Fusibacter tunisiensis sp. nov., isolated from an anaerobic reactor used to treat olive-mill wastewater

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Strain BELH1^T, a novel mesophilic, anaerobic, halotolerant, rod-shaped bacterium, was isolated from a Tunisian wastewater digester. The cells of the strain are motile, measure 0.5×2–5 µm, and occur singly or in pairs. The strain reduced thiosulfate and elemental sulfur (but not sulfate or sulfite) into sulfide. It grew at 15–40 °C (optimum 30 °C), pH 5.8–8.4 (optimum 7) and with 0–10 % (w/v) NaCl (optimum 3.0 %). The genomic DNA G+C content of strain BELH1^T was 38.2 mol% and the strain's predominant cellular fatty acids were C_{14:0}, a summed feature that contained iso-C_{17:1} and/or anteiso-C_{17:1} B, and C_{16:0}. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the novel strain was most closely related to *Fusibacter paucivorans* (94.8 % sequence similarity). Based on phenotypic, phylogenetic and taxonomic characteristics, strain BELH1^T represents a novel species of the genus *Fusibacter*, for which the name *Fusibacter tunisiensis* sp. nov. is proposed. The type strain is BELH1^T (=DSM 24436^T=JCM 17481^T).

The genus *Fusibacter*, first proposed by Ravot *et al.* (1999), belongs to the phylum *Firmicutes*, order *Clostridiales*. To date, the genus contains only one recognized species, *Fusibacter paucivorans*, an anaerobic heterotrophic and thiosulfate-reducing bacterium isolated from a sample of reservoir water collected from an offshore oil-producing well in Congo, Central Africa (Ravot *et al.*, 1999).

In the present study, a polyphasic approach was used for the taxonomic characterization of a novel mesophilic and halotolerant bacterium isolated from a digester used to treat wastewater from a Tunisian olive mill. The novel strain, which was able to use thiosulfate as a terminal electron acceptor, possessed phenotypic and phylogenetic traits that allowed its assignment as a novel species of the genus *Fusibacter*.

Samples were collected in Tunisia, from the sludge of a 2-litre anaerobic sequencing batch reactor, which was run at 35 °C and pH 7.5, with a flow rate of 100 ml day⁻¹, and fed with olive-mill wastewater (100 ml l⁻¹) and phosphogypsum (10 g l⁻¹). The samples were collected under anaerobic conditions and transported to the research laboratory at ambient temperature. Strict anaerobic procedures

were followed for the microbial isolation and culture (Hungate, 1969).

The basal medium used for the isolation contained (l⁻¹): 1.0 g NH₄Cl, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 0.1 g KCl, 0.1 g CaCl₂·2H₂O, 23 g NaCl, 1.0 g yeast extract (Difco), 1.0 g tryptone (Panreac Quimica), 0.5 g cysteine-HCl, 1.6 g Na₂S₂O₃ and 1 ml Widdel trace element solution (Widdel & Pfennig, 1982). The medium was adjusted to pH 7 with 10 M KOH solution before being boiled and 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 then 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, 0.1 ml 15 % (w/v) MgCl₂·6H₂O and 0.1 ml 1 M glucose, all from sterile stock solutions.

Inoculated tubes were incubated at 30 °C. Cultures were purified by repeated use of the Hungate roll-tube method (Hungate, 1969) and medium solidified with 1.6 % (w/v) agar, before transfer of each isolate back into liquid medium.

The pH, temperature and NaCl concentration ranges for growth were determined using basal medium supplemented with 20 mM glucose. Initial pH was varied, from 5.8

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to 8.4, by injecting 0.1 M HCl, 10 % (w/v) NaHCO₃ or 8 % (w/v) Na₂CO₃ (each from a sterile anaerobic stock solution) into Hungate tubes that each contained 5 ml medium. In other experiments, incubation temperatures were varied from 10 °C to 45 °C. For the tests of tolerance to NaCl, the NaCl was added to dry Hungate tubes before the medium was dispensed into the tubes and autoclaved.

Cultures were subcultured into fresh medium at least twice, under the same experimental conditions, before determining growth rates and substrate use.

