Characterization of *Desulfovibrio biadhensis* sp. nov., isolated from a thermal spring

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A novel anaerobic, mesophilic, slightly halophilic sulfate-reducing bacterium, designated strain Khaled BD4^T, was isolated from waters of a Tunisian thermal spring. Cells were vibrio-shaped or sigmoids (5–7×1–1.5 μm) and occurred singly or in pairs. Strain Khaled BD4^T was Gram-stain-negative, motile and non-sporulated. It grew at 25–45 °C (optimum 37 °C), at pH 5.5–8.3 (optimum pH 7.0) and with 0.5–8 % NaCl (optimum 3 %). It required vitamins or yeast extract for growth. Sulfate, thiosulfate, sulfite and elemental sulfur served as terminal electron acceptors, but not fumarate, nitrate or nitrite. Strain Khaled BD4^T utilized H_2 in the presence of 2 mM acetate (carbon source), but also lactate, formate, pyruvate and fumarate in the presence of sulfate. Lactate was incompletely oxidized to acetate. Amongst substrates used, only pyruvate was fermented. Desulfoviridin and *c*-type cytochrome were present. The G+C content of the DNA was 54.6 mol%. The main fatty acids were anteiso-$C_{15:0}$, iso-$C_{18:0}$, iso-$C_{17:0}$ and iso-$C_{14:0}$. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain Khaled BD4^T had *Desulfovibrio giganteus* DSM 4123^T (96.7 % similarity) as its closest phylogenetic relative. On the basis of 16S rRNA gene sequence comparisons together with genetic and physiological characteristics, strain Khaled BD4^T is assigned to a novel bacterial species, for which the name *Desulfovibrio biadhensis* sp. nov. is proposed. The type strain is Khaled BD4^T (=DSM 28904^T=JCM 30146^T).

Tunisia has numerous terrestrial thermo-mineral springs, the majority of which are used for public baths (hammams), swimming pools and for medical treatments provided by commercial thermal establishments. Previous studies have shown that the location and chemical composition of these spring waters are influenced strongly by the regional geology (Meddeb, 1993; Sadki, 1998). However, while geology and tectonism in Tunisia have long been a subject of interest (Fourré *et al.*, 2011), little work has been conducted to investigate the microbial biodiversity associated with these thermal springs. It is only recently that Sayeh *et al.* (2010) reported on the microbial diversity of Tunisian thermal springs based on molecular approaches. These studies revealed the presence of a wide range of aerobic and anaerobic micro-organisms occupying these terrestrial ecological niches. Amongst the former, evidence of numerous sulfate-reducing bacteria (SRB), which are known to be of geomicrobiological significance in these ecosystems (Macfarlane *et al.*, 2007; Moura *et al.*, 2007; Ollivier *et al.*, 2007; Barton *et al.*, 2014), was provided. They include members of the genera *Desulfopila*, *Desulfoglaeba* and *Desulfosporosinus*. Using cultivation-independent techniques, Haouari *et al.* (2008a, b) characterized two thermophilic SRB, *Desulfotomaculum hydrothermale* and *Thermodesulfovibrio hydrogeniphilus*, isolated from thermal terrestrial springs, located in northern Tunisia.

In this study, we report on the isolation of a mesophilic, slightly halophilic SRB from water samples of a Tunisian thermal spring which is proposed to represent a novel species of the genus *Desulfovibrio*.

Water samples were collected under anaerobic conditions from Hammam Biadha in the region of El Krib, Siliana, south-west Tunisia (35.77524° N 9.26674° E, temperature: 45 °C, pH: 7.0, sodium: 19 g l^{-1}, calcium: 1.5 g l^{-1}, sulfate: 3.8 g l^{-1}, bicarbonate: 1.6 g l^{-1}) and transported to the laboratory at ambient temperature. Micro-organisms were isolated and cultivated under strict anaerobiosis, according to the Hungate technique (Hungate, 1969).

