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Carnobacterium pleistocenium sp. nov., a novel psychrotolerant, facultative anaerobe isolated from permafrost of the Fox Tunnel in Alaska

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A novel, psychrotolerant, facultative anaerobe, strain FTR1^T, was isolated from Pleistocene ice from the permafrost tunnel in Fox, Alaska. Gram-positive, motile, rod-shaped cells were observed with sizes 0·6–0·7 × 0·9–1·5 μm. Growth occurred within the pH range 6·5–9·5 with optimum growth at pH 7·3–7·5. The temperature range for growth of the novel isolate was 0–28 °C and optimum growth occurred at 24 °C. The novel isolate does not require NaCl; growth was observed between 0 and 5 % NaCl with optimum growth at 0·5 % (w/v). The novel isolate was a catalase-negative chemoorganoheterotroph that used as substrates sugars and some products of proteolysis. The metabolic end products were acetate, ethanol and CO_2. Strain FTR1^T was sensitive to ampicillin, tetracycline, chloramphenicol, rifampicin, kanamycin and gentamicin. 16S rRNA gene sequence analysis showed 99·8 % similarity between strain FTR1^T and *Carnobacterium alterfunditum*, but DNA–DNA hybridization between them demonstrated 39 ± 1·5 % relatedness. On the basis of genotypic and phenotypic characteristics, it is proposed that strain FTR1^T (= ATCC BAA-754^T = JCM 12174^T = CIP 108033^T) be assigned to the novel species *Carnobacterium pleistocenium* sp. nov.

The genus *Carnobacterium* presently includes eight species: *Carnobacterium divergens*, *C. gallinarum*, *C. mobile*, *C. inhibens*, *C. funditum*, *C. alterfunditum*, *C. viridans* and *C. maltaromaticum*. All of these species are capable of growing at low temperatures and most of them were isolated from refrigerated food, except for the species *C. funditum* and *C. alterfunditum*, which were isolated from anoxic waters in Ace Lake, Antarctica (Franzmann *et al.*, 1991). Some of these species are known as fish pathogens (Hiu *et al.*, 1984). The species *Carnobacterium piscicola* (Collins *et al.*, 1987) and *Lactobacillus maltaromicus* (Miller *et al.*, 1974) were recently identified as synonyms and the two species were transferred to *C. maltaromaticum* (Mora *et al.*, 2003). All species of the genus are facultative anaerobes and are capable of reducing resazurin in aerobic media during growth. In nature, they may act as primary agents responsible for the reduction of oxygen levels in ecosystems and for the creation of advantageous conditions for the development of obligately anaerobic micro-organisms (Franzmann *et al.*, 1991).

In this article we describe a novel psychrotolerant, facultatively anaerobic bacterium isolated from the permafrost tunnel in Fox, Alaska. In previous work, we described in detail the isolation of the novel bacterium and characterized the ecosystem in which it was found (Hoover *et al.*, 2002; Pikuta & Hoover, 2003). Since the geological age of the sample is dated as the Pleistocene epoch, we suggest the name *Carnobacterium pleistocenium* sp. nov. for the novel species.

Strain FTR1^T was isolated from an ice core sample collected from the lower level of a frozen lenticular ice lens (Fig. 1) associated with a Pleistocene thermokarst pond (age ~32 000 years) in the CRREL (Cold Regions Research and Engineering Laboratory) Fox Permafrost Tunnel, which is approximately 15 km north of Fairbanks, Alaska (Hoover & Gilichinsky, 2001).

The sample was kept in a frozen state during transportation

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FTR1^T is AF450136.

