Desulfotomaculum hydrothermale sp. nov., a thermophilic sulfate-reducing bacterium isolated from a terrestrial Tunisian hot spring

Olfa Haouari,^{1,2} Marie-Laure Fardeau,¹ Jean-Luc Cayol,¹ Corinne Casiot,³ Françoise Elbaz-Poulitchet,³ Moktar Hamdi,² Manon Joseph¹ and Bernard Ollivier¹

Correspondence

Bernard Ollivier
bernard.ollivier@univmed.fr

¹IRD, UMR 180, Microbiologie et Biotechnologie des Environnements Chauds, IFR-BAIM, ESIL, Universités de Provence et de la Méditerranée, 163 Avenue de Luminy, F-13288 Marseille Cedex 09, France

²Laboratoire d'Écologie et de Technologie Microbiennes INSAT, 1080 Tunis, Tunisia

³Laboratoire Hydrosociences Montpellier, UMR 5569, Universités Montpellier 2 et 1, CNRS, IRD, Place E. Bataillon, Case MSE, 34095 Montpellier Cedex 05, France

A novel strictly anaerobic, moderately thermophilic, sulfate-reducing bacterium, designated strain Lam5^T, was isolated from a hot spring in north-east Tunisia and was characterized phenotypically and phylogenetically. The isolate stained Gram-negative but had a Gram-positive-type cell wall. The strain comprised endospore-forming, slightly curved rod-shaped cells with peritrichous flagella. It did not possess desulfoviridin. Strain Lam5^T grew anaerobically at 40–60 °C (optimally at 55 °C) and at pH 5.8–8.2 (optimally at pH 7.1); it did not require NaCl but tolerated concentrations up to 1.5 % (w/v). It utilized lactate, pyruvate, formate, ethanol, butanol, glycerol, propanol and H₂ (plus acetate) as electron donors. Lactate was oxidized and pyruvate was fermented to acetate. Sulfate, sulfite, thiosulfate, As(V) and Fe(III) (but not elemental sulfur, fumarate, nitrate or nitrite) were used as electron acceptors. The G + C content of the genomic DNA was 46.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing showed that strain Lam5^T was a member of the genus *Desulfotomaculum*, with *Desulfotomaculum putei* as its closest relative (96 % similarity to the type strain). On the basis of genotypic, phenotypic and phylogenetic data, strain Lam5^T represents a novel species of the genus *Desulfotomaculum*, for which the name *Desulfotomaculum hydrothermale* sp. nov. is proposed. The type strain is Lam5^T (=DSM 18033^T =JCM 13992^T).

Micro-organisms associated with hot springs in geothermal areas have attracted considerable interest in recent years. Because the primary production in these extreme environments is probably sustained by hydrogen-oxidizing bacteria, special attention has been paid to the microaerophilic members of the order *Aquificales* (Aguilar *et al.*, 2004), which are recognized as dominant hydrogen oxidizers within terrestrial hot springs (Reysenbach *et al.*, 1994; Hugenholtz *et al.*, 1998; Reysenbach & Shock, 2002). In addition to hydrogen oxidation performed under microaerophilic conditions by members of the *Aquificales*, hydrogen oxidation occurring under anaerobiosis is also important in hot springs, as temperature and sulfide

concentrations can be involved in maintaining a low redox potential *in situ*. Under these physicochemical conditions, sulfate-reducing bacteria (SRB) may therefore contribute significantly to hydrogen oxidation in these extreme environments, where sulfate is not limiting (Fishbain *et al.*, 2003). It is therefore not surprising that several cultivation-based and culture-independent molecular phylogenetic surveys have provided evidence of a wide diversity of SRB inhabiting geothermal terrestrial hot springs (Blank *et al.*, 2002; Ferris *et al.*, 2003; Fishbain *et al.*, 2003; Hugenholtz *et al.*, 1998; Meyer-Dombard *et al.*, 2005; Skirnisdottir *et al.*, 2000). Most of the SRB isolated so far from terrestrial hot springs, with the exception of the crenarchaeote *Caldivirga maquilingensis* (Itoh *et al.*, 1999), have been reported to oxidize hydrogen. They comprise bacterial members of (i) the genus *Thermodesulfobacterium* (Zeikus *et al.*, 1983; Sonne-Hansen & Ahring 1999), representing one of the deepest branches within the