The basal culture medium (BM) for isolation included (g l^{-1}, except where indicated): NH_4Cl (1), K_2HPO_4 (0.3),

Abbreviation: SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Khaled BD4^T is LM999902.

KH_2PO_4 (0.3), KCl (0.1), $MgCl_2 . 6H_2O$ (0.5), NaCl (2.0), yeast extract (1), $CaCl_2 . 2H_2O$ (0.1), cysteine-HCl (0.5), Na_2SO_4 (4), Widdel trace element solution (10 ml) (Widdel & Pfennig, 1981) and 1 ml of 0.1 % resazurin.

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, degassed 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 of 10 % (w/v) $NaHCO_3$, 0.1 ml of 2 % (w/v) $Na_2S . 9H_2O$ and 20 mM lactate were injected from sterile stock solutions into the tubes.

Enrichments were performed in Hungate tubes or serum bottles inoculated with 10 % of sample and incubated at 37 °C. The culture was purified by repeated use of the Hungate roll tubes method, using agar solid medium (0.8 %), and transferred to liquid medium.

The pH, temperature and NaCl concentration ranges for growth were determined using BM supplemented with 20 mM lactate as electron donor. The pH (from 5 to 9) of the culture medium was adjusted by injecting aliquots of anaerobic stock solution of 100 mM HCl in Hungate tubes (low pH), 10 % $NaHCO_3$ or Na_2CO_3 (high pH). Water baths were used for incubating bacterial cultures from 15 to 60 °C. The NaCl requirement was determined by directly weighing NaCl in 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 by insertion of Hungate tubes into the cuvette holder of a spectrophotometer (Cary 50; Varian). H_2S production was determined photometrically as colloidal CuS following the method described by Cord-Ruwisch (1985). End products of metabolism were measured by HPLC after 2 weeks of incubation at 37 °C (Fardeau *et al.*, 1997).

Morphological characteristics and purity of strains were checked under an Optiphot (Nikon) phase-contrast microscope. For transmission electron microscopy studies, cell preparations were negatively stained with sodium phosphotungstate, as previously described (Fardeau *et al.*, 1997). The presence of spores was analysed by phase-contrast microscopic observations of cultures and after pasteurization tests performed at 80, 90 and 100 °C for 10 and 20 min.

Cultures of the isolate (strain Khaled BD4^T) and of *Desulfovibrio giganteus* DSM 4123^T, its closest phylogenetic relative, were driven and stopped at the end of exponential phase and sent to the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) for fatty acid analysis. Fatty acids were extracted using the method of Miller (1982), with the modifications of Kuykendall *et al.* (1988), and the profile of cellular fatty acids was analysed by GC using the Microbial Identification System (MIDI, Sherlock Version 6.1; database, TSBA40; gas chromatograph, model 6890N; Agilent Technology).

Determination of the G+C content of the DNA and DNA–DNA hybridization experiments were performed at the DSMZ. Genomic DNA for analysis of the base composition and DNA–DNA hybridization studies was isolated after disruption of bacterial cells by using a French press (Thermo Spectronic) and purified by chromatography on hydroxyapatite using the procedure of Cashion *et al.* (1977). The G+C content was determined by using HPLC as described by Mesbah *et al.* (1989). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS spectrophotometer (Varian).

The use of substrates was tested in the presence of 1 g yeast extract l^{-1}. The substrates included formate, lactate, pyruvate, fumarate, acetate, malate, succinate, methanol, ethanol, propanol, 2-propanol, glycerol, l-pentanol, D-glucose, D-fructose, propionate, butyrate, benzoate, Casamino acids, palmitate and H_2/CO_2 (80/20, v/v) with or without acetate (2 mM) as carbon source. Each substrate was tested at a final concentration of 20 mM for sugars and organic acids, 8 mM for palmitate, 10 g l^{-1} for Casamino acids and 2 bar for H_2. Elemental sulfur (1 %, w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM), nitrate (10 mM) and nitrite (2 mM) were tested as potential terminal electron acceptors.

