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Desulfovibrio tunisiensis sp. nov., a novel weakly halotolerant, sulfate-reducing bacterium isolated from exhaust water of a Tunisian oil refinery

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A novel weakly halotolerant, sulfate-reducing bacterium, designated strain RB22^T, was isolated from exhaust water of a Tunisian oil refinery. Cells of strain RB22^T were Gram-negative, motile, vibrio-shaped or sigmoid and non-spore-forming, and occurred singly or in chains. Strain RB22^T grew between 15 and 45 °C (optimum, 37 °C) and at pH 4.5 to 9 (optimum, pH 7). NaCl was not required for growth, but the strain tolerated high NaCl concentrations (up to 70 g l^{-1}) with an optimum of 40 g l^{-1}. Sulfate, thiosulfate, sulfite and elemental sulfur served as electron acceptors, but not fumarate. Nitrate and nitrite were not reduced. Strain RB22^T utilized lactate, formate, fumarate, succinate, glycerol, H_2+CO_2 and methanol as substrates. The DNA G+C content was found to be 59.6 mol%. Phylogenetic analysis based on the 16S rRNA gene revealed that the isolate was a member of the genus *Desulfovibrio*, with no close relatives at the species level (16S rRNA gene sequence similarity of less than 95 %). Strain RB22^T exhibited levels of 16S rRNA gene sequence similarity of 94.6 and 94.12 % to the type strains of the closely related species *Desulfovibrio aespoeensis* and *Desulfovibrio dechloracetivorans,* respectively. On the basis of genotypic and phylogenetic characteristics, and significant phenotypic differences, we suggest that strain RB22^T represents a novel species, for which the name *Desulfovibrio tunisiensis* sp. nov. is proposed. The type strain is RB22^T (=NCIMB 14400^T=JCM 15076^T=DSM 19275^T).

Sulfate-reducing bacteria (SRB) cause great economic damage, particularly in the oil industry, owing to their ability to produce hydrogen sulfide. Crude oil that contains elevated amounts of hydrogen sulfide has reduced commercial value: the presence of hydrogen sulfide makes the separation of water from oil less efficient, and ferrous sulfide precipitates can clog drilling and pumping equipment (Cord-Ruwisch, 1985). In addition, the toxicity of hydrogen sulfide is an occupational health and safety hazard for workers. SRB are the most potent contributors to the anaerobic corrosion of metal, which causes costly failures of equipment and pipelines (Hamilton, 1985). An increasing number of species of SRB from oil wells and from oilfield production fluids are being investigated and many mesophilic species of SRB from oilfields and production waters have been described in detail (Magot *et al.*, 1992). There is thus a pressing need to investigate the bacterial communities of oil wells and oilfield production fluids in various geographical locations, in order to identify the complex processes that lead to adverse effects in the industrial environment, and to find a mean of controlling microbial growth (Dang *et al.*, 1996; Ben Ali Gam *et al.*, 2007). Species of the genus *Desulfovibrio* have been isolated frequently from marine environments, including *Desulfovibrio frigidus* (Vandieken *et al.*, 2006), *Desulfovibrio alkalitolerans* (Abildgaard *et al.*, 2006), *Desulfovibrio bizertensis* (Haouari *et al.*, 2006) and *Desulfovibrio 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 *Desulfovibrio longus* (Magot *et al.*, 1992), considered as

Abbreviation: SRB, sulfate-reducing bacteria.

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halotolerant, and *Desulfovibrio marinus* (Ben Dhia-Thabet *et al.*, 2007), *Desulfovibrio bastinii* (Magot *et al.*, 2004), '*Desulfovibrio capillatus*' (Miranda-Tello *et al.*, 2003), *Desulfovibrio indonesiensis* (Feio *et al.*, 1998, 2004) and *Desulfovibrio gabonensis* (Tardy-Jacquenod *et al.*, 1996), considered as moderate halophiles.

In the present study, we undertook microbiological studies to isolate SRB that can tolerate crude oil in the culture medium, obtained from various exhaust waters of a Tunisian oil refinery located at Bizerte (Tunisia). Only members of the genus *Desulfovibrio* were isolated. One of these organisms, isolated from exhaust water of the oil refinery, was designated strain RB22^T, and is proposed to represent a novel species of the genus *Desulfovibrio*.

