Defluviitoga tunisiensis gen. nov., sp. nov., a thermophilic bacterium isolated from a mesothermic and anaerobic whey digester

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Strain SulfLac1^T, a thermophilic, anaerobic and slightly halophilic, rod-shaped bacterium with a sheath-like outer structure (toga), was isolated from a whey digester in Tunisia. The strain's non-motile cells measured 3–30×1 μm and appeared singly, in pairs or as long chains. The novel strain reduced thiosulfate and elemental sulfur, but not sulfate or sulfite, into sulfide. It grew at 37–65 °C (optimum 55 °C), at pH 6.5–7.9 (optimum pH 6.9) and with 0.2–3 % (w/v) NaCl (optimum 0.5 %). The G+C content of the strain's genomic DNA was 33.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain SulfLac1^T was most closely related to *Petrotoga mobilis* (91.4 % sequence similarity). Based on phenotypic, phylogenetic and chemotaxonomic evidence, strain SulfLac1^T represents a novel species of a new genus within the order *Thermotogales*, for which the name *Defluviitoga tunisiensis* gen. nov., sp. nov. is proposed. The type strain of the type species is SulfLac1^T (=DSM 23805^T =JCM 17210^T).

The cultivated representatives of the order *Thermotogales* are thermophilic anaerobes that are known to thrive in geothermally heated environments (Huber & Hannig, 2006). Members of this order include, for example, species of the genera *Thermotoga*, *Marinitoga*, *Thermosipho* and *Fervidobacterium* that were isolated from hot locations on the seafloor (Huber *et al.*, 1986, 1989; Urios *et al.*, 2004) such as deep-sea hydrothermal chimneys (Antoine *et al.*, 1997; Wery *et al.*, 2001; Postec *et al.*, 2005), from a hot inland oil well (Fardeau *et al.*, 2009) or from a hot spring (Patel *et al.*, 1985). The species belonging to the other genera in the order *Thermotogales* (i.e. *Petrotoga*, *Geotoga*, *Kosmotoga*, *Thermococcoides* and *Oceanotoga*) were all isolated from oilfield ecosystems (Davey *et al.*, 1993; Miranda-Tello *et al.*, 2004, 2007; DiPippo *et al.*, 2009; Feng *et al.*, 2010; Jayasinghearachchi & Lal, 2011). Curiously, some mesothermic ecosystems, such as anaerobic waste digesters and contaminated sediments, contain bacteria that have 16S rRNA gene sequences similar to those of thermophilic members of the order *Thermotogales* (van Houten *et al.*, 2009; Briones *et al.*, 2007; Chouari *et al.*, 2005). Whether such bacteria, called 'mesotoga', are truly mesophilic remains a matter of debate, however, as none of them has yet been cultivated (Nesbø *et al.*, 2006, 2010). The order *Thermotogales* comprises anaerobic, chemo-organotrophic, non-sporulating, rod-shaped bacteria that are characterized by a sheath-like outer structure known as a 'toga' (Huber *et al.*, 1986; Miranda-Tello *et al.*, 2004, 2007).

The present study represents a taxonomic characterization of a novel thermophilic bacterium that was isolated from a mesothermic digester used to process whey. The bacterium was found to show phenotypic and phylogenetic traits that led to its assignment to a novel species of a novel genus within the order *Thermotogales*.

Samples were collected from the sludge of a two-litre, anaerobic, continuously stirred tank reactor in Tunisia. The reactor had been inoculated with a mixture of marine sediments and sludge from a refuse dump and wastewater treatment plant in Tunisia. The reactor was fed with cheese whey (500 ml l^{-1}) and sulfate (5 g l^{-1}) with a flow rate of 100 ml per day and a temperature of 35 °C and at pH 8. The samples were collected under anaerobic conditions and transported to the research laboratory at ambient temperature.

