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

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Strain BELH1T, 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 BELH1T was 38.2 mol% and the strain’s predominant cellular fatty acids were C14 : 0, a summed feature that contained iso-C17 : 1 and/or anteiso-C17 : 1B, and C16 : 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 BELH1T represents a novel species of the genus Fusibacter, for which the name Fusibacter tunisiensis sp. nov. is proposed. The type strain is BELH1T (= DSM 24436T = JCM 17481T).

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 .2 H2O, 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
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 BELH¹T 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 BELH¹T 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 BELH¹T, 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 BELH¹T, were similar in morphology and phylogeny (99–100% 16S rRNA gene sequence similarity) and produced the same end products from glucose metabolism. Strain BELH¹T was selected for further characterization.

The cells of strain BELH¹T 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 BELH¹T was anaerobic but tolerated up 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...
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 BELH1T 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 BELH1T (1506 nt) was found to be most similar to the corresponding sequence of F. paucivorans SEBR 4211T (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 4211T and strain BELH1T was obtained and used for fatty acid analysis. The results are shown in Table 1. Strain BELH1T 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 BELH1T 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).

<table>
<thead>
<tr>
<th>Fatty acid</th>
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<tr>
<td>C₁₀:₀</td>
<td>0.9</td>
<td>–</td>
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<tr>
<td>C₁₂:₀</td>
<td>9.1</td>
<td>1.9</td>
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<tr>
<td>C₁₃:₁</td>
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</tr>
<tr>
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<td>Unknown ECL*</td>
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</tr>
<tr>
<td>C₁₄:₀iso5c</td>
<td>–</td>
<td>1.5</td>
</tr>
<tr>
<td>C₁₆:₀</td>
<td>9.3</td>
<td>6.3</td>
</tr>
<tr>
<td>C₁₆:₁iso9c</td>
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<tr>
<td>C₁₆:₁iso5c</td>
<td>–</td>
<td>0.6</td>
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<tr>
<td>Summed features†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>–</td>
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<tr>
<td>3</td>
<td>2.7</td>
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<td>iso-C₁₈:₁H</td>
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<tr>
<td>C₁₈:₁iso7c</td>
<td>–</td>
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*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 C₁₂:₀ ALDE. Summed feature 3 contained C₁₆:₀iso7c and/or iso-C₁₅:₀ 2-OH. Summed feature 4 contained iso-C₁₇:₁ and/or anteiso-C₁₇:₁ B.

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, and so on.
ribose, xylose, acetate, lactate, propionate, Casamino acids, methanol, bio-Trypcase (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 C₁₄:0, a summed feature that contained iso-C₁₇:1 and/or anteiso-C₁₇:1 B, and C₁₆:0-

The type strain, BELH1T (=DSM 24436T=JCM 17481T) 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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References


