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Characterization of *Desulfovibrio salinus* sp. nov., a slightly halophilic sulfate-reducing bacterium isolated from a saline lake in Tunisia

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Abstract

A novel slightly halophilic sulfate-reducing bacterium, designated strain P1BSR^T, was isolated from water of a saline lake in Tunisia. Strain P1BSR^T had motile (single polar flagellum), Gram-negative, rod-shaped, non-spore-forming cells, occurring singly or in pairs. Strain P1BSR^T grew at temperatures between 15 and 45 °C (optimum 40 °C), and in a pH range between 6 and 8.5 (optimum pH 6.7). The strain required NaCl for growth (1 % w/v), and tolerated high NaCl concentration (up to 12 % w/v) with an optimum of 3 % (w/v). Sulfate, thiosulfate and sulfite served as terminal electron acceptors, but not elemental sulfur, fumarate, nitrate and nitrite. Strain P1BSR^T utilized lactate, pyruvate, formate, D-fructose and glycerol as carbon and energy sources. The main cellular fatty acid was $C_{16:0}$ (50.8 %). The genomic DNA G+C content was 47.7 mol%. Phylogenetic analysis of 16S rRNA gene sequence similarity indicated that strain P1BSR^T was affiliated to the genus *Desulfovibrio*, with the type strains *Desulfovibrio salexigens* (96.51 %), *Desulfovibrio zosterae* (95.68 %), *Desulfovibrio hydrothermalis* (94.81 %) and *Desulfovibrio ferrireducens* (94.73 %) as its closest phylogenetic relatives. On the basis of genotypic, phenotypic and phylogenetic characteristics, it is proposed to assign strain P1BSR^T to a novel species of the genus *Desulfovibrio*, *Desulfovibrio salinus* sp. nov. The type strain is P1BSR^T (=DSM 101510^T=JCM 31065^T).

Sulfate-reducing prokaryotes (SRPs) are mainly chemoheterotrophs, both *Bacteria* and *Archaea* (250 species of 65 genera), that can use sulfate as a terminal electron acceptor in their energy metabolism [1, 2]. Owing to their broad metabolic capacities, sulfate-reducing bacteria (SRB) are important in the mineralization of organic matter in anoxic marine sediments. Dissimilatory sulfate reduction has been observed in various hypersaline environments such as salterns, the Dead Sea and the Great Salt Lake [3]. However, most of the halophilic SRPs isolated so far are marine or slightly halophilic microorganisms [with optimum salinity ranging from 1 to 4 % (w/v) NaCl] belonging to several genera of SRPs, including *Desulfovibrio*, *Desulfonatronovibrio* and *Desulfonatronobacter* species [3–6]. The first SRB species belonging to the genus *Desulfovibrio* isolated from a hypersaline environment was *Desulfovibrio salexigens*, which did not grow at NaCl concentrations higher than 12 % (w/v) [7]. Cord-Ruwish [8] then isolated several strains of SRBs from hypersaline oilfield water containing about 10 % (w/v) NaCl. One isolate grew slowly up to 27 % (w/v) NaCl but has not been described in more detail since. One year later, Trüper and Galinski [9] isolated a few SRB strains from hot brines in the Red Sea that were similar to *Desulfovibrio halophilus*, a moderately halophilic sulfate reducer isolated by Caumette *et al.* [10] from the hypersaline Solar Lake in Sinai. The latter isolate grew in salinity ranging from 3 to 18 % (w/v), and optimally at 6–7 % (w/v) NaCl. However, since 1991, no novel SRB species belonging to the genus *Desulfovibrio* has been characterized that grows in salt concentrations above 10 % NaCl (w/v). No pure culture of extreme halophilic SRB (i.e. growing under saturating-salt conditions) has been isolated until now.

In this study, we report the isolation and characterization of a mesophilic, slightly halophilic SRB isolated from water samples of a Tunisian saline lake that is able to grow in up to 12 % NaCl (w/v), but grows optimally at 3 % (w/v). This SRB isolate is proposed to represent a novel species of the genus *Desulfovibrio*.

Water samples were collected from a continental saline lake located in the middle-east of Tunisia and transported to the laboratory at ambient temperature. Bacteria were

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Abbreviations: SRB, sulfate-reducing bacteria; SRP, sulfate-reducing prokaryote.
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isolated and cultivated under strict anaerobiosis according to the Hungate technique [11], modified for use with syringes [12, 13].