The extraction and purification of total DNA followed by amplification and sequencing of the 16S rRNA gene were performed as previously described (Khelifi *et al.*, 2010). The 16S rRNA gene sequence was then compared with available sequences in the GenBank database using the BLASTN search (Altschul *et al.*, 1990). The evolutionary history was inferred using the neighbour-joining method (Saitou & Nei, 1987). A multiple alignment was built using the program MUSCLE (Edgar, 2004) implemented in MEGA6 (Tamura *et al.*, 2013). 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 (Tamura *et al.*, 2004). There were a total of 1372 positions in the final dataset. Branch robustness was estimated by the non-parametric bootstrap procedure implemented in MEGA6 (1000 replicates of the original dataset). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site (Felsenstein, 1985).

Several colonies developed after incubation at 37 °C and were picked separately. Colonies were black and circular with diameters ranging from 1.0 to 2.0 mm after 3–5 days of incubation at 37 °C. The process of serial dilution was repeated several times until the isolates were deemed

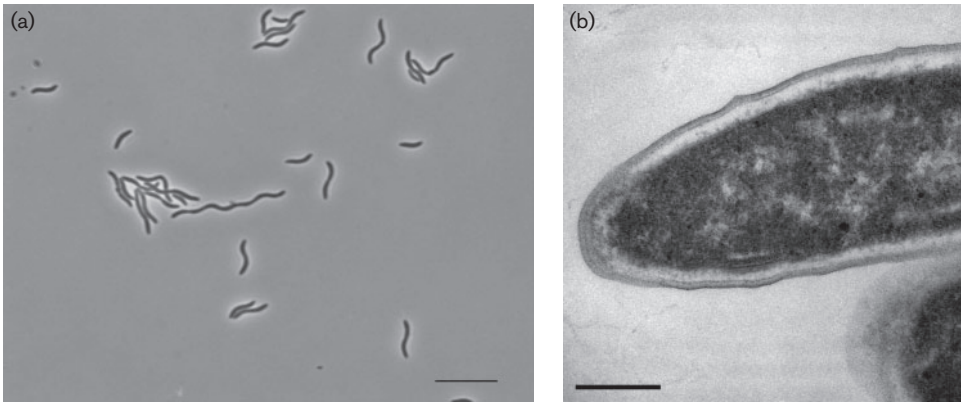

Fig. 1. (a) Phase-contrast photomicrograph showing cells of strain Khaled BD4^T; bar, 10 μm. (b) Transmission electron micrograph of an ultrathin section of a cell of strain Khaled BD4^T showing a thin peptidoglycan layer and an outer membrane; bar, 200 nm.

to be axenic. The purity of the isolates was checked by microscopy and inoculation in sulfate-free media containing yeast extract and sugars to confirm the absence of contamination by fermentative micro-organisms. Several strains were isolated; their morphology, size and metabolic profiles were similar and the same phylogenic inference was obtained for all of them. One strain, designated Khaled BD4^T, was selected and used for further metabolic and physiological characterization.

Cells of strain Khaled BD4^T were Gram-stain-negative, curved rods or vibrios (5–7 × 1–1.5 μm) when grown on medium containing lactate as electron donor and sulfate as terminal electron acceptor (Fig. 1a). Ultrathin sections showed a typical Gram-negative cell wall with a thin peptidoglycan layer and an outer membrane (Fig. 1b). Cells were motile by means of a single polar flagellum (not shown).