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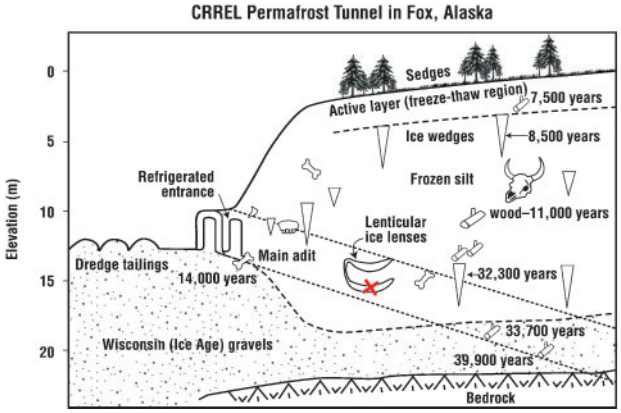

Fig. 1. Schematic image of the geological profile of the CRREL Permafrost Tunnel in Fox, Alaska (modified with permission from Davis, 2004). The red cross designates the sampling site.

to the Astrobiology Laboratory at NSSTC and stored at −20 °C in the laboratory freezer. The permafrost material was melted anaerobically at 2 °C, homogenized and injected into Hungate tubes with an anaerobic medium and was incubated for 2 months at 2 °C. Enrichment cultures were obtained by using anaerobic technique with a medium containing (g l^{-1}): NaCl, 10·0; KCl, 0·3; KH_2PO_4, 0·3; $MgSO_4.7H_2O$, 0·1; NH_4Cl, 1·0; $CaSO_4.7H_2O$, 0·0125; $NaHCO_3$, 0·4; $Na_2S.9H_2O$, 0·4; resazurin, 0·0001; yeast extract, 0·2; peptone, 3·0; 2 ml vitamin solution (Wolin *et al.*, 1963); and 1 ml trace mineral solution (Whitman *et al.*, 1982). The final pH was 7·14–7·2. High-purity nitrogen was used as the gas phase. A pure culture was obtained by the dilution method in Hungate tubes and supported on medium with a modified NaCl concentration of 0·5 % (w/v). The ninth dilution of the morphologically monotypic culture was chosen for growth of colonies on agar medium. Isolation of colonies was performed by the roll-tube method on 3 % (w/v) agar medium. After 10–14 days incubation at 2 °C, colonies of strain FTR1^T were white to cream in colour and round, convex lens-shaped (in deep agar) with a diameter of around 1–2 mm. Colonies grown on the agar surface were conical in shape and the centre had a denser consistency and darker colour than the perimeter. The surface of colonies was granulated and rough with thinner, irregular, torn edges.

Pure cultures in all experiments were incubated at 4–22 °C. The purity of the culture was checked by microscopic control during this study.

The morphology of the novel isolate was examined under a phase-contrast microscope (Fisher Micromaster) and a Hitachi S-4000 field-emission scanning electron microscope was used to examine the ultramicrostructure of the cell surface. An epifluorescence microscope Leitz Diaplan was used with DAPI and BacLite live/dead cell stains. Cells of

