Characterization of *Defluviitalea saccharophila* gen. nov., sp. nov., a thermophilic bacterium isolated from an upflow anaerobic filter treating abattoir wastewaters, and proposal of *Defluviitaleaceae* fam. nov.

Linda Jabari,[1,2] Hana Gannoun,[1,2] Jean-Luc Cayol,[1] Moktar Hamdi,[2] Guy Fauque,[1] Bernard Ollivier[1] and Marie-Laure Fardeau[1]

Correspondence
Marie-Laure Fardeau
marie-laure.fardeau@univ-amu.fr

[1]Université d'Aix-Marseille, Mediterranean Institute of Oceanography (MIO), IRD, MIOUMR 235, CNRS/INSU MIOUMR 7294, 163 Avenue de Luminy, 13288 Marseille Cedex 9, France

[2]Laboratoire d'Ecologie et de Technologie Microbienne, Institut National des Sciences Appliquées et de Technologie, Centre Urbain Nord, BP 676, 1080 Tunis Cedex, Tunisia

A novel thermophilic, anaerobic, Gram-stain-positive, terminal-spore-forming bacterium was isolated from an upflow anaerobic filter treating abattoir wastewaters in Tunisia. This strain, designated LIND6LT2^T, grew at 40–60 °C (optimum 50–55 °C) and at pH 6.0–8.5 (optimum pH 7.0–7.5). It did not require NaCl for growth, but tolerated it up to 2 %. Sulfate, thiosulfate, elemental sulfur, sulfite, nitrate and nitrite were not used as electron acceptors. Growth of LIND6LT2^T was inhibited by sulfite (2 mM). Strain LIND6LT2^T used cellobiose, glucose, mannose, maltose, mannitol, sucrose and xylose as electron donors. The main fermentation products from glucose metabolism were acetate, formate, butyrate and isobutyrate. The predominant cellular fatty acids were $C_{16:0}$ (68.4 %) and $C_{14:0}$ (8.3 %). The G + C content of the genomic DNA was 35.2 mol%. On the basis of its phylogenetic and physiological properties, a new genus and species, *Defluviitalea saccharophila* gen. nov., sp. nov., are proposed to accommodate strain LIND6LT2^T, placed in *Defluviitaleaceae* fam. nov. within the phylum *Firmicutes*, class *Clostridia*, order *Clostridiales*. Strain LIND6LT2^T (=DSM 22681^T =JCM 16312^T) is the type strain of *Defluviitalea saccharophila*, which itself is the type species of *Defluviitalea*.

The anaerobic digestion of organic wastes and industrial wastewaters constitutes a major research focus because of global needs for waste recycling and renewable energy production (Bouallagui *et al.*, 2004; Goberna *et al.*, 2009; LaPara *et al.*, 2000). The use of molecular-biological techniques, especially those directed towards the small-subunit rRNA molecule, has eliminated the need to isolate pure cultures as a means of studying the diversity and structure of microbial communities. However, some authors continue to test different types of bioreactors (Gannoun *et al.*, 2009) and attempt to isolate micro-organisms in pure culture. Clostridia and many other anaerobic species have been found routinely in studies of the decomposition of various organic compounds (Jeong *et al.*, 2004; Warnick *et al.*, 2002). They generally belong to the phylum *Firmicutes*, which accommodates Gram-positive bacteria with a low G + C content (<50 mol%)

and constitutes one of the main phyla within the *Bacteria*. The members of this lineage are highly diverse in their morphology (rod, spiral, coccoid), physiology (aerobic, anaerobic) and lifestyle (non-spore-forming, endospore-forming). At the time of writing, the *Firmicutes* included six classes, *Bacilli*, *Clostridia*, *Erysipelotrichia*, *Mollicutes*, *Negativicutes* and *Thermolithobacteria*, and 362 genera (http://www.bacterio.cict.fr/classifphyla.html#Firmicutes).

