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Desulfovibrio legallis sp. nov.: A Moderately Halophilic, Sulfate-Reducing Bacterium Isolated from a Wastewater Digestor in Tunisia

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Abstract A new moderately halophilic sulfate-reducing bacterium (strain H_1^T) was enriched and isolated from a wastewater digestor in Tunisia. Cells were curved, motile rods (2–3 x 0.5 μm). Strain H_1^T grew at temperatures between 22 and 43°C (optimum 35°C), and at pH between 5.0 and 9.2 (optimum 7.3–7.5). Strain H_1^T required salt for growth (1–45 g of NaCl/l), with an optimum at 20–30 g/l. Sulfate, sulfite, thiosulfate, and elemental sulfur were used as terminal electron acceptors but not nitrate and nitrite. Strain H_1^T utilized lactate, pyruvate, succinate, fumarate, ethanol, and hydrogen (in the presence of acetate and CO_2) as electron donors in the presence of sulfate as electron acceptor. The main end-products from lactate oxidation were acetate with H_2 and CO_2. The G + C content of the genomic DNA was 55%. The predominant fatty acids of strain H_1^T were $C_{15:0}$ iso (38.8%), $C_{16:0}$ (19%), and $C_{14:0}$ iso 3OH (12.2%), and menaquinone MK-6 was the major respiratory quinone. Phylogenetic analysis of the small-subunit (SSU) ribosomal RNA (rRNA) gene sequence indicated that strain H_1^T was affiliated to the genus *Desulfovibrio*. On the basis of SSU rRNA gene sequence comparisons and physiological characteristics, strain H_1^T is proposed to be assigned to a novel species of sulfate reducers of the genus *Desulfovibrio*, *Desulfovibrio legallis* sp. nov. (= DSM 19129^T = CCUG 54389^T).

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Introduction

Sulfate-reducing bacteria (SRB) are anaerobic chemolithotrophic microorganisms, both bacteria and archaea (230 species of 60 genera), that can utilize sulfate as terminal electron acceptor in their energy metabolism [1, 9]. SRB are of major numerical and functional importance in many ecosystems including marine sediments, polluted environments such as anaerobic purification plants, cyanobacterial microbial mats, oil fields environments, rice fieds, deep-sea hydrothermal vents and even in human diseases [1, 9, 10, 16, 19, 22, 23].

In this study, we report on the isolation of a moderately halophilic SRB, strain H_1^T, recovered from a sludge bioreactor treating wastewater in Tunisia. The characteristics of this strain suggest that it is a new species of the genus *Desulfovibrio*, phylogenetically related with the taxon of *Desulfovibrio*: (*D.*) *desulfuricans* [7], *D. fairfieldensis* [17], *D. piger* [18], and *D. intestinalis* [11].

Materials and Methods

Preparation of Media, Isolation and Characterization of the Microorganism

Strain H_1^T was isolated from an anaerobic reactor inoculated with a mixture of two anaerobic sludges obtained from an anaerobic digestor treating urban wastewater and from Tunisian lake sediments [2].

The enrichment culture was performed in 60-ml serum bottles inoculated with 2-ml sample. The basal medium contained (per liter of distilled water): 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 1 g NH_4Cl, 23 g NaCl, 3 g Na_2SO_4, 0.1 g KCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.5 g cysteine. HCl, 0.1 g yeast extract

(Difco Laboratories), 1-ml trace mineral element solution of Widdel and Pfennig [29], and 1 ml of 0.1% resazurin. The pH was adjusted to 7.2 with 10 M KOH solution. 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, degassed under N_2–CO_2 (80:20%, v/v), and subsequently sterilized by autoclaving at 110°C for 45 min. Before culture inoculation, 0.1 ml of 10% (w/v) $NaHCO_3$, 0.1 ml of 2% (w/v) $Na_2S{\cdot}9H_2O$, and 0.1 ml 15% $MgCl_2{\cdot}6H_2O$ were injected from sterile stock solutions into the tubes. The Hungate technique [14] was used throughout this study. A 0.5-ml aliquot of sample was inoculated into Hungate tubes containing 5 ml of basal medium and lactate (20 mM). The tubes were incubated at 37°C. To obtain pure cultures, the enrichment was subcultured several times under the same growth conditions before the isolation. For isolation, the culture was serially diluted tenfold in roll tubes (basal medium containing 1.6% agar); several colonies developed after incubation at 37°C and were picked separately. The process of serial dilution was repeated several times until the isolates were deemed to be axenic. Several strains similar in morphology and phylogeny and growing on lactate were isolated. A strain designated H_1^T was selected and used for further characterization.