Samples were collected in sterile glass bottles and stored in the dark at 4 °C until used. Nine strains were isolated from enrichment cultures initiated with crude oil and lactate under either sulfate or thiosulfate reduction conditions. The Hungate technique (Hungate, 1969) was then used throughout for cultivation. The enrichment medium contained (per litre distilled water): 30 g NaCl, 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 1 g NH_4Cl, 0.5 g $MgCl_2.6H_2O$, 0.2 g $CaCl_2$, 1 g yeast extract, 1 g peptone, 1 g cysteine hydrochloride (all w/v), 10 ml trace element solution (Balch *et al.*, 1979) and 1 ml 0.1 % (w/v) resazurin; the pH was adjusted to 7.2 with 10 M KOH solution. The enrichment medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature and 4.5 ml aliquots were distributed in Hungate tubes under a stream of O_2-free N_2 gas. The N_2 gas phase was replaced by N_2/CO_2 (80 : 20, v/v) and the tubes were autoclaved for 21 min at 121 °C. Before inoculation, 0.1 ml 2 % (w/v) $Na_2S.9H_2O$, 0.1 ml 10 % (w/v) $NaHCO_3$ and 1 % (v/v) crude oil were added.

Enrichments were performed in Hungate tubes containing 4.5 ml enrichment medium and were inoculated with water samples. Lactate and either sulfate or thiosulfate were added at 20 mM before inoculation. The tubes were incubated at 30 °C for 1 week. Four enrichment series were performed. Cultures were purified by repeated use of the Hungate roll-tube method, using enrichment medium without crude oil and solidified with 2 % (w/v) agar (Difco). Several colonies were picked and cultured in the corresponding culture medium. The process of isolation was repeated several times until the isolates were deemed to be axenic. Growth was determined by inserting culture tubes directly into a model Cary 50 Scan spectrophotometer (Varian) and measuring the OD_{580}. Sulfide was assayed photometrically as colloidal CuS according to the method of Cord-Ruwisch (1985). *Desulfovibrio halophilus* DSM 5663^T and '*Desulfovibrio brasiliensis*' DSM 15816 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and cultivated according to the procedures recommended by the DSMZ.

DNA was extracted from various isolates, including strain RB22^T, according to the protocol described for the Wizard Genomic DNA purification kit (Promega). The 16S rRNA genes of these isolates, including strain RB22^T, were amplified using primers Fd1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGCC-3′) and with the following reaction conditions: 1 min at 96 °C, 30 cycles of 20 s at 96 °C, 30 s at 55 °C and 2 min at 72 °C, with a final elongation step for 5 min at 72 °C (Abdelkafi *et al.*, 2006b; Ben Ali Gam *et al.*, 2007). PCR products were then cloned into pGEM-T-easy (Promega) according to the manufacturer's protocol. Recombinant clones with inserts of the expected size were sequenced by using the vector-specific primers SP6 (5′-ATTTAGGTGACACTATAGAA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) (Genome Express). Plasmids containing inserts of the expected size were isolated using a Wizard Plus SV Minipreps DNA purification system (Promega), according to the manufacturer's protocol. Purified plasmids were sent for sequencing to GATC (Konstanz, Germany). Sequence data were imported into the sequence editor BioEdit version 5.0.9 (Hall, 1999); base calls were examined and a contiguous sequence was obtained. The full sequence was aligned using the RDP version 8 Sequence Aligner program (Maidak *et al.*, 2001) and was adjusted manually to conform to the 16S rRNA gene secondary structure model (Lane *et al.*, 1985). A non-redundant BLAST search (Altschul *et al.*, 1997) was used to identify the closest relatives of strain RB22^T. Sequences used in the phylogenetic analysis were obtained from RDP Version 8 (Maidak *et al.*, 2001) and GenBank databases (Benson *et al.*, 1999). Positions of sequence and alignment ambiguity were omitted and pairwise evolutionary distances were calculated using the method of Jukes & Cantor (1969). A dendrogram was constructed using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined using 100 bootstrapped trees (Felsenstein, 1985).

After 2 days of incubation at 30 °C with crude oil, the enrichment culture supported growth. Several strains were isolated from exhaust water of the Tunisian oil refinery in the presence of yeast extract, peptone and lactate as substrates and either sulfate or thiosulfate as terminal electron acceptor.

