

Anaerosalibacter bizertensis gen. nov., sp. nov., a halotolerant bacterium isolated from sludge

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A strictly anaerobic, halotolerant and thermotolerant strain, designated C5BEL^T, was isolated in north Tunisia from storage tanks holding waste generated by the recycling of discarded motor oils. Cells of strain C5BEL^T were Gram-stain-positive, motile by laterally inserted flagella, straight, and spore-forming. Their two major fatty acids were iso-C_{15:0} and iso-C_{15:0} dimethyl acetal. Growth was observed at temperatures of 25–55 °C (optimum, 40 °C) and at pH 6–9 (optimum, pH 7.5). The salinity range for growth was 0–100 g l⁻¹ NaCl (optimum, 5 g l⁻¹). Yeast extract was required for growth. Strain C5BEL^T was heterotrophic, able to use glucose, pyruvate, succinate, yeast extract, bio-trypticase and peptone, but unable to grow on Casamino acids. Sulfate, thiosulfate, sulfite, elemental sulfur, fumarate, nitrate and nitrite were not reduced. The DNA G + C content of strain C5BEL^T was 31.1 mol%. 16S rRNA gene sequence analysis indicated that strain C5BEL^T was a member of the family *Clostridiaceae*, class *Clostridia*, phylum *Firmicutes* and was most closely related to *Sporanaerobacter acetigenes* Lup33^T (=DSM 13106^T) (92.4% similarity). On the basis of 16S rRNA gene sequence comparisons and physiological characteristics, strain C5BEL^T can be classified as a novel species in a new genus, for which the name *Anaerosalibacter bizertensis* gen. nov., sp. nov. is proposed. The type strain of the type species is C5BEL^T (=DSM 23801^T=JCM 17239^T).

The *Clostridia* are a very heterogeneous group of bacteria with respect to metabolic capacities (Schnurer *et al.*, 1996; Collins *et al.*, 1994), and also show a broad variety of physiological characteristics. The order of *Clostridiales* includes a wide range of Gram-negative or Gram-positive, psychrophilic, mesophilic or thermophilic, spore-forming or non-spore-forming, chemo-organoheterotrophic or chemolithotrophic bacteria, found in a variety of habitats (Cato *et al.*, 1986; Wiegel, 2009). Most of the proposed species belonging to the *Clostridiaceae* family are generally obligately anaerobic rods and form endospores, or have been shown to contain sporulation-specific genes. Members of the *Clostridiaceae* family are glycolytic, saccharolytic, peptolytic (including amino acid utilization) and/or chemolithoautotrophic. The fermentation products include various organic acids and alcohols (Wiegel, 2009).

Here we report on the isolation and characterization of a strictly anaerobic, halotolerant, heterotrophic, spore-forming

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of C5BEL^T is HQ534365.

A supplementary figure is available with the online version of this paper.

bacterium, strain C5BEL^T, identified as a member of the family *Clostridiaceae*, in the order *Clostridiales*, originating from waste generated by the recycling of discarded motor oils. Strain C5BEL^T had phenotypic and phylogenetic traits that allowed its assignment as a novel species in a new genus within the order *Clostridiales*.

All samples were collected from the Tunisian lubricants company SoTuLub located in the Bizerte area of north Tunisia. The sludges used for the enrichments originated from storage tanks of SuTuLub holding waste generated by the recycling of discarded motor oils. Samples were collected at a depth of 30 cm into sterile tubes, transferred to 25 ml serum vials closed with butyl rubber stoppers, and stored at 4 °C until processed. The temperature, pH and salinity of the sludge samples were 35 °C, 11.5 and 11 g l⁻¹ NaCl, respectively.