The basal culture medium (BM) for isolation included (per litre): 0.3 g KH_2PO_4, 0.2 g K_2HPO_4, 0.3 g NH_4Cl, 30 g NaCl, 0.5 g KCl, 0.1 g $CaCl_2$, 0.4 g $MgCl_2$, 0.5 g cysteine HCl, 0.5 g yeast extract (Difco), 1 ml mineral element solution and 1 ml 0.1 % (w/v) resazurin [14]. The pH was adjusted to 7.2 with 10 M KOH solution and the medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Aliquots of 5 ml were dispensed into Hungate tubes under N_2/CO_2 (80 : 20, v/v) and subsequently sterilized by autoclaving at 120 °C for 20 min. Before inoculation, 0.1 ml 10 % (w/v) $NaHCO_3$, 0.1 ml 2 % (w/v) $Na_2S.9H_2O$, 20 mM lactate and 20 mM sulfate were injected from sterile stock solutions into the tubes. $Na_2S.9H_2O$ as a reducing agent was omitted when H_2S production was determined [8].

Enrichments were performed in Hungate tubes or serum bottles inoculated with 10 % (v/v) sample and incubated at 30 °C. The culture was purified by repeated use of the Hungate roll tube method, using agar solid medium (0.8 % w/v), and transferred to liquid medium.

The pH, temperature and NaCl concentration ranges for growth were determined in duplicate experiments using BM supplemented with lactate (20 mM) as electron donor and sulfate (20 mM) as previously described [15]. The pH (from 5 to 9) of the culture medium was adjusted by injecting aliquots of anaerobic stock solution into Hungate tubes containing 100 mM HCl for low pH or either 10 % $NaHCO_3$ (w/v) or 8 % Na_2CO_3 (w/v) for high pH. Water baths were used to incubate bacterial cultures from 15 to 50 °C. NaCl requirement was determined by directly weighing NaCl into the Hungate tubes before dispensing the medium. Cultures were subcultured at least twice under the same experimental conditions before determination of growth rates.

Bacterial growth was monitored by measuring the increase in turbidity at 580 nm after inserting Hungate tubes into the cuvette holder of a Cary 50 UV–vis spectrophotometer (Varian). H_2S production was determined photometrically as colloidal CuS following the method described in [8].

Morphological characteristics and purity of strains were checked under an Optiphot (Nikon) phase-contrast microscope. Presence of spores was analysed by phase-contrast microscopy observations of cultures and after pasteurization tests performed for 10 and 20 min at 80, 90 and 100 °C.

Culture of the isolate (strain P1BSR^T) was stopped at the end of the exponential phase and sent to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) for cellular fatty acid analysis. Fatty acids were extracted using the Miller method [16] as modified by Kuykendall *et al.* [17], and the cellular fatty acid profile was analysed by gas chromatography using the Microbial Identification System (MIDI, Sherlock Version 6.1; database, TSBA40; gas chromatograph, model 6890 N; Agilent Technologies). G+C content was determined by HPLC as described by [18].

Substrate utilization was tested in the presence of 0.5 g yeast extract l^{-1}. Substrates (20 mM except where indicated) included lactate, pyruvate, acetate, formate, fumarate, malate, succinate, propionate, methanol (0.1 % w/v), ethanol (0.1 % w/v), butanol (0.1 % w/v), glycerol (0.1 % w/v), D-fructose, D-glucose and H_2/CO_2 (20 : 80 v/v). Elemental sulfur (1 % w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM), fumarate (10 mM), nitrate (10 mM) and nitrite (2 mM) were tested as potential terminal electron acceptors.

Extraction and purification of total DNA followed by amplification and sequencing of the 16S rRNA gene were performed as previously described [15]. The 16S rRNA gene sequence was then compared with available sequences found in GenBank using the BLASTN search [19]. Evolutionary history was inferred using the neighbour-joining method [20]. A multiple alignment was built using the MUSCLE program [21] implemented in MEGA6 [22]. Sequence positions with alignment uncertainty and gaps were omitted from the analysis. Evolutionary analyses were conducted in MEGA6 using the neighbour-joining method. Evolutionary distances were computed using the maximum-composite-likelihood method [23]. The final dataset consisted of a total of 1213 positions. Branch robustness was estimated by the non-parametric bootstrap procedure implemented in MEGA6 (1000 replicates of the original dataset). The tree was

