrogen, peptone or yeast extract as substrates and sulfate as the terminal electron acceptor. All isolates were found to be phylogenetically related to members of the genus Desulfovibrio (Table 1). Strains LB2 and MB2, isolated from marine sediments recovered near Bizerte, were found to be closely related (>99% 16S rRNA gene sequence similarity) to an uncharacterized Desulfovibrio strain, TBP-1 (Boyle et al., 1999). Strain SIJ23, isolated from marine sediments recovered near Bizerte, were

### Table 1. Desulfovibrio strains isolated from Tunisian marine sediments

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Nearest phylogenetic relative</th>
<th>16S rRNA gene sequence similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB2</td>
<td>Peptone</td>
<td>Desulfovibrio TBP-1</td>
<td>99·3</td>
</tr>
<tr>
<td>MB3⁷</td>
<td>H₂</td>
<td>D. senzii DSM 8436⁷</td>
<td>91·3</td>
</tr>
<tr>
<td>LB2</td>
<td>Peptone</td>
<td>Desulfovibrio TBP-1</td>
<td>99·3</td>
</tr>
<tr>
<td>LB3</td>
<td>H₂</td>
<td>D. senzii DSM 8436⁷</td>
<td>91·3</td>
</tr>
<tr>
<td>LB4</td>
<td>H₂</td>
<td>D. senzii DSM 8436⁷</td>
<td>99·9</td>
</tr>
<tr>
<td>LT3</td>
<td>H₂</td>
<td>D. senzii DSM 8436⁷</td>
<td>91·3</td>
</tr>
<tr>
<td>KM2</td>
<td>Peptone</td>
<td>D. senzii DSM 8436⁷</td>
<td>93·8</td>
</tr>
<tr>
<td>SIJ23</td>
<td>Yeast extract</td>
<td>D. alaskensis NCIMB 13491⁷</td>
<td>99·7</td>
</tr>
</tbody>
</table>

Fig. 1. Neighbour-joining phylogenetic dendrogram based on 16S rRNA gene sequence comparison indicating the position of strain MB3T among the most closely related members of the genus Desulfovibrio. Desulfonatronum lacustre DSM 10312T was used as an outgroup. Bootstrap values based on 100 resamplings are given at nodes. Bar, 2 % sequence divergence.
phylogenetically related (99–100 % similarity) to *Desulfovibrio alaskensis* and *Desulfovibrio senezii*, respectively (Feio et al., 2004; Tsu et al., 1998). Four other strains isolated from marine sediments recovered near Korbous (strain KM2), Bizerte (strains LB3 and MB3<sup>T</sup>) and Tunis (strain LT3) also had *D. senezii* as their closest phylogenetic relative, but with low 16S rRNA gene sequence similarity (<97 %), indicating that they may represent novel species of the genus *Desulfovibrio*. Only strain MB3<sup>T</sup> was characterized further.

Strain MB3<sup>T</sup> was strictly anaerobic and mesophilic; optimal temperature for growth was 40 °C (range 15–45 °C). For pH studies, the medium was adjusted to the desired pH using anaerobically prepared stock solutions of NaHCO<sub>3</sub> (10 %) or Na<sub>2</sub>CO<sub>3</sub> (10 %). Strain MB3<sup>T</sup> was neutrophilic; the optimum pH for growth was 7 and growth occurred between pH 6·0 and 8·1. For determination of NaCl requirements, NaCl was weighed directly in the tubes at concentrations of 0–130 g l<sup>−1</sup> before dispensing to NaCl-free basal medium. The isolate was slightly halophilic and grew in the presence of NaCl concentrations ranging from 5 to 50 g l<sup>−1</sup>, with optimum growth at 20 g l<sup>−1</sup>.

The following substrates (20 mM) were used as carbon and energy sources: lactate, ethanol, pyruvate, malate, fumarate, succinate, H<sub>2</sub>/CO<sub>2</sub> with acetate (2 mM), butanol and propanol. Acetate, propionate, butyrate, Casamino acids (2 mM). Strain MB3<sup>T</sup> fermented pyruvate into acetate, hydrogen and CO<sub>2</sub>. Fumarate was fermented only weakly. Fermentation products were determined as described by Farneau et al. (1993). The end products from lactate metabolism in the presence of thiosulfate as terminal electron acceptor were acetate, CO<sub>2</sub> and H<sub>2</sub>S.

Growth of strain MB3<sup>T</sup> was inhibited by the addition of chloramphenicol (50 µg ml<sup>−1</sup>), ampicillin (100 µg ml<sup>−1</sup>) and vancomycin (300 µg ml<sup>−1</sup>).