Abbreviation: SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Lam5^T and Lam7 are EF081293 and EF494253, respectively.

phylogenetic tree, (ii) the genus *Thermodesulfovibrio* (Henry *et al.* 1994; Sonne-Hansen & Ahring 1999), belonging to the phylum 'Nitrospirae', (iii) the genus *Thermodesulfobium*, peripherally related to the phylum 'Nitrospirae', (iv) the genus *Desulfomicrobium* (Thevenieau *et al.*, 2007), a representative of the *Deltaproteobacteria*, and (v) the genus *Desulfotomaculum* (Goorissen *et al.*, 2003; Liu *et al.*, 1997), belonging to the low-G+C Gram-positive group. Within this latter genus, only two species have been isolated from terrestrial hot springs: *Desulfotomaculum luciae*, isolated in Canada, and *Desulfotomaculum solfataricum*, isolated in Iceland.

Here, we report on the isolation of three hydrogenotrophic, thermophilic SRB originating from a terrestrial hot spring in Tunisia and belonging to the genera *Thermodesulfovibrio* and *Desulfotomaculum*. We also propose the assignment of one of these isolates to a novel species of the genus *Desulfotomaculum*.

Samples were collected from the Hamam Essalhine hot spring, located 10 km north of Fernana on the Fernana–Ben Mtir road in north-east Tunisia at 280 m elevation. The hot-spring water is discharged into the River Wad'Ellil. The water temperature at the sampling site was 73 °C, the pH was 6.9 and the salinity was approximately 0.5 % NaCl. The sulfate concentration in the water was 70 mg l⁻¹. SRB were isolated from mixed sediment/water samples collected in sterile plastic bottles in January 2005 and kept at 4 °C until used.

The Hungate technique (Hungate, 1969) was used throughout for cultivation. The basal medium contained the following (l⁻¹ distilled water): 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 1.0 g NH₄Cl, 1.0 g NaCl, 3.0 g Na₂SO₄, 0.1 g KCl, 0.1 g CaCl₂·2H₂O, 0.5 g MgCl₂·6H₂O, 0.1 g yeast extract (Difco), 0.5 g cysteine hydrochloride, 1 ml trace mineral element solution (Widdel & Pfennig, 1981) and 1 ml 0.1 % resazurin. The pH was adjusted to 7.2 with 10 M KOH. The basal medium was boiled under a stream of O₂-free N₂ gas and then cooled to room temperature and 5 ml aliquots were distributed into Hungate tubes under a stream of O₂-free N₂ gas. The N₂ gas phase was replaced with N₂/CO₂ (80:20, v/v) and the tubes were autoclaved for 45 min at 110 °C. Prior to inoculation, 0.1 ml 2 % Na₂S·9H₂O and 0.1 ml 10 % NaHCO₃ were added.

Enrichments were performed in Hungate tubes containing 5 ml medium and inoculated with 0.5 ml sample. The following were used as sole substrates: H₂ from a mixture of H₂/CO₂ [80:20 (v/v), 200 kPa], acetate (20 mM) and lactate (20 mM). Acetate (2 mM) was added as the carbon source in the presence of hydrogen as the electron donor. Three enrichment series were performed in the same medium before isolation. The cultures were purified by repeated use of the Hungate roll-tube method with medium solidified with 2.5 % (w/v) Noble agar (Difco) or with 0.8 % Phytagel (Sigma) for cultures incubated at 55 and 70 °C, respectively. Several of the colonies that developed were picked and cultured in the corresponding

culture medium. The process of isolation was repeated several times until isolates were deemed to be axenic. Cultures were routinely checked by microscopy for contamination, using media containing glucose in the presence of yeast extract as the substrate.