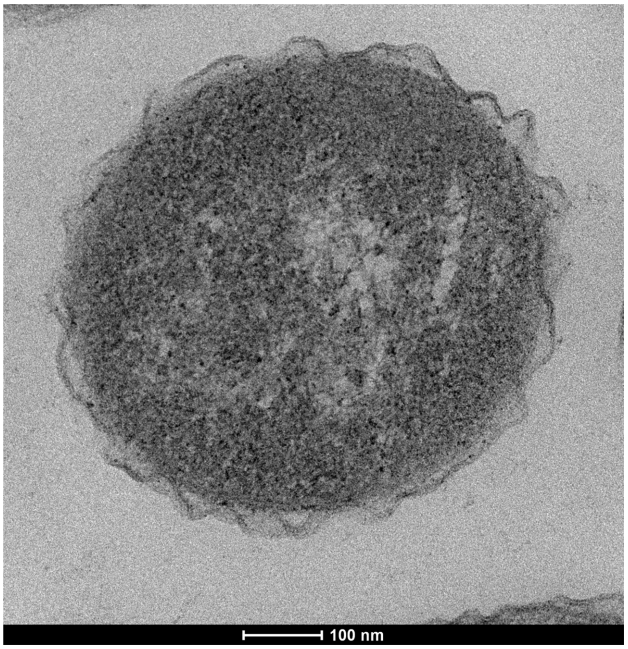

Fig. 1. Transmission electron micrograph of an ultrathin section of a cell of strain P1BSR^T showing a thin peptidoglycan layer and an outer crenated membrane; bar, 100 nm.

drawn to scale, with branch lengths measured in number of substitutions per site [24].

Several colonies developed after incubation at 30 °C and were picked separately. Colonies were black and circular with diameters ranging from 1.0 to 2.0 mm after 1 week of incubation at 30 °C. The serial dilution process was repeated several times until the isolates were deemed to be axenic. Purity of the isolates was checked by microscopy and inoculation in sulfate-free medium containing yeast extract and D-glucose to confirm the absence of contamination by fermentative microorganisms. Several strains were isolated that showed similar morphology, size and metabolic profiles, and the same phylogenic inference was obtained for all of them. One strain, designated P1BSR^T, was selected and used for further metabolic and physiological characterization.

Cells of strain P1BSR^T were Gram-stain-negative rods (0.7–0.9×2.3 µm) when grown on medium containing lactate as an electron donor and sulfate as a terminal electron acceptor. Ultrathin sections showed a typical Gram-negative cell wall with a thin peptidoglycan layer and a crenated outer membrane (Fig. 1).

The optimal temperature for growth was 40 °C (range 15–45 °C) and the optimum pH was 6.7 (range pH 6–8.5). The strain required a minimum of 1 % (w/v) NaCl for growth, and tolerated up to 12 % (w/v); optimum growth occurred with 3 % (w/v) NaCl. All substrates were used by strain P1BSR^T with incomplete oxidation. Amongst the substrates tested, only lactate, pyruvate, formate, glycerol and D-fructose were used as electron donors. End products of pyruvate, fumarate and malate were not fermented. Thiosulfate, sulfate and sulfite, but not fumarate, elemental sulfur, nitrate or nitrite served as terminal electron acceptors. Malate, succinate, methanol, ethanol, butanol, D-glucose, acetate, propionate and H_2/CO_2 did not support growth. The presence of yeast extract in minimal medium with lactate as the only energy and carbon source was required for growth.

Based on the nearly complete (1536 bp) 16S rRNA gene sequence of strain P1BSR^T, the phylogenetic tree obtained by the neighbour-joining method, as shown in Fig. 2, confirms that strain P1BSR^T is an SRB belonging to the genus *Desulfovibrio*, sharing 96.51 % 16S rRNA gene sequence identity with its closest phylogenetic relatives, i.e. *Desulfovibrio salexigens* DSM 2638^T [4, 7, 25] isolated from sling mud (British Guiana), 95.68 % with *Desulfovibrio zosterae*