Morphological characteristics and purity of strain H_1^T were observed with an Optiphot (Nikon) phase-contrast microscope. For transmission electron microscopy studies, cell preparations were negatively stained with sodium phosphotungstate, as previously described [8].

Growth experiments were performed in duplicate, using Hungate tubes containing basal medium. The basal medium was the same as enrichment medium with 20 mM sodium lactate as substrate; it was used to determine the pH, temperature, and NaCl concentration ranges for growth of strain H_1^T. The pH of the medium was adjusted by injecting in Hungate tubes aliquots of anaerobic stock solutions of 1 M HCl (for acidic pHs), 10% $NaHCO_3$, or Na_2CO_3 (for alkaline pHs). Water baths were used to obtain incubation temperatures from 15 to 55°C. For studies of NaCl requirements, NaCl was weighed directly in the tubes before the medium was dispensed. The strain was subcultured under the same experimental conditions before the growth rates were determined. Each substrate was tested in basal medium at a final concentration of 20 mM. Elemental sulfur (1% w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM), nitrate (10 mM), and nitrite (2 mM) were tested as terminal electron acceptors. H_2S production was determined photometrically as colloidal CuS following the method described by Cord-Ruwisch [6].

The sensitivity of strain H_1^T to three antibiotics (chloramphenicol, penicillin, and streptomycin) was tested until 500 μg/ml. Controls with ethanol (solvent for chloramphenicol) were included. The presence of spores was checked by microscopic observation of cultures and pasteurization tests performed at 80, 90, and 100°C for 10 and 20 min. End-products of lactate and pyruvate metabolism were measured by high pressure liquid chromatography (HPLC) and gas chromatography (for the gaseous products) after 2 weeks of incubation at 37°C as previously described [13]. Cytochromes and desulfoviridin (dissimilatory bisulfite reductase) were determined on the crude bacterial extract according to the methods described by Postgate [24].

Bacterial growth was monitored by measuring the increase in turbidity at 580 nm by insertion of Hungate tubes into the cuvette holder of a spectrophotometer (Cary 50, Varian).

The G + C content of DNA of strain H_1^T was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. DNA was isolated and purified by chromatography on hydroxyapatite using the procedure of Cashion et al. [4], and the G + C content was determined using HPLC as described by Mesbah et al. [21]. Cellular fatty acids of strain H_1^T were determined from cultures in late-exponential growth phase in basal medium supplemented with lactate and sulfate. Fatty acids were extracted and analyzed by gas chromatography at the DSMZ following the method described by Vainshtein et al. [27]. Menaquinones were extracted and identified at the DSMZ, Braunschweig, Germany.

DNA Extraction, PCR Amplification, and DNA Sequencing

The genomic DNA of strain H_1^T was extracted using the Wizard Genomic DNA Purification kit, according to the manufacturer's protocol (Promega). The universal primers Fd1 (5′-CAGAGTTTGATCCTGGCTCAG-3′) and R6 (5′-TACGGTTACCTTGTTACGAC-3′) were used to amplify the SSU rRNA gene. Direct sequencing of PCR product was performed by GATC (Germany).

The 16S rRNA gene sequence of the strain H_1^T was imported into the sequence editor BioEdit version 5.0.9 [12]. Reference sequences were obtained from the Ribosomal Database Project II [20] and GenBank databases [3]. Position of sequence and alignment uncertainty were omitted from the analysis. The sequence position and pairwise evolutionary distances based on 1107 unambiguous nucleotides were computed using the method of Jukes and Cantor [15]. Dendrograms were constructed using the neighbor-joining method [25]. Its topology was also supported using the maximum-parsimony and maximum-likelihood algorithms. Confidence in the tree topology was determined by bootstrap analysis using 100 resamplings of the sequences.

