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Fusibacter fontis sp. nov., a sulfur-reducing, anaerobic bacterium isolated from a mesothermic Tunisian spring

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Strain KhalAKB1^T, a mesophilic, anaerobic, rod-shaped bacterium, was isolated from water collected from a mesothermic Tunisian spring. Cells were Gram-staining-positive rods, occurring singly or in pairs and motile by one lateral flagellum. Strain KhalAKB1^T grew at 15–45 °C (optimum 30 °C), at pH 5.5–8.5 (optimum pH 7.0) and in the presence of 0–35 g NaCl l⁻¹ (optimum 1 g NaCl l⁻¹). It fermented yeast extract and a wide range of carbohydrates including cellobiose, D-glucose, D-ribose, sucrose, D-xylose, maltose, D-galactose and starch as electron donors. Acetate, ethanol, CO₂ and H₂ were end products of glucose metabolism. It reduced elemental sulfur, but not sulfate, thiosulfate or sulfite, into sulfide. The DNA G + C content was 37.6 mol%. The predominant cellular fatty acids were C₁₄ : 0 and C₁₆ : 0. Phylogenetic analysis of the 16S rRNA gene sequence suggested *Fusibacter bizertensis* as the closest relative of this isolate (identity of 97.2 % to the type strain). Based on phenotypic, phylogenetic and genotypic taxonomic characteristics, strain KhalAKB1^T is proposed to be assigned to a novel species within the genus *Fusibacter*, order *Clostridiales*, *Fusibacter fontis* sp. nov. The type strain is KhalAKB1^T (=DSM 28450^T=JCM 19912^T).

The genus *Fusibacter* belongs to the phylum *Firmicutes*, class *Clostridia*, order *Clostridiales*. Only three species of this genus have been reported so far. They are *Fusibacter paucivorans*, isolated from reservoir water collected from an offshore oil-producing well in Congo (Ravot *et al.*, 1999), *Fusibacter tunisiensis*, isolated from a digester treating wastewater originating from a Tunisian olive mill (Ben Hania *et al.*, 2012), and *Fusibacter bizertensis*, isolated recently from a drain at the bottom of a corroded kerosene storage tank in Bizerte, northern Tunisia (Smii *et al.*, 2015). These members of the genus *Fusibacter* were recognized as slightly halophilic, fermentative anaerobes with the ability to use thiosulfate and elemental sulfur as terminal electron acceptors, producing sulfide.

In this study, we report on the isolation and characterization of a novel mesophilic, sulfur-reducing bacterium (strain KhalAKB1^T) belonging to the genus *Fusibacter*,

originating from a mesothermic Tunisian spring, which is proposed to be assigned to a novel species of this genus.

Water samples were collected from Kebrite spring in the region of Kef, north-west Tunisia (36° 10' 27" N 8° 42' 17" E; temperature 25 °C, pH 7.0, 0.25 g Na⁺l⁻¹) and transported to the laboratory under anaerobic conditions at ambient temperature. Enrichments and isolation of micro-organisms were performed under strict anaerobiosis, according to the Hungate technique (Hungate, 1969).

The selective medium for isolation contained (unless indicated, g l⁻¹) NH₄Cl (1), K₂HPO₄ (0.3), KH₂PO₄ (0.3), KCl (0.1), MgCl₂·6H₂O (0.5), NaCl (2.0), yeast extract (1), CaCl₂·2H₂O (0.1), cysteine hydrochloride (0.5), trace element solution (10 ml) (Widdel & Pfennig, 1982) and 0.1 % resazurin (1 ml). The medium was adjusted to pH 7.2 with 10 M KOH and the culture medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature. 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, 0.1 ml of 10 % (w/v) NaHCO₃, 0.1 ml of 2 % (w/v) Na₂S·9H₂O and substrates

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KhalAKB1^T is LM999901.

A supplementary figure is available with the online Supplementary Material.

were injected from sterile stock solutions into tubes containing the basal culture medium (BCM).