In this paper, we describe a novel thermophilic, Gram-stain-positive, spore-forming, anaerobic bacterium (strain LIND6LT2^T) isolated from an upflow anaerobic filter treating wastewaters (Gannoun *et al.*, 2009). It had phenotypic and phylogenetic characteristics that allowed its assignment to a novel species and genus within a novel family of the order *Clostridiales* of the phylum *Firmicutes*.

Strain LIND6LT2^T was isolated from an upflow anaerobic filter in Tunisia in which the performance of an efficient biological pretreatment for solubilization of the suspended

solids (proteins and fats) and the anaerobic digestion of biologically pretreated abattoir wastewater were evaluated under mesophilic and thermophilic conditions (Gannoun *et al.*, 2009). The sample was taken during the thermophilic phase.

For the enrichment culture, 0.5 ml sludge from the anaerobic digestor was used to inoculate 5 ml medium. The basal medium contained (per litre distilled water) 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 1 g NH_4Cl, 1 g NaCl, 0.1 g KCl, 0.1 g $CaCl_2 . 2H_2O$, 1 g $MgCl_2 . 6H_2O$, 0.5 g cysteine hydrochloride, 2 g yeast extract (Difco Laboratories), 10 ml oligoelements solution (Balch *et al.*, 1979) and 1 ml 0.1 % resazurin. The medium was adjusted to pH 7.2 with 10 M KOH, 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) $Na_2S . 9H_2O$ and substrates (20 mM lactate or 20 mM acetate) were injected from sterile stock solutions into the tubes. The Hungate technique (Hungate, 1969) was used throughout this study.

Growth experiments were performed in duplicate using Hungate tubes containing basal medium. pH, temperature and NaCl concentration ranges for growth were determined over the ranges pH 5–9, 30–80 °C and 0–50 g NaCl l^{-1} using basal medium supplemented with 20 mM glucose. The pH of the medium was adjusted with anaerobic, sterile stock solutions of 1 M HCl (acidic pH), 1 M $NaHCO_3$ or 1 M Na_2CO_3 (alkaline pH) and the pH was verified after inoculation. Water baths were used to obtain incubation temperatures of up to 80 °C. For studies of NaCl requirements, NaCl was weighed directly in the tubes prior to the medium being dispensed. The strains were subcultured at least once under the same experimental conditions prior to determination of growth rates. In the presence of 2 g yeast extract l^{-1} as a growth factor, the following substrates were tested: acetate, pyruvate, lactate, glucose, maltose, xylose, peptone, lactose, mannose, raffinose, sucrose, arabinose, cellobiose, glycerol, mannitol, rhamnose, Casamino acids, H_2/CO_2 (80/20, v/v) and H_2/CO_2 (80/20, v/v) plus acetate (2 mM). Each substrate was tested in basal medium at a final concentration of 20 mM for sugars and organic acids. 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. The presence of spores was analysed by phase-contrast microscopic observations of cultures and pasteurization tests performed at 80, 90 and 100 °C for 10 and 20 min. The effect of different quantities of yeast extract was tested in a medium prepared without yeast extract.

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 55 °C (Fardeau *et al.*, 2000).

Morphological characteristics and purity of strains were checked under a Nikon Optiphot phase-contrast microscope. For transmission electron microscopy studies, cell preparations were negatively stained with sodium phosphotungstate, as described previously (Fardeau *et al.*, 1997).

The $G+C$ content of the DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ). DNA was isolated and purified by chromatography on hydroxyapatite using the procedure of Cashion *et al.* (1977) and the $G+C$ content was determined by using HPLC as described by Mesbah *et al.* (1989). The determination of cellular fatty acid compositions was performed by the Identification Service of the DSMZ after extraction using the method of Miller (1982) as modified by Kuykendall *et al.* (1988). Fatty acids were separated using the MIDI Microbial Identification system (version 4.0; MIS operating manual, March 2001) (Sasser, 1990). Polar lipid analyses were carried out by the Identification Service of the DSMZ and Dr B. J. Tindall.