Duplicate experiments were performed to determine the physiological conditions (temperature, pH and NaCl concentration) for optimal growth. These experiments were conducted in basal medium with the addition of lactate (20 mM) as the electron donor and sulfate (20 mM) as the electron acceptor. The ability to utilize organic substrates was tested in basal medium supplemented with autoclaved or filter-sterilized substrates. Butyrate, succinate, propionate, fumarate, pyruvate, lactate, acetate, malate, formate, butanol, propanol, pentanol, methanol, glycerol, ethanol, lactose, glucose, fructose and mannose were tested as energy sources, in each case at a final concentration of 20 mM. Casamino acids were tested at 0.25 %. The use of H₂ (200 kPa) as an electron donor was determined in the presence and absence of acetate as a carbon source, whereas CO oxidation was tested at concentrations of 25, 50 and 100 % in the gas phase. Utilization of lactate, pyruvate, formate, ethanol, butanol, glycerol and propanol was tested in the absence of sulfate as a terminal electron acceptor. To test for electron acceptors, sodium thiosulfate (final concentration 20 mM), sodium sulfate (20 mM), sodium sulfite (2 mM), elemental sulfur (2 %, w/v), sodium fumarate (20 mM), sodium nitrate (20 mM) and sodium nitrite (2 mM) were added separately to the medium. Sensitivity to antibiotics was determined by transferring an exponentially growing culture into basal medium containing filter-sterilized antibiotics (penicillin, streptomycin and chloramphenicol) at 25, 50, 100, 200, 300, 500, 750 and 1000 µg ml⁻¹.

Phase-contrast microscopy (Eclipse E600; Nikon) was used for routine examination of the cultures and to obtain photomicrographs. Light and electron microscopy were performed as described previously (Miranda-Tello *et al.*, 2004). Growth was measured by inserting tubes directly into a model Cary 50 Scan spectrophotometer (Varian) and measuring the OD₅₈₀. Sulfide was determined photometrically, as colloidal CuS, using the method of Cord-Ruwisch (1985). The end products of organic-compound metabolism were measured as described previously (Haouari *et al.*, 2006), using HPLC after 2 weeks incubation at optimal temperature and pH. Cytochromes and desulfovibrin were determined as described by Postgate (1959).

The G+C content of the DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany), using the method of Mesbah *et al.* (1989). The whole-cell fatty acid composition was also determined at the DSMZ, using cell material grown under identical conditions (with lactate and sulfate as the electron donor and acceptor, respectively).

Analysis and determination of arsenic species [As(V) and As(III)] were performed using HPLC coupled to hydride generation atomic fluorescence spectrometry. The method, described by Bohari *et al.* (2001), has a detection limit of 2.3 nM for As(III) and 6.1 nM for As(V). The total dissolved iron content was determined by using flame atomic absorption spectrometry. Fe(II) was determined using colorimetry at 510 nm after complexing with 1,10-phenanthroline chloride solution in buffered samples (pH 4.5) (Rodier *et al.*, 1996).

The methods used for the purification and extraction of DNA and the amplification and sequencing of the 16S rRNA gene were as described previously by Miranda-Tello *et al.* (2003), except that primer Rd1 (5'-AAGGAGGTGATCCAGCC-3') was used instead of primer R6.

The 16S rRNA gene sequences of strains Lam5^T, Hbr5 and Hbr7 were imported into the sequence editor BioEdit, version 5.0.9 (Hall, 1999); the base calling was examined and a contiguous consensus sequence was obtained for each isolate. A non-redundant BLASTN search (Altschul *et al.*, 1997) of the full sequences through GenBank (Benson *et al.*, 1999) identified their closest relatives. Sequences used in the phylogenetic analysis were obtained from the Ribosomal Database Project and GenBank and were aligned using programs provided by Ribosomal Database Project II (Maidak *et al.*, 2001). The position of the sequences and pairwise evolutionary distances based on 1138 unambiguous nucleotides were calculated using the method of Jukes & Cantor (1969). Dendrograms were constructed using the neighbour-joining method (Saitou & Nei, 1987). DNA–DNA hybridization was performed at the DSMZ.