Growth experiments were performed in duplicate using Hungate tubes containing 5 ml BCM. Ranges of temperature, pH and NaCl concentration (15–55 °C, pH 5.5–9, 0–200 g NaCl l⁻¹) for growth were determined using BCM supplemented with glucose at 20 mM (final concentration). The pH of the culture medium was adjusted with anaerobic sterile stock solutions of 1 M HCl (acidic pH), 10 % NaHCO₃ or 8 % Na₂CO₃ (alkaline pH) and the pH was always checked after each inoculation. Water baths were used to maintain the incubation temperature. For studies of NaCl requirements, NaCl was weighed directly in the tubes prior to the medium being dispensed. The isolate was subcultured at least once under the same experimental conditions prior to determination of growth rates to minimize the stress induced by the change in culture conditions.

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.

For substrate utilization tests, BCM without glucose was used. Substrates (D-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, melibiose, raffinose, D-rhamnose, D-ribose, D-sorbose, sucrose, trehalose, D-xylose, cellulose, starch, acetate, butyrate, lactate,

fumarate and propionate) were injected from autoclaved stock solutions into Hungate tubes to a final concentration of 20 mM. Formate and methanol were added to obtain a final concentration of 40 mM; Casamino acids and gelatin were added at a final concentration of 5 g l⁻¹.

To test the use of terminal electron acceptors, 20 mM sodium thiosulfate, 20 mM sodium sulfate, 2 mM sodium sulfite and 2 % (w/v) elemental sulfur were added to the medium. H₂S production was determined spectrophotometrically (Cord-Ruwisch, 1985). The end products of substrate metabolism were measured by HPLC after 2 weeks of incubation at 30 °C (Fardeau *et al.*, 1997).

The presence of spores was determined by microscopic examination of cultures that had been grown at different growth phases. The presence of heat-resistant spores was determined by heating at 80 °C for 10 and 20 min, at 90 °C for 5 and 10 min and at 100 °C for 10 min.

Gram staining was performed on heat-fixed cultures using a Difco Gram staining kit as recommended by the manufacturer. The morphology of cells in the exponential phase of growth was investigated with 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).

Growth was measured by inserting Hungate tubes directly into a spectrophotometer (Cary 50; Varian) and measuring

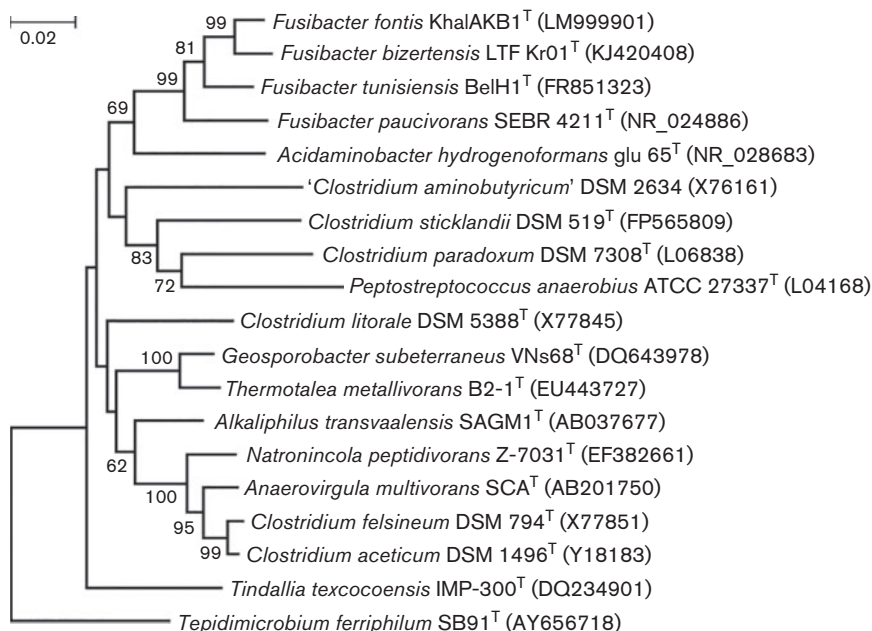


Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain KhalAKB1^T among related representatives of the genus *Fusibacter*. Bootstrap values of 70 % or higher (based on 1000 repetitions) are shown as percentages at branch nodes. *Tepidimicrobium ferriphilum* SB91^T was used as an outgroup. Bar, 2 substitutions per 100 nucleotide positions.

the OD₅₈₀. Hydrogen was measured as described by Fardeau *et al.* (1993).