The Hungate technique (Hungate, 1969) for anaerobic cultures was used throughout this study. Enrichment and isolation were performed using an anaerobic enrichment medium containing (per litre of distilled water): 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 0.5 g NH₄Cl, 2 g NaCl, 0.1 g KCl,

0.1 g CaCl₂, 0.06 g MgCl₂, 0.5 g cysteine HCl, 1 g yeast extract (Difco), 3 g glucose (all w/v), 1 ml mineral element solution (Widdel & Pfennig, 1981) and 1 ml 0.1% (w/v) resazurin. The pH was adjusted to 8.3 with 10 M KOH. This enrichment medium was boiled under a stream of O₂-free N₂ gas, and cooled to room temperature; 5 ml aliquots were distributed into Hungate tubes under a stream of O₂-free N₂ gas. The N₂ gas phase was replaced with N₂/CO₂ (80:20, v/v) and the tubes were autoclaved. Before inoculation, 0.1 ml 2% Na₂S·9H₂O and 0.1 ml 10% NaHCO₃ were added. Enrichments were performed in Hungate tubes containing 5 ml medium and inoculated with sample diluted to 10%. Yeast extract (1 g l⁻¹) and glucose (20 mM) were used as substrates. The tubes were incubated at 37 °C for 48 h. Cultures were purified by repeated use of the Hungate roll-tube method using medium solidified with 2% (w/v) agar (Difco). Several colonies that developed from this method were harvested and cultured in the corresponding culture medium. The isolation process was repeated several times until isolates were deemed axenic. Optimal physiological growth conditions were determined in duplicate experiments conducted in basal medium containing yeast extract (1 g l⁻¹) and glucose (20 mM). For pH growth experiments, the culture medium was adjusted to the desired pH using anaerobically prepared stock solutions of NaHCO₃ (10%) or Na₂CO₃ (8%). The temperature range for growth was determined using the same medium adjusted to the optimum growth pH. For studies of NaCl requirements, NaCl was weighed directly into the tubes at concentrations ranging from 0 to 150 g per litre before dispensing basal medium without NaCl. The tubes were incubated at 37 °C. Growth was measured by inserting tubes directly into a spectrophotometer (Cary 50 Scan; Varian) and measuring the optical density at 580 nm.

Genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega) according to the manufacturer's instructions. The 16S rRNA gene sequence was amplified by using primers Fd1 (5'-AGAGTTTGCCTGGCTCAG-3') and Rd1 (5'-AAGGAGGTGATCCAGCC-3'), and the resulting nucleotide sequence (1517 bp) was manually aligned, using the BioEdit sequence alignment editor software (Hall, 1999). Reference sequences were obtained from the Ribosomal Database Project II (Maidak *et al.*, 2001) and GenBank databases (Benson *et al.*, 1999). Pairwise evolutionary distances based on 1262 unambiguous nucleotides were computed by the Jukes & Cantor (1969) method. The phylogenetic tree obtained by the neighbour-joining method (Saitou & Nei, 1987) is shown in Fig. 1. The topology of the tree was also supported by maximum-parsimony and maximum-likelihood algorithms.

Enrichment cultures were positive (OD₅₈₀ > 0.5) after 48 h incubation at 37 °C, and microscopic examination revealed the presence of motile rod-shaped bacteria. Several strains similar in morphology were isolated, and one strain, named C5BEL^T, was used for further characterization.

The cells of strain C5BEL^T were strictly anaerobic rods, 0.5–1 µm wide and 3–20 µm long, and occurred singly or in pairs. The cells were motile by two or three laterally inserted flagella (Fig. S1a, available in IJSEM online). Strain C5BEL^T was Gram-stain-positive, with spherical and terminal spores that appeared mainly in old cultures. Electron microscopy analysis of strain C5BEL^T showed a thick, multi-layered, typical Gram-positive-type cell wall structure, composed of three dense layers (two thick and a thinner middle layer) separated by two light spaces (Fig. S1b).

The fatty acid composition was determined at the Identification Service of DSMZ after extraction using the modifications (Kuykendall *et al.*, 1988) to the method of Miller (1982). Fatty acids were separated using the MIDI Microbial Identification system (version 4.0, MIS operating manual March 2001) (Sasser, 1990). The major fatty acids detected were iso-C_{15:0} (60.1%) and C_{15:0} dimethyl acetal (22.0%), and one major component that remains unidentified, thought to be iso-C_{15:0} aldehyde (17.8%). Very small proportions of various other fatty acids were also detected.