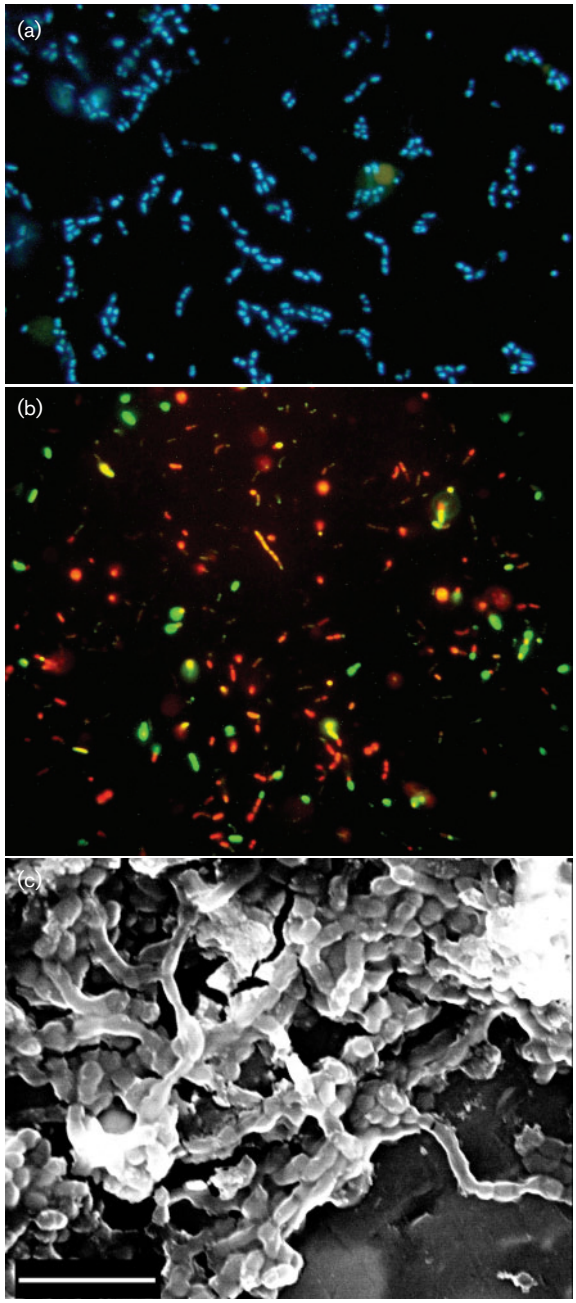

Fig. 2. Images of strain FTR1^T: (a) DAPI-stained cells under a Leitz Diaplan epifluorescence microscope; (b) BacLite live/dead stain; (c) Hitachi S-4000 field emission scanning electron microscope (bar 5 μm).

strain FTR1^T were small, short rods with rounded ends and were 0·7–0·8 μm wide and 1·0–1·5 μm long (Fig. 2). Cells were motile and stained Gram-positive. Spores were never observed. Cells occurred singly, in pairs or in short, irregular curved chains.

Growth of the culture was determined by direct cell counting under a phase-contrast microscope or by measuring the

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OD at 595 nm (Genesis 5; Spectronic Instruments). Catalase activity was determined by the reaction with hydrogen peroxide (Gerhardt *et al.*, 1981). All substrates were added to the medium, containing 0·1 g yeast extract l^{-1}, at concentrations of 3 g l^{-1}. End products of peptone fermentation in the liquid phase were determined by HPLC. Separation was done on an Aminex HPX-87H (Bio-Rad) column with 5 mM H_2SO_4 as the mobile phase. Gases were measured using a gas chromatograph 3700 (Varian) equipped with a Porapak Q column and TCD detector. Nitrogen was used as the gas carrier.

The novel isolate was a facultative anaerobe and grew well under aerobic and anaerobic conditions. During growth under aerobic conditions resazurin was reduced (i.e. became colourless). Strain FTR1^T had a negative catalase reaction. The novel isolate could grow without NaCl with a longer lag phase (three passages on a medium in which all sodium salts were replaced by potassium salts and chloride salts were replaced by sulfates). The NaCl range for growth was 0–5 % (w/v), with optimum growth at 0·5 % (w/v). At 7 % NaCl, growth was absent. Strain FTR1^T grew in pH range 6·5–9·5 and had optimum growth at pH 7·3–7·5. The temperature range for growth of the novel isolate was 0–28 °C with optimal growth at 24 °C.

Strain FTR1^T had a chemoorganoheterotrophic metabolism and was capable of growth on the following substrates: D-glucose, D-fructose, D-mannose, D-maltose, sucrose, lactose, starch, D-mannitol, peptone, Bacto tryptone, Casamino acids and yeast extract. The best growth was observed on D-trehalose and the weakest growth was on D-arabinose. No growth was observed with formate, acetate, lactate, pyruvate, propionate, butyrate, citrate, ethanol, methanol, glycerol, acetone, betaine, trimethylamine or triethylamine.

Metabolic end products of culture on glucose were acetate (1·2 mM) and ethanol (6·3 mM) in the liquid phase and traces of CO_2 in the gas phase.

Concentrations of antibiotics used for testing were 250 μg ml^{-1} for ampicillin, kanamycin, gentamicin, tetracycline and rifampicin and 125 μg chloramphenicol ml^{-1}. Strain FTR1^T was sensitive to all antibiotics tested.

For extraction of fatty acid methyl esters, the culture was incubated for 4 days at 22 °C on the medium described above. The extraction procedure and instruments were described previously (Pikuta *et al.*, 2003). The major fatty acids for strain FTR1^T were $C_{14:0}$, $C_{16:1}$*cis*7, $C_{16:0}$, $C_{18:1}$*cis*9 and $C_{18:0}$ (Table 1).