Exponential-phase cultures of strain KhalAKB1^T were sent to the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) for fatty acid analysis. Fatty acids were extracted using the method of Miller (1982), with the modifications of Kuykendall *et al.* (1988), and the profile of cellular fatty acids was analysed by gas chromatography using the Microbial Identification System (MIDI, Sherlock version 6.1; database, TSBA40; gas chromatograph, Agilent Technologies model 6890N).

The G+C content of the genomic DNA of strain KhalAKB1^T was also determined at the DSMZ. DNA of strain KhalAKB1^T 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). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

The methods of extraction and purification of genomic DNA from strain KhalAKB1^T, and those for the amplification and sequencing of the 16S rRNA gene, were described by Khelifi *et al.* (2010). The 16S rRNA gene sequence was then compared with available sequences in the GenBank database using the BLASTN search (Altschul *et al.*, 1990). A multiple alignment was built using the MUSCLE program (Edgar, 2004) implemented in MEGA5 (Tamura *et al.*, 2011). Sequence positions with alignment uncertainty and gaps were omitted from the analysis (Saitou & Nei, 1987). Evolutionary analyses were conducted in MEGA5 using the neighbour-joining method. Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004). The analysis involved 19 nucleotide sequences. There were a total of 1130 positions in the final dataset. Branch robustness of the resulting tree was estimated by the non-parametric bootstrap procedure implemented in MEGA5 (1000 replicates of the original dataset). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

To obtain an enrichment culture, a 0.5 ml aliquot of the water sample was injected into a Hungate tube containing 5 ml liquid BCM and glucose as energy source and was serially diluted up to 10⁻⁶ under the same culture conditions before incubation. The tubes were then stored at 30 °C for 10 days. To obtain pure cultures from the last positive dilution tube (10⁻⁶), subcultures were undertaken several times under the same growth conditions prior to isolation. For isolation, the culture was serially diluted tenfold in roll tubes. Several colonies that developed were picked separately. The colonies obtained in roll tubes were round and white and about 0.5–2 mm in diameter

after 7 days of incubation. The process of serial dilution was repeated several times until the isolates were deemed to be axenic. Several phylogenetically closely related strains (16S rRNA gene sequence similarity greater than 99 %), similar in morphology and producing the same end products from glucose metabolism, were isolated. One strain designated KhalAKB1^T was chosen for further characterization.

Cells of strain KhalAKB1^T were motile, Gram-staining-positive, small rods, 0.5–2 µm in diameter and 2–12 µm long, and occurred singly or in pairs (Fig. S1A). Transmission electron microscopy of ultrathin sections revealed the typical Gram-positive cell-wall structure with an electron-dense layer and the absence of an outer membrane (Fig. S1B). Spores were not observed under any growth conditions.

Strain KhalAKB1^T was anaerobic. It was mesophilic, growing at 15–45 °C with an optimum at 30 °C. It grew at NaCl

Table 1. Cellular fatty acid profiles of strain KhalAKB1^T and related strains

Strains: 1, KhalAKB1^T; 2, *F. bizertensis* LTF Kr01^T (data from Smii *et al.*, 2015); 3, *F. tunisiensis* BELH1^T (Ben Hania *et al.*, 2012); 4, *F. paucivorans* SEBR 4211^T (Ben Hania *et al.*, 2012). Values are percentages of total fatty acids. ECL, Equivalent chain length; –, not detected/not reported. Major fatty acids (>10 %) of KhalAKB1^T and its closest relative are highlighted in bold.

Fatty acid	1	2	3	4
C ₁₂ :0	3.8	5.9	9.1	1.9
iso-C ₁₃ :0	1.9	–	–	–
C ₁₃ :1	–	4.1	7.9	0.5
C ₁₄ :0	14.9	35.8	30.4	8.3
Unknown ECL 14.959	2.4	2.8	8.3	15.0
anteiso-C ₁₅ :0	4.6	–	–	–
iso-C ₁₅ :0	6.3	–	–	–
C ₁₆ :0	39.4	13.2	9.3	6.3
C ₁₆ :1 ω 9c	–	8.8	3.4	10.7
Summed feature 1*	0.7	6.4	–	–
Summed feature 2*	0.2	1.6	1.5	–
Summed feature 3*	–	9.5	2.7	12.5
Summed feature 4*	9.7	8.2	12.7	35.3
C ₁₈ :0	4.1	1.7	1.1	–
C ₁₈ :1 ω 6c	1.0	–	–	–
C ₁₈ :1 ω 9c	1.3	–	–	–
iso-C ₁₉ :1 I	0.3	1.2	–	2.6
iso-C ₁₇ :0	2.3	–	–	–
C ₁₈ :1 ω 9c	–	–	1.4	1.4