Purification of the DNA, PCR amplification and sequencing of the 16S rRNA gene were performed as described previously (Thabet *et al.*, 2004). The partial sequences generated were assembled using BioEdit version 5.0.9 (Hall, 1999) and the consensus sequence of 1495 nt was corrected manually for errors. The most closely related sequences in GenBank (version 178), the Ribosomal Database Project (release 10) identified using BLAST (Altschul *et al.*, 1990) and the Sequence Match program (Cole *et al.*, 2009) were extracted and aligned. The consensus sequence was then adjusted manually to conform to the 16S rRNA secondary structure model (Winker & Woese, 1991). Nucleotide ambiguities were omitted and evolutionary distances were calculated using the Jukes and Cantor option (Jukes & Cantor, 1969). Dendrograms were constructed with the TREECON program using the neighbour-joining method (Saitou & Nei, 1987). Tree topology was re-examined by the bootstrap method (1000 replications) of resampling (Felsenstein, 1985). Its topology was also supported using the maximum-parsimony and maximum-likelihood algorithms.

Aliquots (0.5 ml) of the sample were inoculated into Hungate tubes containing 5 ml basal medium containing 20 mM lactate as substrate and the tubes were incubated at 55 °C. To obtain pure cultures, the enrichment was subcultured several times under the same growth conditions prior to isolation. For isolation, the culture was serially diluted tenfold in basal medium containing agar (2 % w/v) in roll tubes (Miller & Wolin, 1974); several colonies developed after incubation at 55 °C and they were picked separately in a glove box. The process of serial dilution was repeated several times until the isolates were deemed to be axenic. Several strains similar in morphology and phylogeny were isolated. None of these strains was
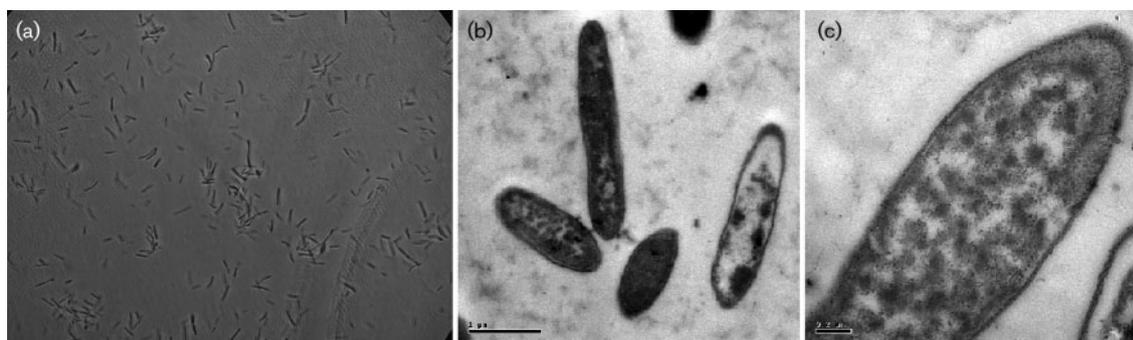
Fig. 1. (a) Phase-contrast photomicrograph showing cells of strain LIND6LT2[T]. (b, c) Thin-section electron micrographs of strain LIND6LT2[T] showing the Gram-positive cell wall. Bars, 1 μm (b) and 0.2 μm (c).

shown to oxidize lactate, suggesting that the primary source of energy in the enrichment was yeast extract and not lactate. A strain designated LIND6LT2[T] was selected and used for further physiological and metabolic characterization.

The colonies obtained in roll tubes were round and pale yellow. They were 1–2 mm in diameter after 3–5 days of incubation at 55 °C. Cells of strain LIND6LT2[T] were non-motile rods, occurring singly or in pairs, approximately 5–10 μm long and about 0.5 μm in diameter (Fig. 1a). Cells stained Gram-positive. Growth was also obtained after heat treatment of cultures at 80, 90 and 100 °C for 10 and 20 min. The strain was sporulating and sporulating cells reached 50 μm in length and formed round, terminal spores. Sections for electron microscopy revealed a cell wall with a structure of three thin layers (Fig. 1b, c).