Strain Khaled BD4^T was anaerobic but tolerated up to 1 % O_2. The physiological optimal growth conditions were determined in duplicate experiments conducted in BM containing lactate (20 mM) as previously described (Fardeau *et al.*, 2000). The optimal temperature for growth was 37 °C (range 25–45 °C). Optimum pH was 7 (range pH 5.5–8.3). The strain required a minimum of 5 g NaCl l^{-1} for growth, and tolerated up to 80 g l^{-1}; optimum growth occurred with 30 g NaCl l^{-1}. Amongst substrates tested, only lactate, pyruvate, fumarate, formate and H_2 were oxidized. The presence of acetate as carbon source (2 mM) was necessary for oxidizing hydrogen. Acetate, CO_2 and H_2S were end products resulting from lactate oxidation. Only pyruvate was fermented, yielding acetate, H_2 and CO_2. Thiosulfate, sulfate, elemental sulfur and sulfite, but not fumarate, nitrate or nitrite served as terminal electron acceptors. Sulfur compounds were reduced to sulfide. Malate, succinate, methanol, ethanol, propanol, 2-propanol, glycerol, l-pentanol, D-glucose, D-fructose, acetate, propionate, butyrate, benzoate, Casamino acids and palmitate did not support growth.

Growth did not occur in minimal medium with lactate as the only energy and carbon source. The presence of vitamins (Balch *et al.*, 1979) or yeast extract, or biotrypticase was required for growth.

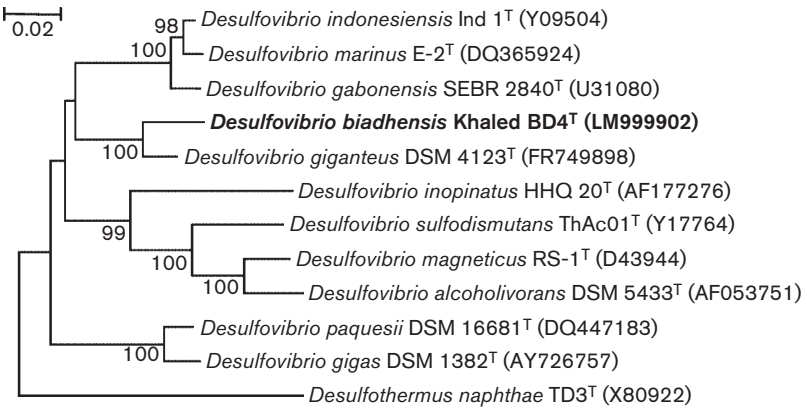

Fig. 2. Phylogenetic position of strain Khaled BD4^T within the genus *Desulfovibrio* based on 16S rRNA gene sequence analysis using the neighbour-joining method. The sequence of *Desulfothermus naphthae* TD3^T was used as outgroup. There were a total of 1372 positions in the final dataset. Numbers at nodes indicate bootstrap values above 70 % (1000 replicates). Bar, 0.02 substitutions per site.

Table 1. Comparison of the main characteristics of strain Khaled BD4^T and *Desulfovibrio giganteus*

Data for *Desulfovibrio giganteus* are from Esnault *et al.* (1988).

Characteristic	Khaled BD4^T	*Desulfovibrio giganteus*
Source	Thermal spring	Coastal lagoon
Temperature growth range (optimum) (°C)	25–45 (37)	15–40 (35)
pH growth range (optimum)	5.5–8.5 (7)	6.5–8.5 (7.5)
Salinity growth range (optimum) (%)	0.5–8 (3)	0.2–8 (0.2–2.5)
DNA G+C content (mol%)	54.6	55.5
Electron acceptors		
Sulfur	+	−
Electron donors		
Methanol	−	+
Ethanol	−	+
Propanol	−	+
2-Propanol	−	+
Glycerol	−	+
Pentanol	−	+

Visible absorption spectra of a cell-free extract of strain Khaled BD4^T showed the presence of low redox potential *c*-type cytochrome (the tetrahaeme cytochrome c_3) with absorption peaks at 522, 551 and 418 nm in the dithionite reduced form. The characteristic absorption band (at 628 nm) of desulfoviridin (the dissimilatory high-spin bisulfite reductase characteristic of the genus *Desulfovibrio*) (Fauque & Barton, 2012) was detected in the cell-free extract.