*Summed features represent groups of two or more fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1 contained iso-C₁₅:1H and/or C₁₃:0 3-OH. Summed feature 2 contained C₁₂:0 ALDE?. Summed feature 3 contained C₁₆:1 ω 7c and/or iso-C₁₅:0 2-OH. Summed feature 4 contained iso-C₁₇:1 I and/or anteiso-C₁₇:1 B.

concentrations ranging from 0 to 35 g l⁻¹ with an optimum at 1 g NaCl l⁻¹ and at pH 5.5–8.5, with the optimum for growth being pH 7.0. Yeast extract was required for growth and could not be replaced by bio-Trypticase.

Strain KhalAKB1^T grew on cellobiose, D-ribose, sucrose, D-xylose, maltose, D-galactose, D-glucose, starch and yeast extract as energy sources in the presence and absence of elemental sulfur. The following substrates were not used: D-arabinose, D-fructose, lactose, D-mannose, trehalose, melibiose, raffinose, rhamnose, D-sorbose, mannitol, acetate, butyrate, lactate, propionate, formate, methanol, Casamino acids and gelatin. H₂, acetate, ethanol and CO₂ were the only end products of glucose fermentation. The strain used elemental sulfur, but not sulfate, thiosulfate or sulfite, as a terminal electron acceptor, producing sulfide.

The DNA G + C content of strain KhalAKB1^T was 37.6 mol%.

Phylogenetic analysis of the 16S rRNA gene sequence, containing 1524 nt, revealed that strain KhalAKB1^T represents a novel species of the genus *Fusibacter* within cluster XI of the order *Clostridiales* (Collins *et al.*, 1994), with all three algorithms that we used (neighbour-joining, maximum-parsimony and maximum-likelihood) (Fig. 1). Strain

KhalAKB1^T shared 97.2, 94.4 and 93.0 % 16S rRNA gene sequence identity, respectively, with its three closest anaerobic relatives, the type strains of *F. bizertensis*, *F. tunisiensis* and *F. paucivorans* (Smii *et al.*, 2015; Ben Hania *et al.*, 2012; Ravot *et al.*, 1999).

The fatty acids of strain KhalAKB1^T were found to be slightly different from those of the three other species of the genus *Fusibacter*, obtained under the same conditions, as reported previously (Ravot *et al.*, 1999; Ben Hania *et al.*, 2012; Smii *et al.*, 2015) (Table 1). Indeed, while the major fatty acids of strain KhalAKB1^T were C_{16:0} and C_{14:0}, those of *F. bizertensis*, its closest relative, were C_{14:0} and C_{16:0}.

Beside phylogenetic considerations, assignment of this strain to the genus *Fusibacter* was confirmed by its DNA G + C content, which was similar to that of the type strains of *F. bizertensis* and *F. tunisiensis* but differed markedly from that of the type strain of *F. paucivorans* (Table 2). In contrast to *F. tunisiensis* and *F. paucivorans*, strain KhalAKB1^T used a wider range of substrates and was less tolerant of the presence of NaCl in the culture medium, similar to *F. bizertensis* (Table 2). In contrast to the three

Table 2. Characteristics that differentiate strain KhalAKB1^T from related strains

Strains: 1, KhalAKB1^T; 2, *F. bizertensis* LTF Kr01^T (data from Smii *et al.*, 2015); 3, *F. tunisiensis* BELH1^T (data from Ben Hania *et al.*, 2012); 4, *F. paucivorans* SEBR 4211^T (Ravot *et al.*, 1999).