Strain LIND6LT2[T] was thermophilic and grew at 40–60 °C, with an optimum between 50 and 55 °C. The pH range for growth was 6.0–8.5, with an optimum at pH 7.0–7.5. The isolate grew in the presence of NaCl concentrations ranging from 0 to 2 %, with an optimum at 0.5 %.

Thiosulfate, sulfate, sulfite, elemental sulfur, nitrate and nitrite were not used as terminal electron acceptors, and sulfite (2 mM) inhibited growth of the strain. Yeast extract (minimum 0.01 %) was required for growth and could not be replaced by peptides (tryptone; Panreac Quimica) or by the trace mineral element solution of Balch et al. (1979). Growth was enhanced by increasing concentrations of yeast extract.

The substrates used for growth were yeast extract, glucose, cellobiose, maltose, mannose, mannitol, sucrose and xylose. The end products resulting from glucose metabolism were acetate, formate, butyrate and isobutyrate. Under optimal growth conditions on a glucose medium, the growth rate was 0.56 h^{-1}. Strain LIND6LT2[T] was anaerobic.

The major cellular fatty acids present in strain LIND6LT2[T] were C$_{16:0}$ (68.4 %), C$_{14:0}$ (8.3 %), C$_{18:0}$ (7.3 %), C$_{16:1}\omega 7c$ (5.3 %) and C$_{18:1}\omega 7c$ (4.1 %) (Table 1). No respiratory quinones were detected. The polar lipid profile comprised

diphosphatidylglycerol, phosphatidylglycerol, phospholipids, a phosphoglycolipid and a glycolipid.

The G + C content of genomic DNA of strain LIND6LT2[T] was 35.2 mol%.

The 16S rRNA gene sequence obtained contained 1495 nt. A phylogenetic tree was reconstructed based on the neighbour-joining method (Fig. 2). Strain LIND6LT2[T] belongs to the class *Clostridia*, order *Clostridiales*; however, it does not group within any family with a validly published name within the order *Clostridiales*.

Strain LIND6LT2[T] was isolated from an upflow anaerobic filter treating abattoir wastewaters in Tunisia. In the 16S rRNA gene sequence-based phylogenetic tree, it forms a separate branch within the order *Clostridiales*. Based on an inferred phylogenetic tree, the closest cultured relative is *Parasporobacterium paucivorans* DSM 15970[T] (Lomans et al., 2001), a member of the family *Lachnospiraceae* [formerly *Clostridium* cluster III (Collins et al., 1994; Ludwig et al., 2009)]. BLAST analysis yields *Parasporobacterium paucivorans* with about 87.17 % similarity. The second, third and fourth closest relatives of strain LIND6LT2[T] are *Clostridium indolis* (McClung & McCoy, 1957), *Clostridium populeti* (Sleat & Mah, 1985) and *Robinsoniella peoriensis* (Cotta et al., 2009),

Table 1. Cellular fatty acid profile of strain LIND6LT2[T]

Values are percentages of total fatty acids.