Strain C5BEL^T was strictly anaerobic and thermotolerant, growing optimally at 40 °C (range 25–55 °C). The optimum pH for growth was 7.5 (range 6–9). The isolate was halotolerant, growing in the presence of NaCl concentrations ranging from 0 to 100 g l⁻¹, with optimum growth at 5 g l⁻¹. The following substrates (20 mM) were used as carbon and energy sources: glucose, succinate, pyruvate, gelatin, yeast extract, bio-trypticase and peptone. Several other substrates did not support growth, such as fructose, ribose, maltose, galactose, xylose, arabinose, fumarate, acetate, valerate, butyrate, propionate, malate, ethanol, butanol, propanol and Casamino acids. Succinate was totally transformed into propionate. Pyruvate was converted to acetate and CO₂. End products from yeast extract and bio-trypticase fermentation were acetate, propionate, butyrate, isovalerate, isobutyrate and CO₂. Acetate was the major fatty acid produced from glucose metabolism with traces of butyrate. Sulfate (20 mM), thiosulfate (20 mM), elemental sulfur (0.1%), sulfite (2 mM), fumarate (20 mM), nitrate (20 mM) and nitrite (2 mM) were not used as electron acceptors. The sulfur test was carried out photometrically as colloidal CuS (Fardeau *et al.*, 1997) and nitrate and nitrite reduction were assayed using specific sticks (Quantofix, Macherey Nagel). Growth of strain C5BEL^T was inhibited by the addition of chloramphenicol (125 mg ml⁻¹) and ampicillin (250 mg ml⁻¹).

The DNA G+C content determined by the Identification Service of the DSMZ, Braunschweig, Germany was 31.1 mol% based on the method of Mesbah *et al.* (1989). Strain C5BEL^T is an anaerobic, heterotrophic, thermotolerant and halotolerant micro-organism of the domain *Bacteria*. Analysis of the 16S rRNA gene of strain C5BEL^T revealed that it grouped with members of the family *Clostridiaceae* of the order *Clostridiales*, as defined in the

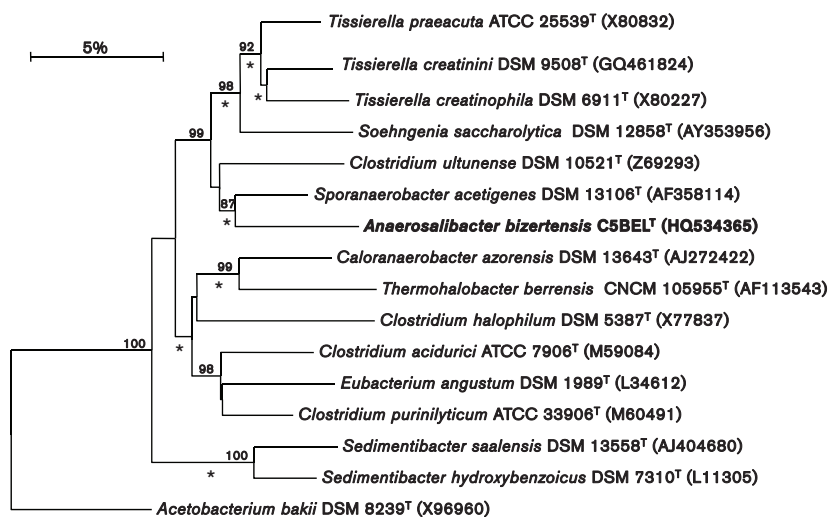


Fig. 1. Phylogenetic dendrogram based on 1262 unambiguous bp of 16S rRNA gene sequence data, showing the position of strain C5BEL^T. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are shown at branch points. Numbers in parentheses are GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences. Bar, 5% estimated sequence divergence.