Genomic DNA was isolated through phenol/chloroform extraction of sonicated biomass followed by ethanol precipitation (Sambrook *et al.*, 1989). The 16S rRNA gene of strain FTR1^T was amplified and sequenced as described previously (Hoover *et al.*, 2003), except for a slight change in the thermal cycling profile (time of initial denaturation at 95 °C was 2 min) and the use of a modified set of 16S rRNA-specific sequencing primers: 5′-GCCAGCAGCCGCGGTAATAC (*Escherichia coli* positions 516–535), 5′-TCGTCAGCTCGTGTCGTGAG (1061–1080), 5′-GGGTTGCGCTCGTTGCGGG (1113–1095) and 5′-GCGCTCGCTTTACGCCCAAT (581–562).

Table 1. Fatty acid compositions of *C. pleistocenium* sp. nov. FTR1^T and *C. alterfunditum* pf4^T

Values are percentages (w/w) of total fatty acids. Summed feature 4 represents unresolved 15:2 FAME and/or 15:1*cis*7. Summed feature 10 represents unresolved 18:1*cis*11/*trans*9/*trans*6. Data for *C. alterfunditum* were taken from Franzmann *et al.* (1991). DMA, Dimethylacetate; FAME, fatty acid methyl ester.

Compound	FTR1^T	pf4^T
12:0 FAME	1·27	0·00
14:0 FAME	7·19	7·10
Summed feature 4	0·00	0·80
16:1*cis*7 FAME	20·73	7·50
16:0 FAME	37·07	40·90
16:0 DMA	0·00	0·80
18:2*cis*9,12 FAME	1·76	0·00
18:1*cis*9 FAME	24·32	7·90
Summed feature 10	1·56	12·1
18:0 FAME	4·83	5·10
20:1*cis*11 FAME	1·28	0·90

The consensus sequence was aligned with eight closely related sequences using CLUSTAL W (Thompson *et al.*, 1994). Pairwise distances were computed with MEGA version 2.1 (Kumar *et al.*, 2001) using the Jukes–Cantor model (Jukes & Cantor, 1969). An unrooted phylogenetic tree was constructed with the same program using the neighbour-joining method (Saitou & Nei, 1987).

A sequence covering 1486 nucleotides of the 16S rRNA gene was obtained, corresponding to positions 28–1492 of the *E. coli* 16S rRNA sequence. No difference was observed between the sequences of the three selected clones. The G+C content of this sequence was 53·5 mol%. The sequence was compared with all sequences presently available in the GenBank database and appeared to be highly similar to sequences from *Carnobacterium* species. A phylogenetic dendrogram was constructed based on 1379 common nucleotides sites, and the dendrogram shows the position of strain FTR1^T among all currently known species of the genus *Carnobacterium* (Fig. 3). According to the pairwise distance table (not shown), based on the same 1379 common nucleotide sites, strain FTR1^T appears to belong to a cluster including *C. alterfunditum*, *C. inhibens* and *C. viridans*, with 99·78, 98·54 and 98·83 % similarity, respectively. Since the similarity of the novel isolate was determined to be highest to *C. alterfunditum* pf4^T, it was suggested that an additional genetic comparison be performed for the two strains.

Melting temperatures (T_m) of total genomic DNA from

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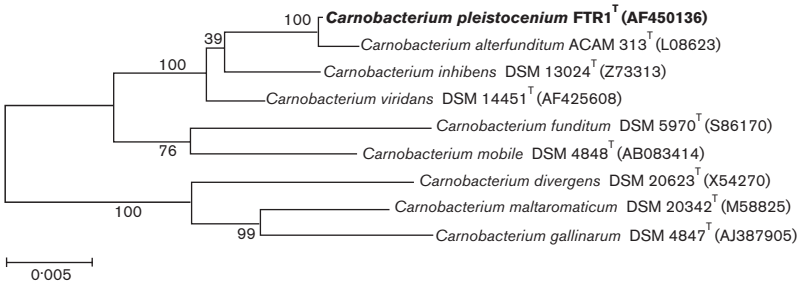