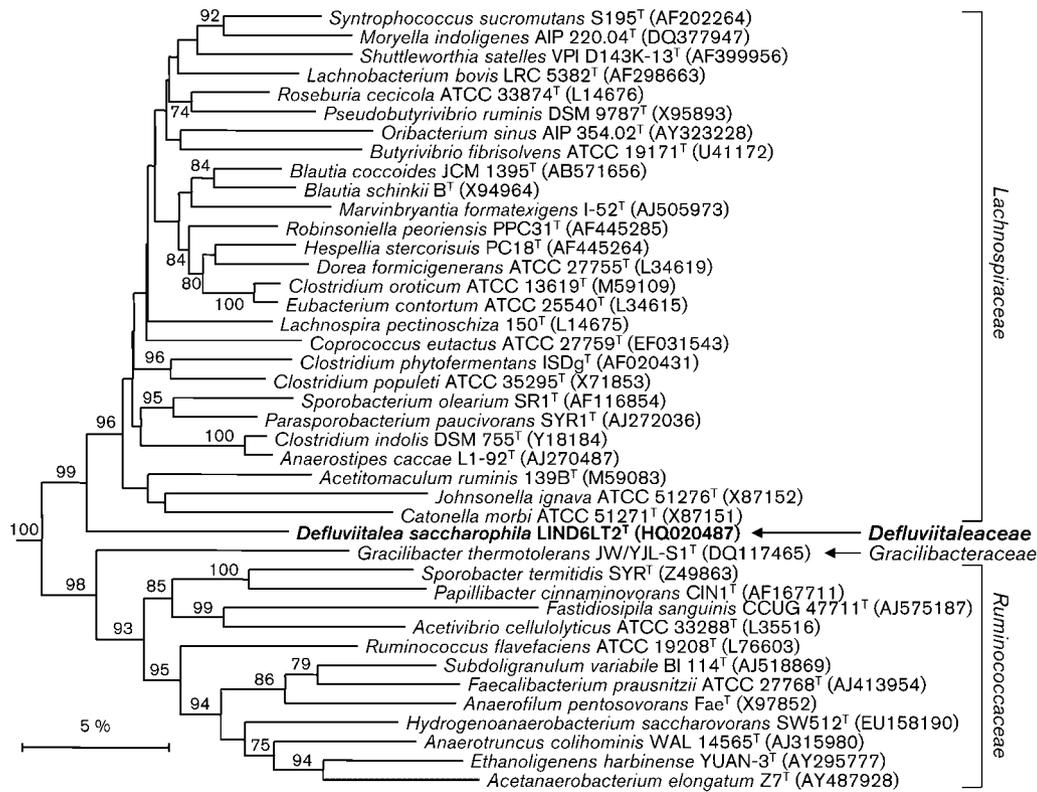| Fatty acid	Content (%)
C$_{16:0}$	68.4
C$_{14:0}$	8.3
C$_{18:0}$	7.3
C$_{16:1}\omega 7c$	5.3
C$_{12:0}$	0.4
C$_{16:0}$ N alcohol	0.7
C$_{18:1}\omega 9c$	0.8
C$_{18:1}\omega 7c$	4.1
Unknown	1.4

Fig. 2. 16S rRNA gene sequence-based phylogenetic analysis showing the relationships between strain LIND6LT2[T] and the genera of the families *Lachnospiraceae*, *Gracilibacteraceae* and *Ruminococcaceae*. The tree was reconstructed from distance matrices using the neighbour-joining method. The type species of each genus is included on the tree with the exception of the genera *Clostridium*, *Eubacterium* and *Lachnospira*. For the latter genus, only a short and low-quality sequence is available for the type strain of the type species *Lachnospira multispira*; a sequence of the type strain of the species *Lachnospira pectinoschiza* (Cornick *et al.*, 1994) has been used to represent this genus. Bar, 5 substitutions per 100 nucleotide positions. Bootstrap values ≥70 % (based on 1000 repetitions) are given at branching points.

respectively with 86.97, 86.29 and 86.20 % similarity. These four strains differ from strain LIND6LT2[T] in physiological and metabolic characteristics. For example, these four strains grow over mesophilic temperature ranges, but strain LIND6LT2[T] grows under thermophilic conditions, between 45 and 60 °C with an optimum at 50–55 °C, and their G+C contents are very different from that of strain LIND6LT2[T] (Table 2).

Parasporobacterium paucivorans has a very limited substrate range, utilizing syringate, trimethoxybenzoate and gallate as sole carbon and energy sources (Lomans *et al.*, 2001). The three other strains utilize common carbohydrates as carbon and energy sources, as do most other closely related micro-organisms of the genera *Clostridium*, *Eubacterium* and *Ruminococcus* (Table 2).

These results indicate that strain LIND6LT2[T] presents phenotypic and phylogenetic characteristics that distinguish it from the other strains of this cluster.

In previous studies, many uncultured bacteria have been detected in bioreactors, for example treating pharmaceutical wastewater under mesophilic (28–32 °C) and thermophilic (50–58 °C) conditions (LaPara *et al.*, 2000), that present similarities to strain LIND6LT2[T].