Strict anaerobic procedures were followed for isolation and culture (Hungate, 1969). The selective medium used for the isolation included (l^{-1}): 1.0 g NH_4Cl, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.1 g KCl, 0.5 g $MgCl_2 . 6H_2O$, 0.1 g $CaCl_2 . 2H_2O$, 1.0 g NaCl, 1.0 g yeast extract (Difco), 0.5 g cysteine hydrochloride, 1.58 g $Na_2S_2O_3$ and 1 ml Widdel trace element solution (Widdel & Pfennig, 1982). The pH of this medium

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SulfLac1^T is FR850164.1.

was adjusted to 7.0 with 10 M KOH before the medium was boiled and then cooled to room temperature under a stream of oxygen-free N_2 gas. 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 with 0.5 ml sample, the basal medium in each tube was supplemented with 0.1 ml 10 % (w/v) $NaHCO_3$, 0.1 ml 2 % (w/v) $Na_2S.9H_2O$ and 0.1 ml 1 M glucose, all from sterile stock solutions.

Inoculated tubes were incubated at 55 °C. Cultures were purified by repeated use of the Hungate roll-tube method and medium solidified with 2.5 % (w/v) agar, before transfer of each isolate back into liquid medium, as previously described (Fardeau *et al.*, 1997).

The pH, temperature and NaCl concentration ranges for growth were determined using the basal medium supplemented with 20 mM glucose. The pH of the medium was varied between 6.4 and 9.0 by the addition of sterile 0.1 M HCl, 10 % (w/v) $NaHCO_3$ or 8 % (w/v) Na_2CO_3. Water baths were used to give incubation temperatures between 25 and 70 °C. For the tests of salt tolerance, NaCl was added to dry Hungate tubes before the medium was dispensed into the tubes and autoclaved. Isolates were subcultured at least twice, under the same conditions, before determining growth rates and the use of substrates.

Gram staining was determined using heat-fixed liquid cultures and the Difco Gram staining kit, according to the kit manufacturer's instructions. The morphology of cells in the exponential phase of growth was investigated in an electron microscope (H 600, Hitachi) at 75 kV, after negative staining with 1 % (w/v) sodium phosphotungstic acid (pH 7.0), as described by Fardeau *et al.* (1997).

Arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, raffinose, ribose, sucrose, xylose, peptone, Casamino acids, acetate, fumarate, lactate, pyruvate, succinate, ethanol, 2-propanol and caproate were each tested as a substrate at a final concentration of 20 mM, in basal medium. Formate and methanol were each tested at 40 mM while microcrystalline cellulose and xylan (a mix of birch-wood and oat-spelt) were each tested at 10 g l^{-1}. Utilization of H_2/CO_2 (80 : 20, v/v), in the absence or presence of 2 mM acetate as carbon source, was tested at a pressure of 2 bars. Sodium thiosulfate (20 mM), sodium sulfate (20 mM), sodium sulfite (2 mM) or elemental sulfur (10 g l^{-1}) was added to the medium as a potential electron acceptor. H_2S production was determined spectrophotometrically by using the method of Cord-Ruwisch (1985). The end products of metabolism were determined, after 2 weeks of incubation at 55 °C, by HPLC (Fardeau *et al.*, 1997).

Genomic DNA was isolated and purified by chromatography on hydroxyapatite, using the procedure of Cashion *et al.* (1977), before its G+C content was determined [at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany] by using HPLC, as described by Mesbah *et al.* (1989). Cultures of strain SulfLac1^T were stopped at the end of the exponential phase and sent to DSMZ for fatty acid analysis. Fatty acids were extracted using the method of Miller (1982), with the modifications of Kuykendall *et al.* (1988), then separated on a gas chromatograph (model 6890N, Agilent Technologies) and identified using version 6.1 of the Sherlock Microbial Identification System (MIDI) and the TSBA40 database (Sasser, 1990).

