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

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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 MB3^T, was characterized further. Cells of strain MB3^T were slender, curved, vibrio-shaped, motile, Gram-negative, non-spore-forming rods. They were positive for desulfovibridin as bisulfite reductase. Strain MB3^T grew at temperatures of 15–45 °C (optimum 40 °C) and at pH 6.0–8.1 (optimum pH 7.0). NaCl was required for growth (optimum 20 g NaCl l⁻¹). Strain MB3^T 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 MB3^T 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 MB3^T (= DSM 18034^T = NCIMB 14199^T) is proposed as the type strain of a novel species, *Desulfovibrio bizertensis* sp. nov.

In marine ecosystems, sulfate-reducing bacteria (SRB) contribute significantly (50 %) to the mineralization of organic matter (Jørgensen, 1982). Hydrogenotrophic SRB of the family *Desulfovibrionaceae* within the *Deltaproteobacteria* have been found as common inhabitants of these ecosystems (Jørgensen & Bak, 1991; Widdel & Hansen, 1992). Within the *Desulfovibrionaceae*, many marine *Desulfovibrio* species have been isolated. They include *Desulfovibrio acrylicus* (van der Maarel *et al.*, 1996), *D. africanus* (Campbell *et al.*, 1966), *D. giganteus* (Esnault *et al.*, 1988), *D. gigas* (Le Gall, 1963) and *D. inopinatus* (Reichenbecher & Schink, 1997), all recovered from shallow marine ecosystems, and *D. profundus* (Bale *et al.*, 1997) and *D. hydrothermalis* (Alazard *et al.*, 2003) recovered from deep marine environments.

We have undertaken microbiological studies to isolate, in particular, hydrogenotrophic SRB from various marine sediments recovered near Tunis, Korbous and Bizerte, Tunisia. In addition to hydrogen, peptone and yeast extract were also used as energy sources in the presence of sulfate as

the terminal electron acceptor. Using these cultures, we isolated only members of the genus *Desulfovibrio*. One of these organisms, designated strain MB3^T, is suggested to represent a novel species of the genus *Desulfovibrio*.

Sediment samples were collected in sterile plastic bottles from the sea off Korbous, Tunis and Bizerte, and kept at 5 °C until inoculation. The *in situ* temperature, pH and conductivity of samples were 30 °C, 7.8 and 62 mS cm⁻¹, respectively.

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 MB3^T is DQ422859.

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

Desulfovibrio strain TBP-1, *D. senexii* DSM 8436^T and *D. alaskensis* NCIMB 13491^T were respectively described by Boyle *et al.* (1999), Tsu *et al.* (1998) and Feio *et al.* (2004).

Strain	Substrate	Nearest phylogenetic relative	16S rRNA gene sequence similarity (%)
MB2	Peptone	<i>Desulfovibrio</i> TBP-1	99.3
MB3 ^T	H ₂	<i>D. senexii</i> DSM 8436 ^T	91.3
LB2	Peptone	<i>Desulfovibrio</i> TBP-1	99.3
LB3	H ₂	<i>D. senexii</i> DSM 8436 ^T	91.3
LB4	H ₂	<i>D. senexii</i> DSM 8436 ^T	99.9
LT3	H ₂	<i>D. senexii</i> DSM 8436 ^T	91.3
KM2	Peptone	<i>D. senexii</i> DSM 8436 ^T	93.8
SIJ23	Yeast extract	<i>D. alaskensis</i> NCIMB 13491 ^T	99.7

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 MB3^T 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 OD₅₈₀. 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 using primers SP6 (5'-ATTTAGGTGACACTATAGAA-3') and T7 (5'-TAATACGACTCACTATAGGG-3') (Genome Express). 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 MB3^T 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 Tunis, and strain LB4, isolated from marine sediments recovered near Bizerte, were

Fig. 1. Neighbour-joining phylogenetic dendrogram based on 16S rRNA gene sequence comparison indicating the position of strain MB3^T among the most closely related members of the genus *Desulfovibrio*. *Desulfonatronum lacustre* DSM 10312^T 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^T) 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^T was characterized further.

Strain MB3^T 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 NaHCO₃ (10 %) or Na₂CO₃ (10 %). Strain MB3^T 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⁻¹ 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⁻¹, with optimum growth at 20 g l⁻¹.

The following substrates (20 mM) were used as carbon and energy sources: lactate, ethanol, pyruvate, malate, fumarate, succinate, H₂/CO₂ with acetate (2 mM), butanol and propanol. Acetate, propionate, butyrate, Casamino acids (0.1 %), fructose, glucose and methanol (40 mM) were also tested but did not support growth. Sulfate (20 mM), thiosulfate (20 mM), elemental sulfur (0.1 %), sulfite (2 mM) and fumarate (20 mM) were used as terminal electron acceptors, but not nitrate (20 mM) or nitrite (2 mM). Strain MB3^T fermented pyruvate into acetate, hydrogen and CO₂. Fumarate was fermented only weakly. Fermentation products were determined as described by

Fardeau *et al.* (1993). The end products from lactate metabolism in the presence of thiosulfate as terminal electron acceptor were acetate, CO₂ and H₂S.

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

The presence of bisulfite reductase (desulfovibridin) 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^T revealed that it grouped with members of the family *Desulfovibrionaceae*, order *Desulfovibrionales*, in the *Deltaproteobacteria*. The phylogenetic tree constructed is shown in Fig. 1. As indicated above, strain MB3^T clustered with *D. senezii* DSM 8436^T, 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^T 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^T 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^T and *D. senezii* DSM 8436^T

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

Characteristic	Strain MB3 ^T	<i>D. senezii</i> DSM 8436 ^T
Cell size (µm)	0.5 × 2.0–3.0	0.3 × 1.0–1.3
Temperature range for growth (°C)	15–45 (40)	25–45 (37)
pH range for growth	6.0–8.1 (7.0)	6.4–8.3 (7.6)
Salinity range for growth (% w/v NaCl)	0.5–5.0 (2.0)	0–12.5 (2.5)
DNA G+C content (mol%)	51	62
Utilization of electron donors		
Ethanol	+	–
Fumarate	+	–
Malate	+	–
Cysteine	–	+
Succinate	+	–
Elemental sulfur as electron acceptor	+	–

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 MB3^T 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-spore-forming rods, approximately 2.0–3.0 µm in length and 0.5 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, biotrypcase and yeast extract are not required for growth. Strictly anaerobic. Reduces sulfate, sulfite, thiosulfate, elemental sulfur and fumarate. Nitrate and nitrite are not used as terminal electron acceptors. Substrates that are oxidized via sulfate reduction are lactate, ethanol, pyruvate, malate, fumarate, succinate, H₂ 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, MB3^T (=DSM 18034^T=NCIMB 14199^T), was isolated from marine sediment recovered near Bizerte, Tunisia.

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