currently proposed taxonomy scheme in *Bergey's Manual of Systematic Bacteriology*, and placed the isolate close to *Sporanaerobacter acetigenes*, *Clostridium ultunense*, *Tissierella praeacuta*, *Tissierella creatinini* and *Tissierella creatinophila*. The two closest relatives with validly published names were *Sporanaerobacter acetigenes* Lup33^T (=DSM 13106^T) and *Clostridium ultunense* BS^T (=DSM 10521^T) with 16S rRNA sequence similarity values of 92.4% and 89.4%, respectively. The phylogenetic tree constructed from 16S rRNA gene sequences is shown in Fig. 1. Other closely related strains with validly published names, including three members of the genus *Tissierella* (*Tissierella praeacuta* ATCC 25539^T, *Tissierella creatinini* DSM 9508^T and *Tissierella creatinophila* DSM 6911^T), showed 90% 16S rRNA gene sequence similarity with strain C5BEL^T.

Strain C5BEL^T differs morphologically and physiologically from the phylogenetically closest strains with validly published names (Table 1). Unlike the three species of the genus *Tissierella* (*T. praeacuta*, *T. creatinini* and *T. creatinophila*), which are non-spore-forming bacteria, strain C5BEL^T produces spherical and terminal spores. Also, these three species of the genus *Tissierella* show similarities in being non-fermentative and unable to degrade carbohydrates and sugars, whereas strain C5BEL^T is able to grow on one sugar (glucose) and two organic acids (pyruvate and succinate). Both strain C5BEL^T and *T. creatinophila* DSM 6911^T are Gram-stain-positive, but *T. creatinophila* DSM 6911^T exhibits an angular shape (2–6 µm long and 0.7–1.1 µm wide) (Harms *et al.* 1998), whereas strain C5BEL^T is rod-shaped. *T. praeacuta* NCTC 11158^T is Gram-negative and unable to use sugars, while strain C5BEL^T is Gram-stain-positive and able to grow on glucose. Cells of *T. creatinini* DSM 9508^T are non-motile, Gram-positive and surrounded by a slim capsule (Farrow *et al.*, 1995), whereas strain C5BEL^T is motile by laterally inserted flagella and has a typical Gram-positive-type cell wall structure.

Some morphological characteristics markedly distinguish strain C5BEL^T from the phylogenetically closely related neighbour *Clostridium ultunense* BS^T (Schnurer *et al.*, 1996). Firstly, both strain C5BEL^T and *C. ultunense* BS^T are Gram-stain-positive, but *C. ultunense* BS^T cells are straight or slightly curved rods with somewhat pointed ends and measure 0.5–7 µm by 0.5–0.7 µm, whereas strain C5BEL^T is rod-shaped with straight cells measuring 3–20 µm by 0.5–1 µm. *C. ultunense* BS^T also has a pleomorphic growth cycle (coccioid, longer cells and long cell chains). In addition, in the early exponential growth phase, cells of *C. ultunense* BS^T are motile by a polar flagellum, but later the cells are non-motile, unlike the cells of strain C5BEL^T, which are motile by laterally inserted flagella throughout their growth cycle. Secondly, strain C5BEL^T and *C. ultunense* BS^T are also different in their growth conditions. Strain C5BEL^T, a thermotolerant bacterium, grew between 25 °C and 55 °C (optimum 40 °C), whereas *C. ultunense* BS^T, as a mesophilic bacterium, cannot grow above 50 °C (optimum 37 °C). Furthermore, in contrast to *C. ultunense* BS^T, which was not able to grow in NaCl concentrations above 29 g l⁻¹, strain C5BEL^T was halotolerant and able to grow in the presence of NaCl concentrations ranging from 0 to 100 g l⁻¹. Thirdly, *C. ultunense* BS^T and strain C5BEL^T also differ in utilization of substrates and formation of end products. Unlike strain C5BEL^T, where two organic acids were utilized (succinate and pyruvate), *C. ultunense* BS^T is unable to use succinate but can utilize pyruvate. Strain C5BEL^T did not utilize amino acids, whereas *C. ultunense* BS^T grows on cysteine. *C. ultunense* BS^T produces acetate and formate from glucose fermentation, while strain C5BEL^T produces acetate and butyrate. *C. ultunense* BS^T also produces hydrogen, but strain C5BEL^T does not.