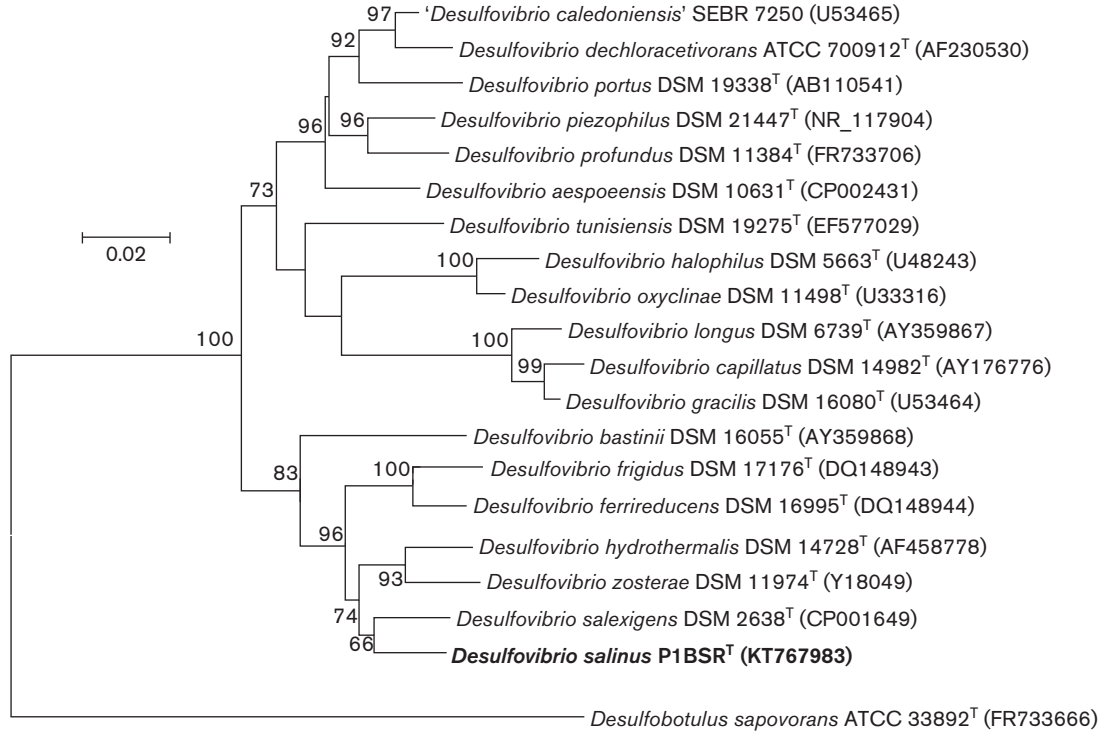

Fig. 2. Phylogenetic dendrogram based on 1368 unambiguous base pairs of 16S rRNA gene sequence data, indicating the position of strain P1BSR^T and its closest relative sequences validated at a species level belonging to the genus *Desulfovibrio*. The tree was reconstructed using the neighbour-joining algorithm based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 2000 replications) greater than 60 % are shown at branch points. *Desulfobotulus sapovorans* 33892^T was used as outgroup. Numbers in parentheses are Genbank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences. Bar, 2 % estimated sequence divergence.

DSM 11974^T [26], 94.81 % with *Desulfovibrio hydrothermalis* DSM 14728^T [27] and 94.73 % with *Desulfovibrio ferrireducens* DSM 16995^T [28], thus indicating that strain P1BSR^T represents a novel species of the genus *Desulfovibrio*. Further evidence resulted from several phenotypic and biochemical differences, including cell morphology, but also comparison of the utilization of carbon and energy sources with its two most-closely-related *Desulfovibrio* species (*Desulfovibrio salexigens* DSM 2638^T and *Desulfovibrio zosterae* DSM 11974^T) (Table 1). Indeed, in contrast to *Desulfovibrio salexigens* showing typically vibrio-shaped cells, as observed for most *Desulfovibrio* species, P1BSR^T cells were rod-shaped and occurred singly or in pairs. These three *Desulfovibrio* strains were mesophilic with different optimum temperatures (Table 1). Strain P1BSR^T and *Desulfovibrio salexigens* DSM 2638^T have similar NaCl requirement (0.6 to 1 % w/v) and can tolerate up to 12 % (w/v) NaCl (Table 1). The three strains share similar genomic DNA G+C content (42.7 to 47.7mol%). The three strains differed slightly in the utilization of growth substrates and terminal electron acceptors (Table 1). Strain P1BSR^T was unable to use H_2/CO_2 and ethanol as electron donors in contrast to *Desulfovibrio salexigens* DSM 2638^T. Strain P1BSR^T was unable to use fumarate and malate as electron donors, in contrast to *Desulfovibrio salexigens* DSM 2638^T and *Desulfovibrio zosterae* DSM 11974^T (Table 1). All the three strains reduced sulfate, thiosulfate and sulfite, but in contrast to P1BSR^T, *Desulfovibrio salexigens* DSM 2638^T utilized fumarate and nitrate as terminal electron acceptors (Table 1). The main cellular fatty acids of strain P1BSR^T were $C_{16:0}$ (50.8 %), iso-$C_{15:0}$ (9.4 %), iso-$C_{17:1}$ C (9 %) and $C_{16:1}$*cis*9 (8.8 %) (Table 2). The fatty acid pattern of strain P1BSR^T clearly distinguished it from its closest relative *Desulfovibrio salexigens* (Table 2). *Desulfovibrio salexigens* showed a predominance of iso-$C_{17:1}$ C (25.5 % versus 9 % in strain P1BSR^T), whereas strain P1BSR^T showed a predominance of $C_{16:0}$ (50.8 vs 21.6 % in *Desulfovibrio salexigens*) (Table 2).