Total DNA was extracted and purified so that the 16S rRNA gene of each of the novel strains could be amplified and sequenced, as previously described (Khelifi *et al.*, 2010). The 16S rRNA gene sequence of strain SulfLac1^T was then compared with sequences in the GenBank database, by using a BLAST search (Altschul *et al.*, 1990). A multiple sequence file was generated using version 5.0.9 of BioEdit (Hall, 1999). Alignments were made using the MUSCLE program (Edgar, 2004) before being manually refined using BioEdit. Phylogenetic trees were then reconstructed by using TREECON (Van de Peer & De Wachter, 1994) and the neighbour-joining method with Kimura's two-parameter correction, the maximum-parsimony method, and the maximum-likelihood method (Felsenstein, 1981; Saitou & Nei, 1987). Bootstrap values were based on 500 replications (Felsenstein, 1985).

In the initial isolation, the colonies obtained in roll tubes were round, white–blue and measured 0.5–1 mm in diameter after incubation for 1 month at 55 °C. After the process of serial dilution was repeated several times, each of the isolates recovered was deemed to be axenic. Several strains that were similar in terms of morphology, 16S rRNA gene sequences (99–100 % sequence similarity) and the end products of their glucose metabolism were isolated. One such strain, designated SulfLac1^T, was selected for further characterization.

Strain SulfLac1^T was a non-motile, rod-shaped bacterium. Its cells, which measured 1 μm in width and 3–30 μm in length, occurred singly, in pairs or in sheaths that each contained up to 10 cells (Fig. 1a). Spores were not observed. Although its cells were Gram-staining-positive, the multilayered cell wall of strain SulfLac1^T is typical of Gram-negative bacteria (Fig. 1b).

Strain SulfLac1^T was anaerobic but tolerated up to 0.5 % (v/v) O_2. It grew at 37–65 °C (optimum 55 °C) but not at 30 °C or 70 °C, with 0.2–3 % (w/v) NaCl (optimum 0.5 %), and at pH 6.7–7.9 (optimum pH 6.9). Yeast extract was required for growth. Elemental sulfur and thiosulfate, but not sulfate or sulfite, were used as terminal electron acceptors, and growth was improved in the presence of elemental sulfur or thiosulfate. Strain SulfLac1^T utilized arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, microcrystalline cellulose, xylan and yeast extract but not mannitol, peptone, Casamino acids, acetate, lactate, fumarate, pyruvate, succinate, ethanol, methanol, 2-propanol, caproate, H_2/CO_2 or formate, even if acetate (2 mM) was present as a carbon source or thiosulfate was present as a

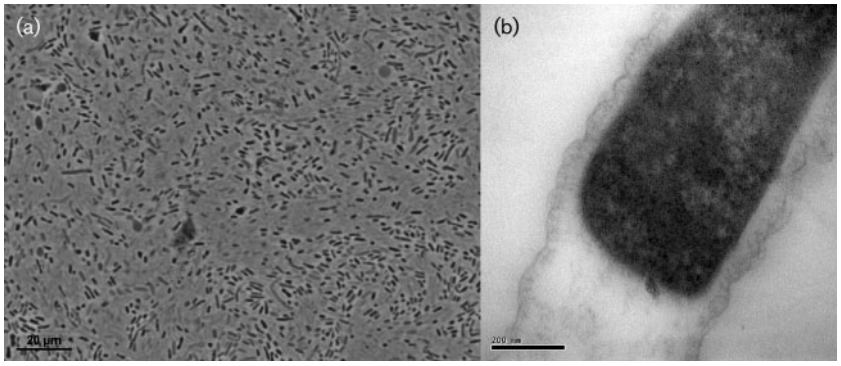

Fig. 1. (a) Phase-contrast photomicrograph showing cells of strain SulfLac1^T. (b) Transmission electron micrograph of a cell of strain SulfLac1^T showing an outer sheath-like structure. Bars, 10 μm (a) and 0.2 μm (b).

terminal electron acceptor. The end products of glucose metabolism were acetate (2 moles mol^{-1} of glucose used), hydrogen and carbon dioxide.