Gram staining was determined using heat-fixed liquid cultures and the Difco Gram staining kit, according to the manufacturer's instructions. The morphology of cells in the exponential phase of growth was investigated with an electron microscope (H600; Hitachi) at 75 kV, after negative staining with 1 % (w/v) sodium phosphotungstic acid (pH 7.0), as described by Fardeau *et al.* (1997).

Substrate utilization was tested by using basal medium containing the potential substrate at 20 mM (arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, melibiose, raffinose, rhamnose, ribose, sorbitol, sorbose, sucrose, trehalose, xylose, formate, acetate, butyrate, lactate, propionate, Casamino acids, methanol and gelatin) or at 10 g l⁻¹ (tryptone). To test for electron acceptors, sodium thiosulfate (20 mM), sodium sulfate (20 mM), sodium sulfite (2 mM) or elemental sulfur (10 g l⁻¹) were added to the medium. H₂S production was determined spectrophotometrically, as described by Cord-Ruwisch (1985). The end products of glucose metabolism were measured by HPLC, after incubation at 30 °C for 2 weeks (Fardeau *et al.*, 1997).

Cultures of strain BELH1^T were stopped at the end of exponential phase and sent to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), in Braunschweig, Germany, 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 (6890N; Agilent Technologies) and identified using version 6.1 of the Sherlock Microbial Identification System (MIDI) and the TSBA40 database (Sasser, 1990).

The G + C content of the genomic DNA of strain BELH1^T was also determined at the DSMZ; DNA was isolated and purified by chromatography on hydroxyapatite, using the procedure of Cashion *et al.* (1977), before the G + C content was determined by HPLC, as described by Mesbah *et al.* (1989).

The extraction and purification of total DNA from strain BELH1^T, followed by the amplification and sequencing of the strain's 16S rRNA gene, were performed as described by Khelifi *et al.* (2010). The 16S rRNA gene sequence 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 constructed 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 seen in roll tubes were round and brown and measured about 2 mm in diameter after 12 days of incubation at 30 °C. Serial dilution was repeated until each of the several isolates was deemed to be axenic. Three of the isolated strains, designated BELA1, BELB2 and BELH1^T, were similar in morphology and phylogeny (99–100 % 16S rRNA gene sequence similarity) and produced the same end products from glucose metabolism. Strain BELH1^T was selected for further characterization.

The cells of strain BELH1^T were motile, Gram-staining-positive rods that measured 0.5 × 2–5 µm and occurred singly or in pairs (Fig. 1a). Transmission electron microscopy of ultrathin sections revealed the typical cell wall of a Gram-positive bacterium, with an electron-dense layer (Fig. 1b). Spores were not observed.

Strain BELH1^T was anaerobic but tolerated up to 1 % (v/v) O₂. It was also mesophilic, growing at 15–40 °C (optimum 30 °C) but not at 10 °C or 45 °C. The strain grew with

Fig. 1. (a) Phase-contrast photomicrograph showing cells of strain BELH1^T. (b) Transmission electron micrograph of an ultrathin section of a cell of strain BELH1^T, showing the Gram-positive-type of cell wall. Bars, 10 µm (a) and 0.2 µm (b).

Fig. 2. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain BELH1^T among members of cluster XI of the order *Clostridiales*. Bootstrap values (expressed as percentages of 500 replications) are shown at branch points. *Tepidimicrobium ferriphilum* SB91^T, belonging to cluster XII, was used as outgroup. Bar, 0.05 substitutions per nucleotide position.

0–10% (w/v) NaCl (optimum 3%), and at pH 5.8–8.4 (optimum pH 7). Yeast extract was required for growth, and growth was enhanced in the presence of yeast extract and/or tryptone. Elemental sulfur and thiosulfate were used as terminal electron acceptors, but not sulfate or sulfite. Strain BELH1^T grew on glucose, maltose, sucrose and trehalose, both in the presence and absence of thiosulfate. Arabinose, cellobiose, fructose, galactose, lactose, mannose, rhamnose, ribose, sorbose, xylose, formate, acetate, lactate, propionate, Casamino acids, methanol, tryptone and gelatin were not utilized. The fermentation products detected in cultures grown on glucose were hydrogen, carbon dioxide and acetate, with a good carbon recovery. Thiosulfate reduction increased cellular yields and growth rates.

