Desulfovibrio marinus sp. nov., a moderately halophilic sulfate-reducing bacterium isolated from marine sediments in Tunisia

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Two novel sulfate-reducing bacterial strains, designated E-2^T and IMP-2, were isolated from geographically distinct locations. Strain E-2^T was recovered from marine sediments near Sfax (Tunisia), whereas strain IMP-2 originated from oilfield production fluids in the Gulf of Mexico. Cells were Gram-negative, non-sporulated, motile, vibrio-shaped or sigmoid. They were strictly anaerobic, mesophilic and moderately halophilic. Sulfate, sulfite, thiosulfate and elemental sulfur served as electron acceptors, but not nitrate or nitrite. H₂ (with acetate as carbon source), formate, fumarate, lactate, malate, pyruvate, succinate and fructose were used as electron donors in the presence of sulfate as terminal electron acceptor. Lactate was oxidized incompletely to acetate. Fumarate and pyruvate were fermented. Desulfovibridin and c-type cytochromes were present. 16S rRNA gene sequence analysis of the two strains showed that they were phylogenetically similar (99.0% similarity) and belonged to the genus *Desulfovibrio*, with *Desulfovibrio indonesiensis* and *Desulfovibrio gabonensis* as their closest phylogenetic relatives. The G + C content of the DNA was respectively 60.4 and 62.7 mol% for strains E-2^T and IMP-2. DNA–DNA hybridization experiments revealed that the novel strains had a high genomic relatedness, suggesting that they belong to the same species. We therefore propose that the two isolates be affiliated to a novel species of the genus *Desulfovibrio*, *Desulfovibrio marinus* sp. nov. The type strain is strain E-2^T (=DSM 18311^T =JCM 14040^T).

Sulfate-reducing bacteria (SRB) are widely present in natural habitats with high sulfate concentrations such as marine environments, where they contribute significantly to organic matter mineralization (Fauque & Ollivier, 2004). They are defined by their ability to use sulfate as a terminal electron acceptor during anaerobic respiration. Phylogenetically, they may be divided into four major groups, based on 16S rRNA gene sequence analysis. They include the Gram-negative SRB of the *Deltaproteobacteria*, the Gram-positive spore-forming SRB, the deeply branching thermophilic SRB and the thermophilic archaeal sulfate reducers (Castro *et al.*, 2000; Fauque & Ollivier, 2004; Stackebrandt *et al.*, 1995). The mesophilic members of the *Deltaproteobacteria* represent the largest group of SRB, now including around 40 genera. Members of the genus

Desulfovibrio have been isolated frequently from marine environments, including *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). Halotolerant to halophilic *Desulfovibrio* species have also been recovered from oilfield environments (Birkeland, 2005); these include *Desulfovibrio vietnamensis* (Dang *et al.*, 1996) and *D. longus* (Magot *et al.*, 1992), considered as halotolerant, and *Desulfovibrio gabonensis* (Tardy-Jacquenod *et al.*, 1996), *D. capillatus* (Miranda-Tello *et al.*, 2003), *D. bastinii* (Magot *et al.*, 2004), *D. indonesiensis* (Feio *et al.*, 1998) and *D. gracilis*, considered as moderate halophiles. Although most of these latter species were isolated from oilfield production waters, it is difficult to reach conclusions about the indigenous character of these bacteria with regard to oil reservoirs: are they contaminants or common inhabitants of deep oil reservoirs? This question remains unanswered because of possible contamination through drilling or water-flooding processes

Abbreviation: SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains E-2^T and IMP-2 are respectively DQ365924 and DQ365925.

(Magot, 2005). In this study, we report on the isolation of two moderately halophilic strains of SRB that were recovered from marine sediments contaminated by industrial activities and from an oil–water separation system treating oilfield production fluids. Their phenotypic, genotypic and phylogenetic characteristics suggest that they represent a novel species of the genus *Desulfovibrio*.

Strain E-2^T was isolated from marine sediments contaminated by industrial activities (phosphogypsum disposal resulting from phosphoric acid production) near Sfax (Tunisia), while strain IMP-2 was isolated from an oil–water separation tank treating production fluids from an offshore production platform in the Gulf of Mexico. Samples were collected in sterile glass bottles and kept at room temperature until used. Standard anaerobic techniques were used throughout this study (Balch *et al.*, 1979; Hungate, 1969). Enrichment and isolation were performed using basal SRB growth medium, containing (per litre distilled water) 1 g NH₄Cl, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 0.1 g KCl, 0.1 g CaCl₂, 0.2 g MgSO₄·7H₂O, 30 g NaCl, 0.2 g yeast extract, 0.5 g cysteine hydrochloride, 1 mg resazurin and 10 ml trace mineral element solution (Balch *et al.*, 1979). The pH was adjusted to 7 with 10 M KOH and the medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature. Aliquots were dispensed into Hungate tubes (5 ml) and serum bottles