Therefore, based on its phenotypic, chemotaxonomic and phylogenetic characteristics, we propose strain P1BSR^T as a novel species of the genus *Desulfovibrio*, for which the name *Desulfovibrio salinus* sp. nov. is proposed.

Table 1. Physiological and biochemical characteristics of strain P1BSR^T in comparison with its two most-closely-related *Desulfovibrio* species

Strains: 1, P1BSR^T (data from this study); 2, *Desulfovibrio salexigens* (DSM 2638^T) [4, 7, 25]; 3, *Desulfovibrio zosterae* (DSM 11974^T) [26]. +, Positive; −, negative; NR, not reported.

Characteristic	1	2	3
Shape	Rod	Vibrio	Curved rod
Cell size (μm)	0.7–0.9×2–3	0.5–0.8×1.3–2.5	0.5×3.0
Habitat	Saline lake	Marine or estuarine mud	Roots
Temperature range (°C)	15–45	Up to 45	5–34.5
Optimum temperature	40	30–36	32.5–34.5
pH range	6–8.5	NR	5.5–7.5
Optimum pH	6.7	7.8	6.8–7.3
NaCl range (%)	1–12	0.5–12	0–3.5
Optimum NaCl (%)	3	2.0–4.0	1.2
DNA G+C content (mol%)	47.7	47.1	42.7
Electron donor (with sulfate):			
H_2/CO_2	−	+	−
Lactate	+	+	+
Malate	−	+	+
Fumarate	−	+	+
Formate	+	+	−
Acetate	−	−	−
Propionate	−	−	−
D-Fructose	+	+	+
Ethanol	−	+	+
Glycerol	+	+	NR
Electron acceptor with lactate as energy and carbon source:			
Sulfate	+	+	+
Thiosulfate	+	+	+
Sulfite	+	+	+
Elemental sulfur	−	−	+
Nitrate	−	+	−
Fumarate	−	+	+

Table 2. Comparison of the main fatty acids (%) of strain P1BSR^T with its closest relative *Desulfovibrio salexigens* (Method TSBA40; Calcul method ANAER6)

Strains: 1, P1BSR^T (data from this study); 2, *Desulfovibrio salexigens* (DSM 2638^T) (data from [26]).

Fatty acid	Percentage (w/v) of total fatty acids	
	1	2
$C_{14:0}$	1.6	1.0
iso-$C_{15:0}$	9.4	15.8
iso-$C_{16:1}$ H	1.2	0.3
iso-$C_{16:0}$	1.6	0.3
$C_{16:1}$*cis*9	8.8	9.8
$C_{16:0}$	50.8	21.6
iso-$C_{17:1}$ C	9.0	25.5
anteiso-$C_{17:1}$ C	2.3	4.0
iso-$C_{17:0}$	3.0	5.4
anteiso-$C_{17:0}$	1.0	1.7
$C_{18:1}$*cis*9	0.5	0.3
$C_{18:1}$*cis*11/*trans*9/*trans*6	1.0	5.1
$C_{18:0}$	4.4	1.4

DESCRIPTION OF *DESULFOVIBRIO SALINUS* SP. NOV.

Desulfovibrio salinus (sa.li'nus. N. L. masc. adj. *salinus* salty).

Rod-shaped cells, 0.7–0.9 µm wide and 2–3 µm long, occurring singly or in pairs and motile by a single polar flagellum. pH range for growth: 6–8.5, optimum at pH 6.7. Temperature range for growth: 15–45 °C, optimum at 40 °C. NaCl 1 % (w/v) is required for growth. NaCl range for growth: 1–12% NaCl (w/v), with optimum growth at 3 % (w/v). All substrates are used with incomplete oxidation. Formate, D-fructose, lactate, pyruvate and glycerol are utilized as electron donors. Substrates not utilized include malate, succinate, methanol, ethanol, D-glucose, acetate, propionate and H_2/CO_2. Pyruvate is not fermented. Utilizes sulfate, thiosulfate and sulfite as terminal electron acceptors but not elemental sulfur, fumarate, nitrate or nitrite.

The type strain is P1BSR^T(=DSM 101510^T =JCM 31065^T), isolated from water collected from a saline lake in Tunisia. The G+C content of the DNA of the type strain is 47.7 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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