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

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Strain SulfLac1T, 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 SulfLac1T was most closely related to Petrotoga mobilis (91.4 % sequence similarity). Based on phenotypic, phylogenetic and chemotaxonomic evidence, strain SulfLac1T 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 SulfLac1T (=DSM 23805T =JCM 17210T).

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 (Nesbo 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 NH4Cl, 0.3 g K2HPO4, 0.3 g KH2PO4, 0.1 g KCl, 0.5 g MgCl2·6H2O, 0.1 g CaCl2·2H2O, 1.0 g NaCl, 1.0 g yeast extract (Difco), 0.5 g cysteine hydrochloride, 1.58 g Na2S·9H2O and 1 ml Widdel trace element solution (Widdel & Pfennig, 1982). The pH of this medium...
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 N2 gas. Aliquots of 5 ml were dispensed into Hungate tubes, degassed under N2/CO2 (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) NaHCO3, 0.1 ml 2% (w/v) Na2S, 9H2O 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) NaHCO3 or 8% (w/v) Na2CO3. 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 the exponential phase of growth was investigated in an electron microscope (H 600, Hitachi) at 75 kV, after fixation 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 at 40 mM while microcrystalline cellulose and xylan (a mix of birch-wood and oat-spelt) were each tested at 10 g l⁻¹. 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) NaHCO3 or 8% (w/v) Na2CO3. 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.

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Utilization of H2/CO2 (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. H2S 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 Sulflac1T 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 Sulflac1T 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 TREBEX (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 Sulflac1T, was selected for further characterization.

Strain Sulflac1T 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 Sulflac1T is typical of Gram-negative bacteria (Fig. 1b).

Strain Sulflac1T was anaerobic but tolerated up to 0.5% (v/v) O2. 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 Sulflac1T 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, H2/CO2 or formate, even if acetate (2 mM) was present as a carbon source or thiosulfate was present as a
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 SulfLac\(^{T}\) were C\(_{16:0}\) (42.7%), C\(_{18:1\alpha 9c}\) (30.7%), C\(_{16:1\alpha 9c}\) (10.7%), C\(_{18:0}\) (9.9%) and C\(_{18:1\alpha 7c}\) (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 SulfLac\(^{T}\) 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 SulfLac\(^{T}\) appeared closely related (\(\geq 99\%\) sequence similarity) to several uncultivated strains that have also been considered members of the order Thermotogales (Nesbo 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 SulfLac\(^{T}\) represents the first cultivated member of the monophyletic group that Nesbo 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 SulfLac\(^{T}\) (91.4% sequence similarity). However, in phylogenetic trees based on such sequences, strain SulfLac\(^{T}\) forms a distinct branching lineage (Fig. 2), with <92% sequence similarity with any recognized species. It is therefore proposed that strain SulfLac\(^{T}\) 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 SulfLac\(^{T}\) 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 SulfLac\(^{T}\) represents a novel species of a new genus within the order Thermotogales, for which the name Defluviitoga tunisiensis gen. nov., sp. nov. is proposed.

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Fig. 1. (a) Phase-contrast photomicrograph showing cells of strain SulfLac\(^{T}\). (b) Transmission electron micrograph of a cell of strain SulfLac\(^{T}\) showing an outer sheath-like structure. Bars, 10 \(\mu m\) (a) and 0.2 \(\mu m\) (b).

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Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1190 nt), showing the relationship between strain SulfLac\(^{T}\) 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. Desulfuribaculum thermolithotrophum BSA\(^{T}\) (AJ001049) and Thermodesulfovibrio yellowstonii ATCC 51303\(^{T}\) (AB231858) were used as outgroups (not shown). Bar, 0.1 substitutions per nucleotide position.
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 H2S. 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, H2/CO2 or formate. Acetate, H2 and CO2 are the end products of glucose fermentation. The predominant cellular fatty acids are C16:0 and C18:1ω9c.

The type strain, SulfLac1T (= DSM 23805T = JCM 17210T), was isolated from an anaerobic reactor used to digest whey. The genomic DNA G+C content of the type strain is 33.6 mol%.

Acknowledgements

We thank Dr Jean Euzéby for checking the Latin etymology of the generic and specific names, Manon Joseph for help with the electron microscopy and Dr Pierre Roger for revising the manuscript.

References