Enrichment cultures were performed on H₂/CO₂ (80:20, v/v; 200 kPa) and at atmospheric pressure at 55 and 70 °C on acetate and lactate as energy sources in the presence of sulfate as the terminal electron acceptor. Growth and sulfide production occurred in the presence of hydrogen or lactate, but not in the presence of acetate, as the electron donor. Single, brown, discus-shaped colonies (1 mm in diameter) developed after 8 days incubation at 55 °C (lactate and H₂) and 70 °C (H₂). They were picked and serially diluted in roll-tubes before the culture was considered pure. Two strains (Lam5^T and Hbr5) were isolated at 55 °C and one strain (Hbr7) was isolated at 70 °C. The closest phylogenetic relatives of strains Lam5^T and Hbr7 were the type strains of *Desulfotomaculum putei* (96% similarity) and *Desulfotomaculum australicum* (99% similarity), respectively, whereas the closest relative of strain Hbr5 was *Thermodesulfovibrio yellowstonii* (95% similarity to the type strain). In this respect, the phylogenetic results indicated that strains Lam5^T and Hbr5 should be considered as representatives of novel species of SRB. Only strain Lam5^T was characterized further.

The purity of the strains was confirmed by morphological homogeneity observed under a phase-contrast microscope and by the absence of growth in liquid sulfate-free SRB

medium supplemented with yeast extract (1 g l⁻¹) and glucose (20 mM) under aerobic or anaerobic conditions.

Cells of strain Lam5^T were slightly curved rods, 2–5 µm long and 0.5 µm wide, and usually occurred singly (Fig. 1a). They were motile by means of peritrichous flagella (Fig. 1b). Endospores were observed in preparations stained with malachite green and under phase-contrast microscopy. The spores were oval and subterminal to terminal, and deformed the cells (Fig. 1a). No gas vacuoles were observed. Cells of Lam5^T stained Gram-negative. Electron micrographs of thin sections of cells revealed an atypical Gram-positive cell wall with a periodic arch-like surface layer (data not shown).

Strain Lam5^T was strictly anaerobic, growing optimally at 55 °C (range 40–60 °C). The optimum pH for growth was around 7.1 (range pH 5.8–8.2). It did not require NaCl for growth. The upper NaCl concentration for growth was 1.5% (w/v). The cells contained c₃-type cytochromes, but not desulfoviridin, similarly to members of the genus *Desulfotomaculum*. Strain Lam5^T did not require yeast extract, peptides or vitamins for growth, but 0.1% biotrypcase enhanced growth. Under optimal growth conditions, in the presence of sulfate as an electron acceptor, strain Lam5^T did not support autotrophic growth and used H₂ and formate only in the presence of acetate as the carbon source. Lactate, pyruvate, ethanol, butanol,

Fig. 1. Phase-contrast micrograph (a) and transmission electron photomicrograph (b) of negatively stained cell of strain Lam5^T grown in medium containing lactate and sulfate. Bars, 5 µm (a) and 1 µm (b).

glycerol and propanol were also used as electron donors. The main end products resulting from lactate oxidation were acetate, CO₂ and H₂S. In the absence of sulfate, strain Lam5^T fermented only pyruvate, to acetate, H₂ and CO₂. The following compounds did not support growth of strain Lam5^T: CO, glucose, fructose, mannose, acetate, propionate, butyrate, succinate, fumarate, malate, methanol, glycerol, starch and Casamino acids. Sulfate, sulfite and thiosulfate (but not elemental sulfur, fumarate, nitrate or nitrite) served as electron acceptors in the presence of lactate as the energy and carbon source. Strain Lam5^T also reduced As(V) to As(III) and Fe(III) to Fe(II) in the presence of pyruvate as the electron donor.

Growth was inhibited by chloramphenicol (25 µg ml⁻¹), streptomycin (750 µg ml⁻¹) and penicillin (1000 µg ml⁻¹).

Physiological characteristics of strain Lam5^T (e.g. the temperature optimum for growth, and for spore formation in particular) suggested that this isolate may survive, but not grow, in the Tunisian hot spring studied, as it does not grow at temperatures above 60 °C. This also suggested that strain Lam5^T might play a significant ecological role only in the cooler parts of the thermal spring studied.