On the basis of these physiological, chemotaxonomic and phylogenetic data, strain LIND6LT2[T] is assigned to the new genus and species *Defluviitalea saccharophila* gen. nov., sp. nov.; *Defluviitalea* gen. nov. is proposed as the type genus of the novel family *Defluviitaleaceae* fam. nov. within the order *Clostridiales* of the phylum *Firmicutes*.

Description of *Defluviitalea* gen. nov.

Defluviitalea (De.flu.vi.i.ta′le.a. L. n. *defluvium* sewage, wastewater; L. fem. n. *talea* a rod; N.L. fem. n. *Defluviitalea* a rod isolated from wastewater).

Cells have a Gram-positive type of cell wall and are non-motile, terminal-spore-forming rods. Thermophilic. Fermentative. Anaerobic type of metabolism. Cellobiose, glucose, mannose, maltose, mannitol, sucrose and xylose are used as electron donors. The predominant cellular fatty acids are $C_{16:0}$, $C_{14:0}$ and $C_{18:0}$. The G+C content of the genomic

Table 2. Differential characteristics between strain LIND6LT2[T] and its four phylogenetically closest relatives

Taxa: 1, strain LIND6LT2[T]; 2, *Parasporobacterium paucivorans* (data from Lomans *et al.*, 2001); 3, *Clostridium indolis* (McClung & McCoy, 1957); 4, *Clostridium populeti* (Sleat & Mah, 1985); 5, *Robinsoniella peoriensis* (Cotta *et al.*, 2009). +, Positive; −, negative; ND, no data available.

Characteristic	1	2	3	4	5
Gram stain	+	−	+	−	+
Optimum growth conditions					
Temperature (°C)	50–55	34–37	30–37	35	37
pH	7–7.5	ND	5.3–5.7	7.0	ND
NaCl concentration (%)	0.5	0–1.8	ND	ND	ND
Motility	−	−	+ (peritrichous flagella)	+	−
Morphology	Rods	Double rods	Slightly curved rods	Rods	Short rods
DNA G+C content (mol%)	35.2	ND	44	45.5–46	49
Main substrates used	Carbohydrate	Syringate, gallate	Carbohydrate	Carbohydrate	Carbohydrate
Fermentation end products*	A, B, F, iB	MT, DMS	F, A, EtOH	A, B, L	A, S

*A, Acetate; B, butyrate; DMS, dimethyl sulfide; EtOH, ethanol; F, formate; iB, isobutyrate; L, lactate; MT, methanethiol; S, succinate.

DNA of the type strain of the type species is 35.2 mol%. The type species is *Defluviitalea saccharophila*.

Description of *Defluviitalea saccharophila* sp. nov.

Defluviitalea saccharophila (sac.cha.ro.phi′la. L. n. *saccharum* from Gr. n. *sakchar -aros* sugar; N.L. fem. adj. *phila* from Gr. adj. *philos -ê -on* friendly to, loving; N.L. fem. adj. *saccharophila* sugar-loving).

Displays the following properties in addition to those given in the genus description. Cells are 5–10 μm long. The temperature range for growth is 45–60 °C, with optimum growth between 50 and 55 °C. The optimum pH for growth is 7.0–7.5. Grows in the presence of 0–2 % NaCl (optimum growth at 0.5 %). Yeast extract is required for growth. Cellobiose, glucose, mannose, maltose, mannitol, sucrose and xylose are used as electron donors.

The type strain, LIND6LT2[T] (=DSM 22681[T] =JCM 16312[T]), was isolated from an upflow anaerobic filter treating abattoir wastewaters in Tunisia.

Description of *Defluviitaleaceae* fam. nov.

Defluviitaleaceae (De.flu.vi.i.ta.le.a′ce.a.e. N.L. n. *Defluviitalea* type genus of the family; suff. -*aceae* ending to denote a family; N.L. fem. pl. n. *Defluviitaleaceae* the *Defluviitalea* family).

Display the properties of the type genus. The family belongs to the order *Clostridiales* of the phylum *Firmicutes*. The type genus is *Defluviitalea*.

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