Finally, strain C5BEL^T is markedly distinct from its phylogenetically closest relative *Sporanaerobacter acetigenes* Lup33^T (Hernandez-Eugenio *et al.*, 2002). Strain C5BEL^T exhibits approximately 8% sequence divergence with *S.*

Table 1. Comparison of the morphological and physiological properties of strain C5BEL^T and related species

Strain: 1, C5BEL^T (data from this study); 2, *Sporanaerobacter acetigenes* Lup33^T (=DSM 13106^T) (Hernandez-Eugenio *et al.*, 2002); 3, *Tissierella creatinini* DSM 9508^T (Gauglitz, 1988; Hermann *et al.*, 1992; Farrow *et al.*, 1995); 4, *Tissierella praeacuta* NCTC 11158^T (Tissier, 1908; Cato *et al.*, 1979; Collins & Shah, 1986; Farrow *et al.*, 1995); 5, *Tissierella creatinophila* DSM 6911^T (Harms *et al.*, 1998); 6, *Clostridium ultunense* BS^T (=DSM 10521^T) (Schnurer *et al.*, 1996); 7, *Soehngenia saccharolytica* DSM 12858^T (Parshina *et al.*, 2003). Optimum values are given in parentheses. –, Negative; +, positive; NR, not reported; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Cell shape	Rod	Rod	Rod	Rod	Rod (with angular form)	Rod	Rod
Cell size (µm)	3–20 × 0.5–1	3–5 × 0.4–0.5	1–3.5	2–6 × 0.3–0.6	2–6 × 0.7–1.1	0.5–7 × 0.5–0.7	0.5–0.7 × 2–11
Gram type	+	+	+	–	+	–	+
Temperature range for growth (°C)	25–55 (40)	25–50 (40)	20–39 (37)	NR	10–40 (30–34)	15–50 (37)	15–40 (30–37)
Salinity range for growth (g l ⁻¹)	0–100 (5)	0–40 (0)	NR (3.5)	NR	NR	≤29	NR
pH range for growth	6–9 (7.5)	5.5–8.5 (7.5)	6.7–9.1 (8.3)	NR (7.5)	6.5–8.5 (7.4)	5–10 (7)	6.5–7.5 (7)
DNA G + C content (mol%)	31.1	32.2	32	28	30	32	43
Stickland's reaction	–	+	NR	NR	–	NR	NR
Reduction of S ⁰	–	+	NR	NR	–	–	NR
Substrates utilized							
Casamino acids	–	+	–		NR	–	NR
Arginine	–	+	–	NR	–	–	–
Serine	–	–	–	+	–	–	+
Histidine	–	+	–	NR	–	–	NR
Isoleucine	–	+	–	NR	–	–	NR
Leucine	–	+	–	NR	–	–	–
Methionine	–	+	–	NR	–	–	NR
Phenylalanine	–	+	–	NR	–	–	NR
Tryptophan	–	+	–	NR	–	–	NR
Valine	–	+	–	NR	–	–	NR
Glucose	+	+	–	–	–	+	+
Ribose	–	+	–	–	–	–	+
Succinate	+	–	–	NR	–	–	–
Pyruvate	+	+	–	NR	–	+	+
Gelatin	+	–	–	NR	ND	–	–
End products of glucose metabolism							
Acetate	+	+	–	NR	–	+	+
Butyrate	+	–	–	NR	–	–	–
H ₂	–	+	–	NR	–	+	+