Amongst the strains isolated from exhaust water of the Tunisian oil refinery, four strains (RB21, RB22^T, API4T and API2B) were found to belong to the genus *Desulfovibrio*. Strains API4T and API2B were found to be closely related to *Desulfovibrio senezii* DSM 8436^T (Tsu *et al.*, 1998), with 99.9 % 16S rRNA gene sequence similarity. The closest phylogenetic relative of strains RB22^T and RB21 was *Desulfovibrio aespoeensis* DSM 10631^T (Motamedi & Pedersen, 1998), but with low 16S rRNA gene sequence similarity (<97 %). The gene sequence similarity of strain RB22^T with *D. aespoeensis* DSM 10631^T was determined to be 94.6 %. These results indicated that strains RB21 and RB22^T could represent a novel species of the genus *Desulfovibrio*. Strain RB22^T was chosen as the type strain and was characterized further.

Cells of strain RB22^T were Gram-negative and spores were never observed. Strain RB22^T was mesophilic and strictly anaerobic. Cells were vibrio-shaped or sigmoid, 0.5 × 1–2.5 μm, and occurred singly or in chains. For the determination of the NaCl requirement, various amounts of NaCl were weighed directly in tubes, prior to dispensing NaCl-free enrichment medium, to obtain NaCl concentrations of 0–200 g l^{-1} (Abdelkafi *et al.*, 2006a; Ben Ali Gam *et al.*, 2007). The isolate was regarded as being a weakly halotolerant bacterium and grew in the presence of NaCl concentrations ranging from 0 to 70 g l^{-1}, with optimum growth occurring at 40 g NaCl l^{-1}. Strain RB22^T did not require NaCl for growth. For pH studies, the medium was adjusted to the desired pH using anaerobically prepared stock solutions of $NaHCO_3$ (10 %) or Na_2CO_3 (10 %). The optimum pH for growth was 7.0 and growth occurred between pH 4.5 and 9. The optimum temperature for growth was 37 °C (temperature range for growth was 15–45 °C).

Table 1. Differential physiological and biochemical characteristics of strains RB22^T (*Desulfovibrio tunisiensis* sp. nov.), *D. halophilus* DSM 5663^T and '*D. brasiliensis*' DSM 15816

Data for *D. halophilus* DSM 5663^T and '*D. brasiliensis*' DSM 15816 were taken from Caumette *et al.* (1991) and Warthmann *et al.* (2005), respectively. Data for strain RB22^T were from this study. ND, Not determined.

Characteristic	Strain RB22^T	*D. halophilus* DSM 5663^T	'*D. brasiliensis*' DSM 15816
Temperature for growth (°C)			
Range	15–45	17–42	15–45
Optimum	37	35	33
pH for growth			
Range	4.5–9	5.5–8.5	6.3–9.0
Optimum	7.0	7.0	7.6
NaCl concentration for growth (%)			
Range	0–7	3–18	1–15
Optimum	4	6–7	3–10
Electron donors (with either sulfate or thiosulfate)			
Malate	−	−	+
Butanol	−	−	ND
Ethanol	−	+	−
Fumarate	+	−	+
Formate	+	ND	+
Methanol	+	ND	−
Electron acceptors (with lactate as energy and carbon source)			
Fumarate	−	−	+
Thiosulfate	+	+	−
DNA G+C content (mol%)	59.6	60.7	56.3

Substrate utilization tests were performed with modified enrichment medium (i.e. without crude oil and peptone, with 0.1 g yeast extract and with either sulfate or thiosulfate as a terminal electron acceptor).

Strain RB22^T utilized the following substrates (20 mM) as carbon and energy sources: lactate, $H_2 + CO_2$ (80 : 20, v/v; 2 bars), formate, fumarate, succinate, glycerol and methanol. Acetate, malate, propionate, ethanol, butanol and 2-propanol were also tested but did not support growth. Sulfate (20 mM), thiosulfate (20 mM), elemental sulfur (1 %, w/v), sulfite (2 mM) and bisulfite (0.1 %, w/v) were used as terminal electron acceptors, but not fumarate, nitrate or nitrite (10 mM). 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.

Other characteristics of strain RB22^T, in comparison with those of closely related species of the genus *Desulfovibrio*, are shown in Table 1 and were determined as described previously (George *et al.*, 2008; Alazard *et al.*, 2003).