(20 ml) under a stream of N₂/CO₂ (80:20, v/v) gas and the sealed vessels were autoclaved for 45 min at 110 °C. Prior to inoculation, Na₂S·9H₂O and NaHCO₃ were injected from anaerobic sterile stock solutions to respective final concentrations of 0.04 and 0.2 % (w/v). Liquid cultures were incubated at 35 °C for 1 week. Pure cultures were obtained by repeated application of the agar roll-tube dilution method (Hungate, 1969). Purity of the isolates was checked by microscope observation and inoculation in sulfate-free media containing yeast extract and sugars. *D. gabonensis* DSM 10636^T and *D. indonesiensis* DSM 15121^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and cultivated according to the procedures recommended by the DSMZ. In order to characterize the two isolates phenotypically, standard and specific tests for SRB were performed, including Gram reaction, cell morphology, motility and determination of the electron donors and acceptors used and the presence of desulfoviridin and *c*-type cytochromes (Postgate, 1984), as well as other tests as shown in Table 1 or included in the species description. 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 added to the medium at final concentrations of 20 mM, 20 mM, 2 mM, 2 % (w/v) and 10 mM, respectively.

Table 1. Differentiating physiological and biochemical characteristics of strains E-2^T and IMP-2, *D. gabonensis* DSM 10636^T and *D. indonesiensis* DSM 15121^T

Data for *D. gabonensis* DSM 10636^T and *D. indonesiensis* DSM 15121^T were taken from Tardy-Jacquenod *et al.* (1996) and from Feio *et al.* (1998), respectively, unless indicated. Data for strains E-2^T and IMP-2 were taken from this study. ND, Not determined.

Characteristic	Strain E-2 ^T	Strain IMP-2	<i>D. gabonensis</i> DSM 10636 ^T	<i>D. indonesiensis</i> DSM 15121 ^T
Temperature for growth (°C)				
Range	20–50	25–40	15–40	10–37
Optimum	37	35	30	ND
pH for growth				
Range	6.5–8.5	6.5–8.2	6.4–8.2	6.5–8.5
Optimum	7.0	7.0	6.9–7.3	6.8–7.2
NaCl concentration for growth (%)				
Range	0.5–11	0.5–10	1–17	1–10*
Optimum	5	2	5–6	5–6*
Electron donors				
Malate	+	+	+	—*
Butanol	—	—	+	+/-
Ethanol	+	+	+	—
Fructose	+	+	+	—*
Fumarate fermentation	+	+	+	—*
Electron acceptors				
Elemental sulfur	+	+	+	—*
Fumarate	—	—	+	—*
DNA G + C content (mol%)	60.4	62.7	59.5	58.1*

*Data from this study.

Black, circular colonies, 2.5 mm in diameter, appeared after 1 week of incubation at 37 °C in roll tubes. Cells of strains E-2^T and IMP-2 were vibrioid and motile. The cells were approximately 0.5 µm in diameter and 1.5–2.5 µm long and occurred singly and in chains. Electron microscope observations revealed that both isolates were motile by a polar flagellum. Gram staining was negative and spores were never observed. Strains E-2^T and IMP-2 were strictly anaerobic micro-organisms, slightly to moderately halophilic, growing optimally in media containing 5 and 2.5 % (w/v) salts, respectively. No growth was observed in the absence of NaCl. The optimum pH for both isolates was 7.0. The two isolates grew optimally at 37 °C but they had a slightly different temperature ranges for growth: strain E-2^T grew at 20–50 °C whereas strain IMP-2 grew at 25–40 °C. In the presence of sulfate, both isolates oxidized the following substrates: H₂ (with acetate as carbon source), formate, fumarate, lactate, malate, pyruvate, succinate, ethanol and fructose. Acetate was produced from lactate oxidation. No growth was observed on acetate, benzoate, butyrate, citrate, propionate, valerate, butanol, glycerol, 2-propanol, methanol, glucose, Casamino acids or peptone. Pyruvate was fermented into acetate, H₂ and CO₂, whereas fumarate was disproportionated into acetate and succinate. The isolates used elemental sulfur, sulfate, sulfite and thiosulfate but not fumarate, nitrate or nitrite as electron acceptors. Desulfovibridin was present as bisulfite reductase. Two peaks, at 418 and 550 nm, were detected in cell-free extracts reduced with dithionite, characteristic of *c*-type cytochromes.