Fig. 3. Unrooted phylogenetic tree showing the position of strain FTR1^T among other species of the genus *Carnobacterium*. Numbers at nodes are bootstrap percentages (1000 replicates). Sequence accession numbers are shown in parentheses (note that accession number L08623 for *C. alterfunditum* erroneously gives the strain as ACAM 311, a strain of *Psychrobacter urativorans*; J. P. Bowman, personal communication). Bar, 5 substitutions per 1000 nucleotides.

strain FTR1^T and *C. alterfunditum* pf4^T were determined by procedures previously described (De Ley *et al.*, 1970; Gillis *et al.*, 1970). Purified genomic DNA (200 μg) from strain FTR1^T and *C. alterfunditum* pf4^T was sonicated to generate DNA fragments of 500–700 bp. Any residual RNA and single-stranded DNA was removed by treatment with RNase A and S1 nuclease, respectively (Ausubel *et al.*, 1987). The concentration and purity of the DNA were determined from the A_{260} and the A_{260}/A_{280} ratio using a Shimadzu UV-160 spectrophotometer. DNA (80 μg) from each of these micro-organisms was then denatured in 1× SSC buffer (pH 7·0) by increasing the temperature of the sample from 26 to 100 °C (at a rate of 1 °C min^{-1}) and the A_{260} was recorded. The experiment was conducted in triplicate. The T_m was determined by calculating the temperature at which the hyperchromicity reached half of the value obtained after complete melting. The T_m of the genomic DNA of strain FTR1^T was 62 ± 2 °C (mean ± SD, n = 4), whereas it was 53 ± 3 °C (n = 4) for *C. alterfunditum* pf4^T.

To determine the relatedness of genomic DNA between strain FTR1^T and *C. alterfunditum* pf4^T, DNA–DNA hybridization was performed by DNA reassociation kinetics as described previously (De Ley *et al.*, 1970; Johnson, 1985). Purified, sonicated genomic DNA (80 μg) from each strain was added to 4× SSC buffer (pH 7·0) and 25 % deionized formamide. The DNA was denatured by raising the temperature to 100 °C and cooled to 5 °C above the respective melting temperatures. The temperature was then rapidly (1·5 min) lowered to the reassociation temperature and the A_{270} was recorded at 5 s intervals for a total of 20 min. The initial reassociation kinetics were determined by linear regression analysis. The experiment was conducted in triplicate. The percentage relatedness of the DNA was calculated using the equation described by De Ley *et al.* (1970). All statistical analyses were performed using Microsoft Excel. DNA–DNA hybridization established 39 ± 1·5 % relatedness (mean ± SD, n = 3) between the genomes of strain FTR1^T and *C. alterfunditum* pf4^T.

The genome sizes of strain FTR1^T and *C. alterfunditum* pf4^T were determined from the DNA reassociation kinetics, following the equation described by Gillis *et al.* (1970). The genome sizes were $2·1 \times 10^9$ Da for strain FTR1^T and $1·9 \times 10^9$ Da for *C. alterfunditum* pf4^T.

The G + C content in genomic DNA of strain FTR1^T was determined by following the procedure described by Starr & Mandel (1968) and Franzmann *et al.* (1991). The total G + C content of the purified genomic DNA for strain FTR^T was 42 ± 1·5 mol% (mean ± SD, n = 3) compared with 33–34 mol% for *C. alterfunditum* (Franzmann *et al.*, 1991).