acetigenes Lup33^T. Besides this important phylogenetic divergence, some phenotypic and metabolic characteristics are completely distinct. Firstly, strain C5BEL^T is a halotolerant bacterium capable of growth in the presence of NaCl concentrations ranging from 0 to 100 g l⁻¹, with optimum growth at 5 g l⁻¹, while the upper limit for *S. acetigenes* Lup33^T is 40 g l⁻¹, with optimum growth in the absence of NaCl. Strain C5BEL^T also differs from *S. acetigenes* Lup33^T in its ability to reduce elemental sulfur and the range of sugars and amino acids utilized. Unlike strain C5BEL^T, *S. acetigenes* Lup33^T facultatively utilizes elemental sulfur as a terminal electron acceptor, producing sulfide. Also, while strain C5BEL^T ferments only glucose, *S. acetigenes* Lup33^T grows on glucose and ribose. Furthermore, strain C5BEL^T differs from *S. acetigenes* Lup33^T in its utilization of Casamino acids

and amino acids. Unlike *S. acetigenes* Lup33^T, strain C5BEL^T is unable to grow on Casamino acids, and *S. acetigenes* Lup33^T is able to utilize eight amino acids (arginine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan and valine) for growth, whereas strain C5BEL^T was unable to grow on any of the 20 amino acids tested. *S. acetigenes* Lup33^T also performed the Stickland's reaction. Finally, in contrast to *S. acetigenes* Lup33^T, for which acetate is the only fatty acid produced from glucose metabolism, strain C5BEL^T produces butyrate as well as acetate from glucose fermentation.

Taking into account the phenotypic and phylogenetic characteristics, we suggest that strain C5BEL^T represents a novel species of a new genus of the order *Clostridiales*, family *Clostridiaceae*, for which the name *Anaerosalibacter bizertensis* gen. nov., sp. nov. is proposed.

Description of *Anaerosalibacter* gen. nov.

Anaerosalibacter (An.a.e.ro.sa.li.bac'ter. Gr. pref. *an* not; Gr. n. *aer* *aeros* air; L. n. *sal salis* salt; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Anaerosalibacter* a halotolerant rod not living in air).

Rod-shaped, Gram-stain-positive, motile bacterium, forming terminal spores. Halotolerant and thermotolerant, growing up to 55 °C. Strictly anaerobic. Heterotrophic. Grows on yeast extract, peptone, bio-trypticase, pyruvate, succinate, glucose and gelatin, but not on Casamino acids. Yeast extract is required for growth. Sulfur, sulfate, thiosulfate, nitrate and nitrite are not necessary for growth. 16S rRNA gene sequence comparison places *Anaerosalibacter* in the lineage of Gram-positive bacteria with low G+C content, in the family *Clostridiaceae* of the order *Clostridiales*. The type species is *Anaerosalibacter bizertensis*. The DNA G+C content of the type strain of the type species is 31.1 mol%.

Description of *Anaerosalibacter bizertensis* sp. nov.

Anaerosalibacter bizertensis (bi.zert.en'sis. N.L. masc. adj. *bizertensis* of or belonging to Bizerte, pertaining to the Bizerte area of north Tunisia where the organism was isolated).

Displays the following properties in addition to those described for the genus. Cells are rod-shaped, 3–20 × 0.5–1 µm, occurring singly or in pairs. The major fatty acids are iso-C_{15:0} and iso-C_{15:0} dimethyl acetal and one major component that remains unidentified, with small proportions of various other fatty acids also present. Grows at temperatures ranging from 25 to 55 °C (optimum, 40 °C). Grows in the presence of NaCl at concentrations of 0–100 g l⁻¹ (optimum, 5 g l⁻¹) and at pH 6–9 (optimum, pH 7.5). Requires yeast extract to degrade pyruvate, succinate, glucose and gelatin. Does not utilize elemental sulfur, sulfate, thiosulfate, sulfite, nitrate, nitrite or oxygen as electron acceptors.

The type strain, C5BEL^T (=DSM 23801^T=JCM 17239^T), was isolated from storage tanks holding waste generated by the recycling of discarded motor oils located in the Bizerte area of north Tunisia. The DNA G+C content of the type strain is 31.1 mol% (HPLC).

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