Results and Discussion

The cells of strain H_1^T are Gram-negative, vibrio-shaped, and slightly motile, with one polar flagellum, non-spore-forming-rods (Fig. 1), approximately 2–3 μm in length and about 0.5 μm in diameter, and they occur generally singly. Strain H_1^T was mesophilic and grew at temperatures ranging from 22 to 43°C, with an optimum at 35°C. The isolate was moderately halophilic and grew in the presence of NaCl concentrations ranging from 1 to 45 g/l, with an optimum between 20 to 30 g/l. The optimum pH range for growth was 7.3–7.5 and growth occured between pH 5 and 9.2. The cells contained c-type cytochromes and desulfovidirin as dissimilatory bisulfite reductase. The isolate did not require peptides or vitamins although 0.1% biotrypcase enhanced growth. Sulfate, sulfite, thiosulfate, and elemental sulfur were utilized as terminal electron acceptors, but not nitrate and nitrite. Strain H_1^T grew on lactate, pyruvate, ethanol, fumarate, succinate, and hydrogen (in the presence of acetate and CO_2) as substrates in the presence of sulfate as electron acceptor. The main end-products resulting from lactate oxidation were acetate, CO_2, and H_2. Strain H_1^T fermented fumarate and pyruvate in the absence of sulfate. The products of fumarate fermentation were succinate and acetate. The following compounds did not support growth in the presence of sulfate: glucose, fructose, mannitol, mannose, ribose, xylose, glycerol, formate, acetate, malate, propionate, butyrate, valerate, methanol, and casamino acids. In optimal growth conditions on a lactate sulfate medium, the growth rate was 0.097/h.

Ability of strain H_1^T to grow in the presence of three antibiotics (penicillin, streptomycin, and chloramphenicol) was tested. Antibiotics were added separately to culture medium at different concentrations ranging from 25 to 500 μg/ml. Bacterial growth and microscopic observations after antibiotic addition showed that strain H_1^T tolerated a chloramphenicol concentration up to 200 μg/ml and concentration up to 500 μg/ml for both penicillin and streptomycin. The G + C content of strain H_1^T was 55 mol%.

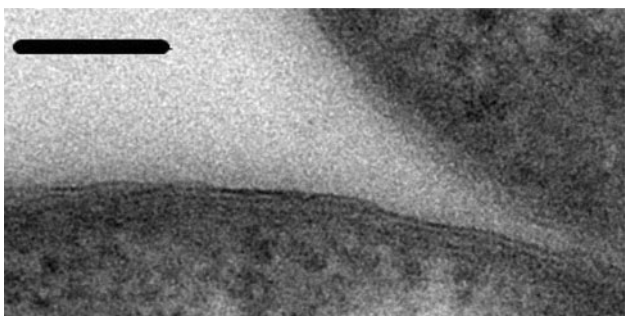

Fig. 1 Electron micrograph of an ultrathin section of a cell of strain H_1^T. Bar = 0.1 μm

Table 1 Cellular fatty acid profile of strain H_1^T

Fatty acids	Strain H_1^T
C14:0	2.5
C15:0 iso	38.8
C15:0 anteiso	3.4
C15.1 w5c	0.9
C14:0 iso 3OH	12.2
C16:0	19.0
Iso 17:1 w9c	1.5
Summed feature 4	4.2
C17:0 iso	7.0
C17:0 cyclo	2.6
C18:0	1.8

Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system; Summed feature 4 is represented by 17:1 ISO I/ANTEI B and 17:1 ANTEISO B/iI

The fatty acid composition of strain H_1^T is represented in Table 1. The predominant fatty acids were $C_{15:0}$ iso (38.8%), $C_{16:0}$ (19%), and $C_{14:0}$ iso 3OH (12.2%). $C_{15:0}$ iso is also one of the major fatty acid in most species of *Desulfovibrio* [27]. The fatty acid composition of *D. desulfuricans* Essex 6 [26] showed the presence of three dominant fatty acids: $C_{16:0}$ (24.1%°), $C_{15:0\ iso}$ (24%), and $C_{17:1\ iso}$ (21.2%). The main difference in these two fatty acid compositions is the total absence of $C_{17:1\ iso}$ in strain H_1^T. Strain H_1^T contained menaquinone MK-6 (98%) as a major component and menaquinone MK-5 (2%) as a minor component.