Analysis of the almost-complete sequence (1565 bp) of the 16S rRNA gene of strain Lam5^T indicated that this novel isolate clusters with the members of the family *Peptococcaceae*, order *Clostridiales*. The phylogenetic tree (Fig. 2) showed that strain Lam5^T clustered with *D. putei* SMCC W459^T, an isolate recovered from a deep terrestrial subsurface in Virginia, USA (Liu *et al.*, 1997), with a 16S rRNA gene sequence similarity of 96 %, thus indicating that the former strain should be assigned to a novel species

of the genus *Desulfotomaculum*. This is confirmed by the DNA–DNA hybridization studies, which revealed a low level of relatedness (6.5 %) between the two strains. Phenotypic and genotypic characteristics of strain Lam5^T, e.g. the DNA G + C content (46.8 mol%), also indicate that it belongs to the genus *Desulfotomaculum*. In addition to the phylogenetic differences observed between strain Lam5^T and *D. putei*, various phenotypic differences were revealed, e.g. with regard to the use of fructose, the capacity for autotrophic growth on hydrogen (Table 1) and the temperature range for growth. In contrast to the thermophile *D. putei*, strain Lam5^T should be considered as a moderately thermophilic SRB. Finally, the results of a comparison between the cellular fatty acid composition of strain Lam5^T (Table 2) and that of its closest phylogenetic relative, *D. putei*, are consistent with the assignment of the isolate to a novel species of the genus *Desulfotomaculum*.

Members of the spore-forming genus *Desulfotomaculum* have been recovered from hot ecosystems on several occasions (Stackebrandt *et al.*, 1997), suggesting a significant ecological role for these micro-organisms in such ecosystems in terms of the oxidization of hydrogen or organic compounds with the concomitant reduction of sulfate and other sulfur oxyanions to sulfide. Despite the wide distribution of thermophilic *Desulfotomaculum* strains within the hot biosphere, few thermophilic SRB belonging to the genus *Desulfotomaculum* have been isolated from terrestrial hot springs. They include *D. luciae* (Liu *et al.*, 1997) and *D. solfataricum* (Goorissen *et al.*, 2003). With the isolation of strain Lam5^T and also Hbr7, we have shown that *Desulfotomaculum* species should be considered as common inhabitants of terrestrial hot

Fig. 2. Neighbour-joining phylogenetic dendrogram, based on 16S rRNA gene sequences, indicating the position of strain Lam5^T with respect to its closest relatives in the genus *Desulfotomaculum*. Bootstrap percentages (based on 100 resamplings) are given at nodes. Bar, 2 % sequence divergence.

Table 1. Characteristics that serve to distinguish strain Lam5^T and *D. putei* DSM 12395^T

Data for *D. putei* DSM 12395^T were taken from Liu *et al.* (1997). To determine the use of electron donors, the two strains were cultivated under the same growth conditions (see basal medium above). –, No growth; +, good growth.

Characteristic	Strain Lam5 ^T	<i>D. putei</i> DSM 12395 ^T
Isolation source	Terrestrial hot spring	Deep terrestrial subsurface
Morphology	Slightly curved rods	Rods
Cell size (µm)	1 × 3–6	1.0–1.1 × 2–5
Spore location	Terminal	Paracentral
Temperature for growth (°C)		
Range	40–60	40–65
Optimum	55	64
pH for growth		
Range	5.8–8.20	6–7.9
Optimum	7	7.5
DNA G + C content (mol%)	46.8	47.1
Utilization of electron donors		
H ₂ (with acetate as carbon source)	–	+
Fructose fermentation	–	+

springs, as confirmed previously in a culture-independent analysis (Fishbain *et al.*, 2003), thus emphasizing their ecological significance not only in subterrestrial ecosystems, but also in the terrestrial hot biosphere.

Table 2. Cellular fatty acid compositions (%) of strain Lam5^T and *D. putei* DSM 12395^T

The two strains were cultivated (by the DSMZ) under the same growth conditions. ECL, Equivalent chain length; ALD, aldehyde; DMA, dimethyl acetal. In both strains, 100 % of the total fatty acids were identified; –, not detected.