The novel isolate is the first representative of this genus to be found alive in ice entrained in Pleistocene permafrost. Detailed characterization of this ecosystem and the importance of its study to paleomicrobiology, biostratigraphy, geocryology, microbial evolution, conservation of the modern gene pool and the long-term viability of micro-organisms in deep anabiosis were described previously (Hoover *et al.*, 2002). Indeed, there is still much less known about the microbiota preserved in Pleistocene permafrost and ice than is known about fossil Pleistocene mammals. In Table 2, distinguishing features of the novel isolate and *C. alterfunditum* pf4^T are shown. Notwithstanding the different sources of isolation for the two strains, a very close phylogeny and physiology is demonstrated, while some metabolic features have significant differences. For example, the sugars that could be used as substrates are different, and lactic acid is absent from the end products of strain FTR1^T. Also, the composition of fatty acid methyl esters in the novel isolate is different: 16 : 0 DMA is absent from strain FTR1^T, but it represents around 0·8 % of the total in *C. alterfunditum* pf4^T; 12 : 0 FAME and 18 : 2*cis*9,12 FAME are present in strain FTR1^T, but they are absent from *C. alterfunditum* pf4^T (Table 1). DNA–DNA hybridization of genomic DNA between strain FTR1^T and *C. alterfunditum* pf4^T exhibited only 39 % relatedness. Also, the melting temperature, the G + C content of the genomic DNA and the genome sizes of the two micro-organisms are different (Table 2).

On the basis of phenotypic and genotypic characteristics (Gram-positive cell wall, facultatively anaerobic and fermentative metabolism, psychrotolerant and slightly alkalitolerant physiology, independence from NaCl, 16S rRNA gene sequence and DNA–DNA hybridization), strain

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Table 2. Comparative characteristics of strain FTR1^T and *C. alterfunditum* pf4^T

Data for *C. alterfunditum* pf4^T were taken from Franzmann *et al.* (1991). (+), Weak growth.

Attribute	FTR1^T	*C. alterfunditum* pf4^T
Temperature range (optimum) (°C)	0–28 (24)	0–28 (23)
NaCl range (optimum) (%)	0·1–5·0 (0·5)	(0·6)
pH range (optimum)	6·5–9·5 (7·3–7·5)	6·0 (7·0–7·4)*
Growth on:		
D-Arabinose	(+)	−
D-Mannitol	+	−
D-Fructose	+	(+)
Maltose	+	(+)
D-Mannose	+	(+)
D-Trehalose	+	−
Lactose	+	−
D-Ribose	+	−
D-Arabinose	+	−
Starch	+	−
Sucrose	+	(+)
End products:		
Lactic acid†	−	+
Aesculin hydrolysis	+	−
DNA G+C content (mol%)	42	33–34
Genome size (Da)	$2·1 \times 10^9$	$1·9 \times 10^9$
T_m (°C)	62	53
Ecology	Permafrost deposits in Fox tunnel, Alaska	Ace Lake, Antarctica

*Minimum pH of 4·9–5·4 achieved on PYG-2 % NaCl medium. No maximum pH for growth was reported.
†Both species produce acetic acid and ethanol.

FTR1^T is identified as representing a novel species of the genus *Carnobacterium*. The name *Carnobacterium pleistocenium* sp. nov. is suggested for this organism, in accordance with the geological age of the sample from which the type strain was isolated, which dated from the Pleistocene epoch.

Description of *Carnobacterium pleistocenium* sp. nov.

Carnobacterium pleistocenium (plei.sto.ce′ni.um. N.L. neut. adj. *pleistocenium* belonging to the Pleistocene, a geological epoch).

Cells are motile, small rods with rounded ends, 0·7–0·8 × 1·0–1·5 μm. Gram-positive. Growth occurs between 0 and 28 °C (optimum 24 °C) and at $pH_{22\,°C}$ 6·5–9·5 (optimum pH 7·3–7·5). Range of NaCl for growth is 0–5 % (w/v); optimum growth at 0·5 % (w/v) NaCl. Facultative anaerobe. Catalase-negative. Heterotrophic growth occurs with D-glucose, D-fructose, maltose, D-mannitol, D-mannose, D-trehalose, lactose, D-ribose, D-arabinose, sucrose, starch, peptone, Bacto tryptone, Casamino acids and yeast extract. End products of growth are acetate, ethanol and traces of carbon dioxide (in gas phase). Sensitive to ampicillin, kanamycin, gentamicin, tetracycline, rifampicin and chloramphenicol.

The type strain, FTR1^T (=ATCC BAA-754^T=JCM 12174^T =CIP 108033^T), was obtained from a sample of permafrost from Fox Tunnel, Alaska.

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