In the phylogenetic analysis, the amplified 16S rRNA gene sequence of strain BELH1^T (1506 nt) was found to be most similar to the corresponding sequence of *F. paucivorans* SEBR 4211^T (94.8% sequence similarity). Thus, in the neighbour-joining tree (Fig. 2), as in the maximum-parsimony and maximum-likelihood trees (data not shown), the novel strain was clustered with *F. paucivorans*.

Under identical growth conditions, biomass of both *F. paucivorans* SEBR 4211^T and strain BELH1^T was obtained and used for fatty acid analysis. The results are shown in Table 1.

Strain BELH1^T could be differentiated from *F. paucivorans* by the range of substrates it could use, its fatty acid composition, its phylogeny, and its lower genomic DNA G+C content (Table 2).

Based on the data obtained in this polyphasic study, strain BELH1^T represented a novel species of the genus *Fusibacter*, for which the name *Fusibacter tunisiensis* sp. nov. is proposed.

Description of *Fusibacter tunisiensis* sp. nov.

Fusibacter tunisiensis (tu.ni.si.en'sis. N.L. masc. adj. *tunisiensis* of or belonging to Tunisia, the country where the bacterium was first recovered).

Round colonies (1 mm in diameter) are present after incubation at 30 °C for 8 days. Cells are motile, measure 0.5 × 2–5 µm and appear singly or in pairs. Grows at 15–40 °C (optimum 30 °C) and at pH 5.8–8.4 (optimum pH 7.0). Halotolerant, growing with 0–10% (w/v) NaCl (optimum 3%). Microaerophile. Utilizes glucose, maltose, sucrose and trehalose, but not arabinose, cellobiose, fructose, galactose, lactose, mannose, sorbose, rhamnose,

Table 1. Cellular fatty acid contents (%) of strain BELH1^T and *F. paucivorans* SEBR 4211^T

Strains: 1, BELH1^T; 2, *F. paucivorans* SEBR 4211^T. —, Not detected.

Fatty acid	1	2
C _{10:0}	0.9	—
C _{12:0}	9.1	1.9
C _{13:1}	7.9	0.5
C _{14:0}	30.4	8.3
Unknown ECL* 14.959	8.3	15.0
C _{14:1ω5c}	—	1.5
C _{16:0}	9.3	6.3
C _{16:1ω9c}	3.4	10.7
C _{16:1ω5c}	—	0.6
Summed features†		
2	1.5	—
3	2.7	12.5
4	12.7	35.3
iso-C _{19:1} I	—	2.6
C _{18:0}	1.1	0.9
iso-C _{18:1} H	—	0.5
C _{18:1ω9c}	1.4	1.4
C _{18:1ω7c}	—	1.0

*Equivalent chain-length.

†Summed features represent groups of two fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained C_{12:0} ALDE?. Summed feature 3 contained C_{16:1ω7c} and/or iso-C_{15:0} 2-OH. Summed feature 4 contained iso-C_{17:1} and/or anteiso-C_{17:1} B.

Table 2. Characteristics that differentiate strain BELH1^T from *F. paucivorans*

Strains: 1, BELH1^T; 2, *F. paucivorans* SEBR 4211^T. +, Positive; –, negative.

Characteristic	1	2
Temperature for growth (°C)		
Range	15–40	20–45
Optimum	30	37
pH for growth		
Range	5.8–8.4	5.7–8.0
Optimum	7.0	7.3
NaCl concentration for growth (% w/v)		
Range	0–10	0–10
Optimum	3	0–3
DNA G + C content (mol%)	38.2	43
Substrates utilized:		
Cellobiose	–	+
Fructose	–	+
Maltose	+	–
Ribose	–	+
Sucrose	+	–
Trehalose	+	–

ribose, xylose, acetate, lactate, propionate, Casamino acids, methanol, bio-Tryptase (bioMérieux) or gelatin. Requires yeast extract for growth. Reduces thiosulfate and elemental sulfur, but not sulfate or sulfite, into sulfide. The end products of glucose fermentation in the presence of yeast extract and tryptone are acetate, CO₂ and H₂. The major fatty acids are C_{14:0}, a summed feature that contained iso-C_{17:1} and/or anteiso-C_{17:1} B, and C_{16:0}.

The type strain, BELH1^T (=DSM 24436^T=JCM 17481^T) was isolated in Tunisia from a digester treating olive-mill wastewater. The genomic DNA G + C content of the type strain is 38.2 mol%.

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