Characteristic	1	2	3	4
Morphology	Rod	Rod	Rod	Spindle-shaped rod
DNA G + C content (mol%)	37.6	37.4	38.2	43
Temperature for growth (°C)				
Range	15–45	15–35	15–40	20–45
Optimum	30	30	30	37
NaCl concentration for growth (g l ⁻¹)				
Range	0–35	0–50	0–100	0–100
Optimum	1	5	30	0–30
pH for growth				
Range	5.5–8.5	5.5–8.2	5.8–8.4	5.7–8.0
Optimum	7.0	7.2	7.0	7.3
Use of thiosulfate as an electron acceptor	–	+	+	+
Substrates utilized				
D-Fructose	–	+	–	+
Cellobiose	+	+	–	+
D-Ribose	+	+	–	+
Sucrose	+	+	+	–
D-Xylose	+	+	–	–
Maltose	+	+	+	–
D-Galactose	+	+	–	–
Trehalose	–	+	+	–
Starch	+	+	–	–
D-Mannitol	–	+	–	+
End products of glucose metabolism*	A, CO ₂ , E, H ₂	A, CO ₂ , H ₂	A, CO ₂ , H ₂	A, B, CO ₂ , H ₂

*A, Acetate; B, butyrate; E, ethanol.

previously described species of the genus *Fusibacter*, strain KhalAKB1^T did not reduce thiosulfate to sulfide.

DNA–DNA hybridization studies were performed with *F. bizertensis* LTF Kr01^T, being the only member of the genus *Fusibacter* that showed more than 97 % 16S rRNA gene sequence similarity to strain KhalAKB1^T, as this value corresponds to the higher threshold ensuring the description of a novel species (Wayne *et al.*, 1987). They revealed low DNA–DNA relatedness (23 %) between strain KhalAKB1^T and *F. bizertensis* LTF Kr01^T, which is significantly below the threshold of 70 % relatedness that would indicate a relationship at the species level (Wayne *et al.*, 1987). Therefore, strain KhalAKB1^T may be clearly distinguished from *F. bizertensis*. While the type strains of *F. paucivorans* and *F. bizertensis* were isolated from hydrocarbon-rich ecosystems (an oil reservoir and a kerosene storage tank), the type strain of *F. tunisiensis* originated from a polluted wastewater environment. Strain KhalAKB1^T is the first member of genus *Fusibacter* to be isolated from a spring, suggesting that these bacteria may occupy a range of ecological niches. The possible ability of members of the genus *Fusibacter* to use elemental sulfur and/or thiosulfate as terminal electron acceptors, thus making them potential participants in the biogeochemical sulfur cycle in ecosystems that they inhabit, is particularly interesting.

Finally, based on comparative physiological and phylogenetic data, we propose that strain KhalAKB1^T should be assigned to a novel species of the genus *Fusibacter*, for which the name *Fusibacter fontis* sp. nov. is proposed.

Description of *Fusibacter fontis* sp. nov.

Fusibacter fontis (fon'tis. L. masc. gen. n. *fontis* of a spring).

Has round colonies after 7 days of incubation at 30 °C. Cells are motile, Gram-positive rods (0.5 × 1 µm). They appear singly or in pairs. No spores are detected. Mesophilic, with growth at 15–45 °C (optimum 30 °C). Optimum growth at 0.1 % NaCl; grows in the presence of 0–3.5 % NaCl. Optimum growth at pH 7.0; grows at pH 5.5–8.5. Yeast extract is required for growth, but not bio-Trypticase. Reduces elemental sulfur to sulfide. Utilizes cellobiose, D-ribose, sucrose, D-xylose, maltose, D-galactose, D-glucose, starch and yeast extract as electron donors, but not D-arabinose, D-fructose, lactose, D-mannose, melibiose, raffinose, D-rhamnose, D-sorbitose, acetate, butyrate, lactate, propionate, formate, methanol, Casamino acids or gelatin. The end products of glucose fermentation are acetate, ethanol, CO₂ and H₂. The major fatty acids are C_{14:0} and C_{16:0}.

The type strain KhalAKB1^T (=DSM 28450^T=JCM 19912^T) was isolated from water collected from a mesothermic Tunisian spring. The genomic DNA G+C content of the type strain is 37.4 mol% (HPLC).

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