The cellular fatty acids detected in strain SulfLac1^T were $C_{16:0}$ (42.7 %), $C_{18:1}\omega9c$ (30.7 %), $C_{16:1}\omega9c$ (10.7 %), $C_{18:0}$ (9.9 %) and $C_{18:1}\omega7c$ (6.0 %). Its genomic DNA G+C content was 33.6 mol%.

The phylogenetic analysis of its amplified 16S rRNA gene sequence (1475 nt) indicated that, even though it was isolated from a mesothermic digester, strain SulfLac1^T belonged to the order *Thermotogales*. The observation of a sheath-like outer structure (toga) under the electron microscope supported this indication. In terms of its 16S rRNA gene sequence, strain SulfLac1^T appeared closely related ($\geqslant$99 % sequence similarity) to several uncultivated strains that have also been considered members of the order *Thermotogales* (Nesbø *et al.*, 2010). The closest GenBank accession (EF558953) was the 16S rRNA gene sequence of an environmental clone isolated from a hot (55 °C) anaerobic digester, although other very similar sequences represented bacteria retrieved from mesothermic terrestrial and subterrestrial environments (Li *et al.*, 2009; Liang *et al.*, 2009; Rivière *et al.*, 2009). Strain SulfLac1^T represents the first cultivated member of the monophyletic group that Nesbø *et al.* (2010) described as a sister group of the *Petrotoga* cluster. Phylogenetic analysis based on 16S rRNA gene sequences indicates that *Petrotoga mobilis* SJ95^T, which was isolated from the hot water of a North Sea oil reservoir (Lien *et al.*, 1998), represents the established species that is most closely related to strain SulfLac1^T (91.4 % sequence similarity). However, in phylogenetic trees based on such sequences, strain SulfLac1^T forms a distinct branching lineage (Fig. 2), with <92 % sequence similarity with any recognized species. It is therefore proposed that strain SulfLac1^T is considered a novel species of a new genus within the order *Thermotogales*. This proposal is supported by some phenotypic characteristics of the novel strain. For example, strain SulfLac1^T appears much less halophilic than members of the genus *Petrotoga* and also differs in terms of its substrate-utilization profile and the end products of its glucose metabolism (Table 1). Based on comparative morphological, physiological and phylogenetic data, strain SulfLac1^T represents a novel species of a new genus within the order *Thermotogales*, for which the name *Defluviitoga tunisiensis* gen. nov., sp. nov. is proposed.

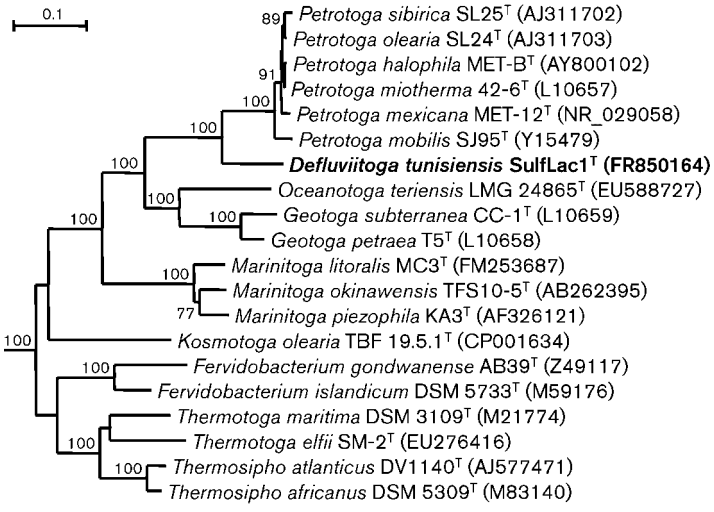

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1190 nt), showing the relationship between strain SulfLac1^T and established members of the order *Thermotogales*. Bootstrap values (expressed as percentages of 500 replications) are shown at branch points. GenBank accession numbers are in parentheses. *Desulfurobacterium thermolithotrophum* BSA^T (AJ001049) and *Thermodesulfovibrio yellowstonii* ATCC 51303^T (AB231858) were used as outgroups (not shown). Bar, 0.1 substitutions per nucleotide position.