The G + C content of the DNA was determined by HPLC at the Identification Service of the DSMZ as 60.4 mol% for strain E-2^T and 62.7 mol% for strain IMP-2. The 16S rRNA genes of strains E-2^T and IMP-2 were amplified and sequenced as described elsewhere (Maidak *et al.*, 2001; Miranda-Tello *et al.*, 2003; Weisburg *et al.*, 1991). Sequences of 1524 and 1478 nucleotides, respectively, of the 16S rRNA genes of strains E-2^T and IMP-2 were determined and aligned manually using the alignment editor BioEdit version 5.0.9 (Hall, 1999). The 16S rRNA gene sequences of the new isolates shared 99.0 % similarity, suggesting that they are closely related phylogenetically. The strains belonged to the genus *Desulfovibrio*, of the class *Deltaproteobacteria*, with *D. indonesiensis* Ind 1^T (98.3 % similarity) and *D. gabonensis* SEBR 2840^T (97.1 % similarity) as their closest phylogenetic relatives (Fig. 1).

DNA–DNA hybridization experiments were carried out at the DSMZ. DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977) and DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) with the modification described by Huß *et al.* (1983) and Escara & Hutton (1980), using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992). A quite high DNA–DNA reassociation value of 64.5 ± 3.8 % (mean of four values) was obtained between strain E-2^T and IMP-2. This value is

Fig. 1. Phylogenetic tree based on a comparison of the 16S rRNA gene sequences of strains E-2^T and IMP-2 and selected members of the genus *Desulfovibrio*. *Desulfovibrio senzeii* DSM 8436^T was taken as an outgroup. Bootstrap values (from 100 replications) are shown at branching points; only values above 80 are shown. Bar, 0.02 substitutions per nucleotide position.

close to the threshold value of 70 % for assignment of strains to the same species (Wayne *et al.*, 1987). Moreover, the few physiological differences that exist between the two isolated strains are not sufficient to differentiate them at the species level (Table 1). Strains E-2^T and IMP-2 should therefore be assigned to the same species of the genus *Desulfovibrio*. DNA–DNA hybridization data revealed relatedness of only 15.7 % between strain E-2^T and *D. indonesiensis* DSM 15121^T and 26.0 % between strain E-2^T and *D. gabonensis* DSM 10636^T. Strain IMP-2 showed 12.4 % relatedness to *D. indonesiensis* DSM 15121^T and 35.2 % relatedness to *D. gabonensis* DSM 10636^T. Among the phenotypic differences that exist between *D. indonesiensis* and the two isolates, the former did not use ethanol, malate or fructose as electron donors or elemental sulfur as a terminal electron acceptor and did not ferment fumarate. In contrast to our isolates, *D. gabonensis* oxidized butanol and used fumarate as an electron acceptor (Table 1). Based on the phenotypic, genotypic and phylogenetic characteristics of our isolates, we propose to assign them to a novel species, *Desulfovibrio marinus* sp. nov. Finally, our results indicated that phylogenetically similar SRB inhabit both marine and oilfield environments. In this respect, marine SRB may be possible contaminants of oil reservoirs through water-flooding processes in particular, thus contributing to oil souring and corrosion problems in the oil industry (Crolet, 2005; Vance & Thrasher, 2005).

Description of *Desulfovibrio marinus* sp. nov.

Desulfovibrio marinus (ma.ri'nus. L. masc. adj. *marinus* of or belonging to the sea, marine).

Cells are strictly anaerobic, vibrio-shaped or sigmoid, 0.5 × 1.5–2.5 µm, occurring singly and in chains. Motile by a polar flagellum. Grows at 20–50 °C, with optimum growth at 37 °C. Grows in the presence of NaCl at 0.5 to 11 %, with optimum growth around 2.5–5 %. The optimum pH for

growth is 7.0; growth occurs at pH 6.5–8.5. Uses H₂, formate, fumarate, lactate, malate, pyruvate, succinate and fructose as electron donors. Lactate is converted to acetate. Substrates that are not used include acetate, benzoate, butyrate, citrate, propionate, valerate, butanol, glycerol, 2-propanol, methanol, glucose, Casamino acids and peptone. Pyruvate and fumarate are fermented. Uses elemental sulfur, sulfate, thiosulfate and sulfite but not fumarate, nitrate or nitrite as electron acceptors. Desulfovibrin and ϵ -type cytochromes are present. The G + C content of DNA of the type strain is 60.4 mol% (HPLC).

The type strain, strain E-2^T (=DSM 18311^T =JCM 14040^T), was isolated from seawater near Sfax (Tunisia). A second strain of the species, IMP-2, was isolated from oilfield production fluids in the Gulf of Mexico.

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