Fatty acid	ECL	Strain Lam5 ^T	<i>D. putei</i> DSM 12395 ^T
14:0	14.0	0.9	3.0
iso-15:0	14.6	15.7	20.5
anteiso-15:0	14.7	2.0	1.2
16:0 ALD	14.9	–	1.1
15:0 DMA	15.1	–	0.6
iso-16:0	15.6	0.9	–
16:1 c7	15.8	1.3	2.4
16:1 c9	15.8	3.6	8.7
16:1 c11	15.9	–	0.6
16:0	16.0	10.7	17.7
16:1 c9 DMA	16.3	0.9	2.0
16:0 DMA	16.5	3.5	7.1
iso-17:0	16.6	35.5	16.9
anteiso-17:0	16.7	4.3	0.5
17:0 cyclo	16.9	5.8	5.9
17:0	17.0	1.0	–
iso-17:0 DMA	17.1	8.5	8.2
anteiso-17:0	17.2	0.6	–
18:1 c9	17.8	0.8	0.7
18:1 c11	17.8	2.1	1.7
18:0	18.0	1.9	1.2

The identification of strain Lam5^T as a novel representative of the SRB belonging to the low-G + C Gram-positive group is of taxonomic significance, but it is also noteworthy that our enrichments using hot-spring water samples at 55 and 70 °C have led to the isolation of hydrogenotrophic SRB (H₂ being generated geothermally from hot springs). In contrast (as in our study), acetate oxidation through sulfate-reduction activity has never been reported to occur in terrestrial hot springs. Therefore, complete oxidation, under anaerobic conditions, of organic matter (when available in these hot ecosystems) should occur as a result of alternative types of metabolism, such as methanogenesis or iron reduction (Chang *et al.*, 2001). Finally, in contrast to *Desulfosporosinus auripigmenti* (formerly *Desulfotomaculum auripigmentum*), strain Lam5^T was shown to reduce As(V) by means of a detoxifying system rather than a respiratory one (Newman *et al.*, 1997).

Taken together, these results emphasize the ecologically significant role that SRB, and particularly members of the genus *Desulfotomaculum*, may play not only in the sulfur cycle but also in the transformation of organic matter through oxidoreductive processes in hot ecosystems.

On the basis of its phenotypic, genotypic and phylogenetic characteristics, strain Lam5^T represents a novel species of the genus *Desulfotomaculum*, for which the name *Desulfotomaculum hydrothermale* sp. nov. is proposed.

Description of *Desulfotomaculum hydrothermale* sp. nov.

Desulfotomaculum hydrothermale (hy.dro.ther.ma'le. Gr. n. *hydro* water; Gr. n. *thermos* heat; N.L. neut. adj. *hydrothermale* from a hydrothermal area).

Anaerobic and moderately thermophilic. Cells are slightly curved rods, about 0.5 µm wide and 2–5 µm long, and

stain Gram-negative. Oval, subterminal to terminal spores are produced that deform the cells. Cells are motile by means of peritrichous flagella. Growth occurs between 40 and 60 °C (optimally at 55 °C). The pH range for growth is 5.8–8.2 (optimum pH 7.1). Growth does not occur at NaCl concentrations above 1.5 % (w/v). Sulfate, thiosulfate and sulfite are used as electron acceptors, but elemental sulfur, fumarate, nitrate or nitrite are not used. As(V) is reduced to As(III) and Fe(III) is reduced to Fe(II). Electron donors utilized in the presence of sulfate as the electron acceptor include lactate, pyruvate, formate, ethanol, butanol, glycerol, propanol and H₂. Lactate is oxidized to acetate. Pyruvate is fermented to acetate, H₂ and CO₂. Fructose is not used. The DNA G+C content of the type strain is 46.8 mol% (by HPLC).

The type strain, Lam5^T (=DSM 18033^T =JCM 13992^T), was isolated from a terrestrial hot spring in Tunisia.

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