Table 1. Characteristics that differentiate strain SulfLac1^T from those species within the genus *Petrotoga* that appear phylogenetically most closely related

Strains: 1, SulfLac1^T (data from this study); 2, *P. mobilis* SJ95^T (Lien *et al.*, 1998); 3, *P. mexicana* MET12^T (Miranda-Tello *et al.*, 2004); 4, *P. halophila* MET-B^T (Miranda-Tello *et al.*, 2007); +, Positive; −, negative.

Characteristic	1	2	3	4
Temperature for growth (°C)				
Range	37–65	40–65	25–65	45–65
Optimum	55	58–60	55	60
pH for growth				
Range	6.5–7.9	5.5–8.5	5.6–8.5	5.6–7.8
Optimum	6.9	6.5–7.0	6.6	6.7–7.2
NaCl concentration for growth (%, w/v)				
Range	0.2–3	0.5–9	1–20	0.5–9
Optimum	0.5	3–4	3	4–6
Motility	−	+	+	−
Electron acceptors used				
Sulfur	+	+	+	+
Thiosulfate	+	+	+	−
Sulfite	−	−	+	−
Substrate utilization				
Mannose	+	−	+	−
Raffinose	+	−	+	−
Cellulose	+	−	−	−
DNA G+C content (mol%)	33.6	34	36.1	34.6
End products of glucose fermentation	Acetate, CO_2, H_2	Acetate, ethanol, H_2, CO_2	Acetate, CO_2, H_2, traces of lactate	Acetate, lactate, CO_2, H_2

Description of *Defluviitoga* gen. nov.

Defluviitoga (De.flu.vi.i.to′ga. L. n. *defluvium* sewage, wastewater; L. fem. n. *toga* a toga; N.L. fem. n. *Defluviitoga*, a toga isolated from wastewater).

Cells are rod-shaped with a sheath-like outer structure. They occur singly, in pairs or in chains. Growth is anaerobic and thermophilic. Chemo-organotrophic, having the ability to ferment a broad spectrum of carbohydrates and yeast extract. Reduces thiosulfate and elemental sulfur to H_2S. Phylogenetic analysis based on 16S rRNA gene sequences locates the genus *Defluviitoga* in the bacterial domain, within the order *Thermotogales* and close to the genus *Petrotoga*. The type species is *Defluviitoga tunisiensis*.

Description of *Defluviitoga tunisiensis* sp. nov.

Defluviitoga tunisiensis (tu.ni.si.en′sis, N.L. fem. adj. *tunisiensis* of or belonging to Tunisia, the country where the bacterium was first isolated).

A mesophilic, slightly halophilic and anaerobic bacterium. Cells are non-spore-forming, Gram-negative rods. Grows at 37–65 °C (optimum 55 °C), at pH 6.7–7.9 (optimum pH 6.9) and with 0.2–3.0 % (w/v) NaCl (optimum 0.5 %). Yeast extract required for growth. Arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, microcrystalline cellulose, xylan and yeast extract are used as electron donors, but not mannitol, Casamino acids, peptone, acetate, lactate, fumarate, pyruvate, succinate, ethanol, H_2/CO_2 or formate. Acetate, H_2 and CO_2 are the end products of glucose fermentation. The predominant cellular fatty acids are $C_{16:0}$ and $C_{18:1}\omega9c$.

The type strain, SulfLac1^T (=DSM 23805^T =JCM 17210^T), was isolated from an anaerobic reactor used to digest whey. The genomic DNA G+C content of the type strain is 33.6 mol%.

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