Phylogenetic analysis of 16S rRNA gene revealed that strain H_1^T was a member of the family *Desulfovibrionaceae* and forms a cluster with species of the genus *Desulfovibrio.* The highest sequence similarity was observed between strain H_1^T and *D. desulfuricans* subsp. *desulfuricans* (strain Essex 6) with a similarity value of 96.00%. Strain H_1^T had also *D. fairfieldensis* (95.61%), *D. intestinalis* (94.57% similarity), and *D. piger* (94.34% similarity) as its closest phylogenetic relatives. This relationship between strain H_1^T and other closest relatives was also highlighted in the phylogenetic tree (Fig. 2). The 16S rRNA gene sequences of strain H_1^T have been deposited in GenBank under accession number FJ225426.

Strains of *Desulfovibrio* species live in sediments or muds, marine environments and the digestive tract of humans or animals [1, 9, 19, 23]. Strain H_1^T was isolated from an upflow anaerobic sludge bioreactor treating wastewater in Tunisia. This strain was shown to oxidize several substrates comprising lactate, pyruvate, succinate, ethanol, fumarate, and hydrogen (with acetate and CO_2). It is a slightly halophile growing optimally at NaCl concentration of 2–3%. All the SRB so far examined contained menaquinones, and the number of isoprenoid units per side

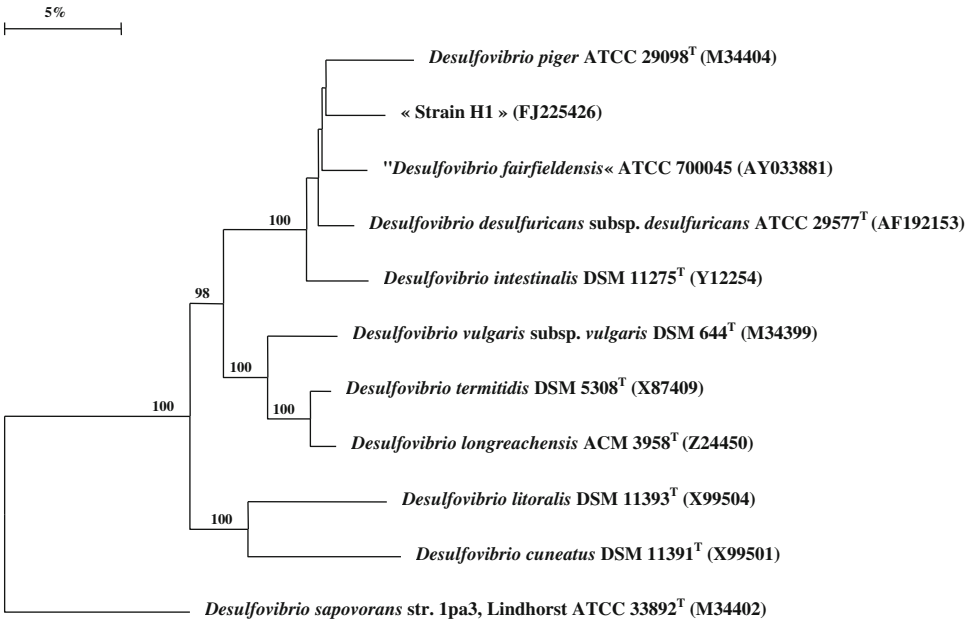

Fig. 2 Phylogenetic tree based on 16S rDNA sequence data indicating the position of strain H_1^T among the closest members of *Desulfovibrio* genus. Accession numbers of 16S rRNA gene sequences of reference organisms are included in the dendrogram. Bar, 5 nucleotide substitution per 100 nucleotides

chain varies from five to nine [5]. The major respiratory quinone of strain H_1^T was menaquinone MK-6 (98%) as it is the case in most *Desulfovibrio* species [5].