The G+C content of the genomic DNA of strain RB22^T was 59.6 mol%, as determined by using the HPLC method (Mesbah *et al.*, 1989). This value fell within the range described for species of the genus *Desulfovibrio* (Claus & Berkeley, 1986).

Phylogenetic analyses, based on 16S rRNA gene sequence data, indicated that strain RB22^T was most closely related to species of the genus *Desulfovibrio* (Fig. 1). Strain RB22^T exhibited levels of 16S rRNA gene similarity of 94.6, 94.12, 93.88 and 93.69 % to the type strains of *D. aespoeensis*, *Desulfovibrio dechloracetivorans*, '*Desulfovibrio caledoniensis*' and *D. halophilus*, respectively. Levels of 16S rRNA gene similarity between strain RB22^T and other strains used in the phylogenetic analysis were less than 95 %. The generally recommended and accepted criteria for delineating bacterial species state that strains with 16S rRNA gene sequence similarities of less than 97 % do not belong to the same species (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987), with no need for DNA–DNA relatedness studies.

Description of *Desulfovibrio tunisiensis* sp. nov.

Desulfovibrio tunisiensis (tu.ni.si.en′sis. N.L. masc. adj. *tunisiensis* of Tunisia, pertaining to Tunisia).

Cells are strictly anaerobic, motile, vibrio-shaped or sigmoid, 0.5 × 1–2.5 μm, and occurr singly and in chains. Grows at 15–45 °C, with optimum growth at 37 °C. Grows in the presence of NaCl at 0–7 % (w/v), with optimum growth around 4 %. Optimum pH for growth is 7.0; growth occurs at pH 4.5–9. Utilizes $H_2 + CO_2$, formate, fumarate, lactate, succinate, glycerol and methanol as electron donors.

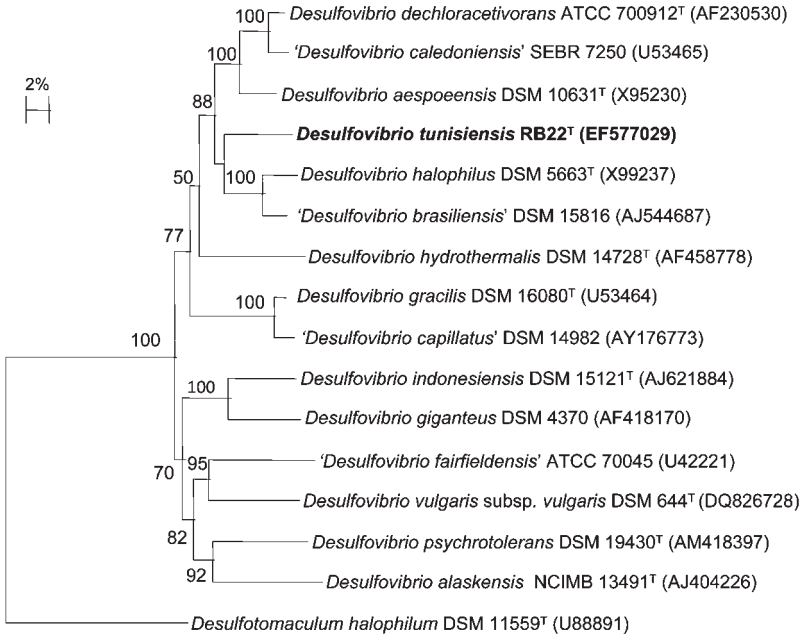

Fig. 1. Phylogenetic dendrogram based on 1347 unambiguous bp of 16S rRNA gene sequences indicating the position of strain RB22^T and closely related species of the genus *Desulfovibrio*. The tree was constructed using the neighbour-joining algorithm. Accession numbers are given in parentheses. Bar, 0.02 substitutions per nucleotide position.

Lactate is converted to acetate. Substrates that are not utilized include acetate, malate, benzoate, propionate, ethanol, butanol, 2-propanol, glucose and peptone. Fumarate and pyruvate are fermented. Utilizes elemental sulfur, sulfate, thiosulfate and sulfite but not fumarate, nitrate or nitrite as electron acceptors. Desulfoviridin and *c*-type cytochromes are present. The G+C content of the DNA of the type strain is 59.6 mol% (HPLC).

The type strain, strain RB22^T (=NCIMB 14400^T=JCM 15076^T=DSM 19275^T), was isolated from exhaust water of a Tunisian oil refinery.

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