The phylogenetic tree obtained by the neighbour-joining method, as shown in Fig. 2, confirms that strain Khaled BD4^T is an SRB belonging to the genus *Desulfovibrio*, sharing 96.7 % sequence similarity with its closest phylogenetic relative, *Desulfovibrio giganteus* DSM 4123^T isolated from Berre Lagune (France). However, DNA–DNA hybridization experiments revealed that strain Khaled BD4^T showed only 19 % reassociation with *Desulfovibrio giganteus* DSM 4123^T, thus indicating that strain Khaled BD4^T represents a novel species of the genus *Desulfovibrio*. This is supported by several phenotypic differences, including the use of elemental sulfur and tolerance to higher NaCl concentration, but also the range of substrates used (Table 1). Indeed, in contrast to *Desulfovibrio giganteus*, strain Khaled BD4^T could not oxidize alcohols (e.g. methanol, ethanol, propanol, 2-propanol and glycerol) (Table 1). Moreover, the profiles of fatty acids were slightly different for both strains when cultivated under the same growth conditions (Table 2). Indeed, while the major fatty acids of strain Khaled BD4^T were anteiso-$C_{15:0}$, iso-$C_{18:0}$, iso-$C_{17:0}$ and iso-$C_{14:0}$, those of *Desulfovibrio giganteus* were anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{14:0}$ (Table 2).

Therefore, based on the phenotypic, chemotaxonomic, phylogenetic and genetic characteristics of strain Khaled BD4^T, we suggest that it to be assigned to a novel species of the genus *Desulfovibrio*, for which the name *Delfovibrio biadhensis* sp. nov. is proposed.

Description of *Desulfovibrio biadhensis* sp. nov.

Desulfovibrio biadhensis (bia.dhen′sis. L. masc. adj. *biadhensis* of or belonging to Biadha).

Cells are vibrio-shaped or sigmoids, 5–7 × 1–1.5 μm, occurring singly or in chains. Motile by means of a single polar flagellum. Grows at 25–45 °C, with optimum growth at 37 °C. Grows in the presence of 0.5–8 % NaCl, with optimum growth at around 3 %. The optimum pH for

Table 2. Cellular fatty acid contents (%) of strain Khaled BD4^T and *Desulfovibrio giganteus* DSM 4123^T cultivated under the same growth conditions

−, Not detected.

Fatty acid	Khaled BD4	*Desulfovibrio giganteus* DSM 4123^T
iso-$C_{13:0}$	1.2	1.2
iso-$C_{14:0}$	9.2	11.2
$C_{14:0}$	0.4	1.0
iso-$C_{15:0}$	4.2	3.5
anteiso-$C_{15:0}$	33.6	35.1
iso-$C_{16:1}$ H	−	1.6
iso-$C_{16:0}$	7.0	12.3
$C_{16:0}$	4.4	10.6
iso-$C_{17:1}\omega 9c$	−	1.7
anteiso-$C_{17:1}\omega 9c$	−	1.1
iso-$C_{17:0}$	11.7	1.8
$C_{17:0}$	0.8	2.1
iso-$C_{18:1}$ H	−	1.1
iso-$C_{18:1}\omega 7c$	−	1.8
$C_{18:0}$	6.0	4.3
iso-$C_{18:0}$	12.3	−
iso-$C_{17:0}$ 3-OH	2.1	1.6
anteiso-$C_{19:0}$	1.1	−

growth is 7.0; growth occurs at pH 5.5–8.3. Utilizes H_2, formate, fumarate, lactate and pyruvate as electron donors. Lactate is converted to acetate and CO_2. Substrates that are not utilized include malate, succinate, methanol, ethanol, propanol, 2-propanol, glycerol, l-pentanol, D-glucose, D-fructose, acetate, propionate, butyrate, benzoate, Casamino acids and palmitate. Pyruvate is fermented to acetate, H_2 and CO_2. Utilizes elemental sulfur, sulfate, thiosulfate and sulfite as electron acceptors but not fumarate, nitrate or nitrite. Desulfoviridin and *c*-type cytochromes are present.

The type strain, Khaled BD4^T (=DSM 28904^T=JCM 30146^T), was isolated from waters collected from a Tunisian thermal spring. The G+C content of the DNA of the type strain is 54.6 mol% (HPLC).

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