Due to morphological and phylogenetical characteristics, strain H_1^T clearly belongs to the genus *Desulfovibrio*, family Desulfovibrionaceae.

Table 2 presents the comparison of metabolic and physiological characteristics between strain H_1^T and four other closest relatives *Desulfovibrio* species (*D. desulfuricans* strain Essex 6, *D piger*, *D fairfieldensis*, and *D intestinalis*). Strain H_1^T and *D. piger* utilized fewer substrates than did *D. desulfuricans*, *D. fairfieldensis*, and *D. intestinalis*. There are several important differences in the metabolic and physiological properties of strain H_1^T compared to its four phylogenetically closest relatives. Taking into account its phenotypic, genotypic, and phylogenetic characteristics, we proposed to assign strain H_1^T (T = type species) to a novel species of SRB of the genus *Desulfovibrio*, *D. legallis* sp. nov.

Description of *Desulfovibrio legallis* sp. nov

Desulfovibrio legallis, le.gal.lis in honor and in memory of the french microbiologist and biochemist Pr. Jean LeGall (Department of Biochemistry, University of Georgia, Athens, USA), who greatly stimulated research on dissimilatory sulfate reduction. Cells are strictly anaerobic, Gram-negative, vibrio-shaped, slightly motile, non-spore-forming-rods, approximately 2–3 μm in length and about 0.5–1 μm in diameter, and they occur generally singly. The temperature range for growth is 22–43°C (optimum 35°C).

Table 2 Comparison of physiological and metabolic properties of strain H_1^T with four other most-closely related *Desulfovibrio* species

Characteristic	Strain H_1^{T1}	*D. desulfuricans*[2]	*D. piger*[3]	*D. fairfieldensis*[4,5]	*D. intestinalis*[6]
Electron donors					
Formate	−	+	−	+	+
Malate	−	+	−	+	+
Fumarate	+	+	−	+	+
Hydrogen	+	+	+	NR*	+
Use of nitrate as electron acceptor	−	+	−	+	−
Morphology	Rod	Vibrio	Rod	Vibrio	Vibrio
Motility	+	+	−	+	+
DNA G + C%	55	59	64	62	55

+: used, −: not used, *NR** not reported

References : *1* this study, *2* Devereux et al. [7], *3* Loubinoux et al. [18], *4* Loubinoux et al. [17], *5* Warren et al. [28], *6* Fröhlich et al. [11]

The optimum NaCl concentration varied between 20 and 30 g/l; the optimum pH is 7.3–7.5. Uses lactate, pyruvate, fumarate, ethanol, succinate, and hydrogen (in the presence of acetate and CO_2) as electron donors in the presence of sulfate as terminal electron acceptor. Lactate is incompletely oxidized into acetate. Substrates that are not utilized include acetate, propionate, malate, valerate, formate, methanol, glycerol, mannitol, mannose, xylose, casamino acids, fructose, glucose, and ribose. Sulfate, sulfite, thiosulfate, elemental sulfur, and fumarate serve as terminal electron acceptors; nitrate and nitrite are not utilized. Desulfoviridin (as dissimilatory bisulfite reductase) and c-type cytochromes are present in the crude bacterial extract. The G + C content of DNA of strain H_1^T is 55 mol %. Strain H_1^T tolerates chloramphenicol (concentration of 200 μg/ml) and concentration of 500 μg/ml for penicillin and streptomycin. The predominant fatty acid is $C_{15:0}$ iso (38.8%), and menaquinone MK-6 (98%) is the major respiratory quinone.

The type strain is H_1^T (= DSM 19129^T = CCUG 54389^T) which was isolated from a sludge bioreactor treating wastewater in Tunisia.

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