The presence of bisulfite reductase (desulfoviridin) was confirmed by measuring the absorbance of cell-free extracts at 630 nm (Badziong et al., 1978). In addition, *c*-type cytochromes were detected by reduction of extracts with sodium dithionite, with two peaks occurring at 418 and 550 nm.

The G+C content of the DNA (determined by the Identification Service of the DSMZ, Braunschweig, Germany) was 51 mol% based on the method of Mesbah et al. (1989).

Analysis of the almost complete sequence (1274 bp) of the 16S rRNA gene of strain MB3<sup>T</sup> revealed that it grouped with members of the family *Desulfovibrioaceae*, order *Desulfovibrionales*, in the *Deltaproteobacteria*. The phylogenetic tree constructed is shown in Fig. 1. As indicated above, strain MB3<sup>T</sup> clustered with *D. senezii* DSM 8436<sup>T</sup>, an isolate recovered from a solar saltern in California (Tsu et al., 1998), with a 16S rRNA gene sequence similarity of 91·3 %. Phenotypic differences were observed between strain MB3<sup>T</sup> and *D. senezii*, including the range of substrates utilized, salt tolerance and DNA G+C content (Table 2). In addition to the taxonomic significance of strain MB3<sup>T</sup> as a novel representative of the SRB within the *Deltaproteobacteria*, it is noteworthy that the enrichments described herein, performed with marine sediment samples originating from different locations in Tunisia, have led to the isolation of only *Desulfovibrio*-like strains when using

### Table 2. Comparison of the morphological and physiological properties of strain MB3<sup>T</sup> and *D. senezii* DSM 8436<sup>T</sup>

Optimum values are given in parentheses. −, No growth; +, good growth. Data for *D. senezii* DSM 8436<sup>T</sup> were taken from Tsu et al. (1998).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain MB3&lt;sup&gt;T&lt;/sup&gt;</th>
<th><em>D. senezii</em> DSM 8436&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0·5–2 0·3–0</td>
<td>0·3 × 1·0–1·3</td>
</tr>
<tr>
<td>Temperature range for growth (°C)</td>
<td>15–45 (40)</td>
<td>25–45 (37)</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>6–0–8·1 (7·0)</td>
<td>6–4–8·3 (7·6)</td>
</tr>
<tr>
<td>Salinity range for growth (% w/v NaCl)</td>
<td>0·5–5·0 (2·0)</td>
<td>0–12·5 (2·5)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>51</td>
<td>62</td>
</tr>
<tr>
<td>Utilization of electron donors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Malate</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Cysteine</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Succinate</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Elemental sulfur as electron acceptor</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>
hydrogen as the substrate. However, the prominent isolation of these Desulfovibrio strains may result from their known ability to grow rapidly when using hydrogen as substrate (Jørgensen & Bak, 1991) as compared with the growth of other hydrogenotrophic SRB species. Interestingly, among the Desulfovibrio strains recovered from Tunisian marine sediments, we have isolated some using peptone as substrate, providing further evidence that SRB may play a decisive role in the regulation of electron flow in protein amino-acid turnover through sulfate reduction in marine ecosystems (Hansen & Blackburn, 1995; Baena et al., 1998).

On the basis of its phenotypic, genotypic and phylogenetic characteristics, strain MB3T is proposed as the type strain of a novel species, Desulfovibrio bizertensis sp. nov.

Description of Desulfovibrio bizertensis sp. nov.

Desulfovibrio bizertensis (bi.zer.ten’sis, N.L. masc. adj. bizertensis from Bizerte, referring to the place of isolation of the type strain).

Cells are Gram-negative, vibrio-shaped, motile, non-sporulating rods, approximately 2.0–3.0 μm in length and 0.5-5 μm in diameter, and occur singly or in pairs. No spores are formed. Strictly anaerobic, mesophilic, neutrophilic and slightly halophilic. The temperature range for growth is 15–45 °C (optimum 40 °C). The optimum pH is 7.0. Vitamins, biotyping and yeast extract are not required for growth. Strictly anaerobic. Reduces sulfate, sulfite, thiosulfate, elemental sulfur and fumarate. Nitrate and thiosulfate are oxidized via sulfate reduction are lactate, ethanol, pyruvate, malate, fumarate, succinate, H$_2$ plus acetate, butanol and propanol. Desulfoviridin-type bisulfite reductase and c-type cytochromes are present. The G+C content of the DNA is 51 mol%.

The type strain, MB3T (˜DSM 18034T=NCIMB 14199T), was isolated from marine sediment recovered near Bizerte, Tunisia.

Acknowledgements

Many thanks are due to G. Fauque for suggested improvements to the manuscript.

References


