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Tumebacillus permanentifrigoris gen. nov., sp. nov., an aerobic, spore-forming bacterium isolated from Canadian high Arctic permafrost

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A Gram-positive, aerobic, rod-shaped bacterium (strain Eur1 9.5^T) was isolated from a 9-m-deep permafrost sample from the Canadian high Arctic. Strain Eur1 9.5^T could not be cultivated in liquid medium and grew over the temperature range 5–37 °C; no growth was observed at 42 °C and only slow growth was observed at 5 °C following 1 month of incubation. Eur1 9.5^T grew over the pH range 5.5–8.9 and tolerated NaCl concentrations of 0–0.5 % (w/v). Eur1 9.5^T grew heterotrophically on complex carbon substrates and chemolithoautotrophically on inorganic sulfur compounds, as demonstrated by growth on sodium thiosulfate and sulfite as sole electron donors. Eur1 9.5^T contained iso-C_{15:0} as the major cellular fatty acid and menaquinone 7 (MK-7) as the major respiratory quinone. The cell-wall peptidoglycan was of type A1 γ . The DNA G + C content was 53.1 mol%. The 16S rRNA gene sequence of strain Eur1 9.5^T was only distantly related ($\leq 87\%$ sequence similarity over 1407 bp) to any recognized bacterial species. Based on physiological and phylogenetic analyses, strain Eur1 9.5^T is suggested to represent a novel species of a new genus, for which the name *Tumebacillus permanentifrigoris* gen. nov., sp. nov. is proposed. The type strain of *Tumebacillus permanentifrigoris* is Eur1 9.5^T (=DSM 18773^T =JCM 14557^T).

A novel Gram-positive bacterium, designated strain Eur1 9.5^T, was isolated during the course of a study on the microbial diversity in a 9-m-deep permafrost sample from Eureka (79° 59' 41" N 85° 48' 48" W), Ellesmere Island, Nunavut, Canada (Steven *et al.*, 2007). Comparative 16S rRNA gene sequence analysis indicated that strain Eur1 9.5^T formed an independent branch in the order *Bacillales*, and data from a polyphasic study was used to define in detail the taxonomic position of this novel isolate.

Strain Eur1 9.5^T was isolated by using standard dilution plate techniques on Difco R2A agar (Becton Dickinson) and, unless otherwise stated, cells were grown on R2A agar (pH 7.0) incubated at room temperature (approximately 22–25 °C). Strain Eur1 9.5^T proved to be recalcitrant to cultivation in liquid media and therefore all experiments were performed on agar media. On R2A agar, strain Eur1

9.5^T formed yellow colonies after about 2 days incubation at room temperature. Cells inoculated into liquid R2A medium produced an insoluble yellow precipitate that appeared to be free of bacterial cells based on microscopic investigations ($\times 1000$ magnification) of recovered precipitate material. Likewise, strain Eur1 9.5^T did not grow in liquid modified sulfur media (composition described below) supplemented with 20 mM sodium thiosulfate or 2.0 g glucose l⁻¹, LB broth (Becton Dickinson) or trypticase soy broth (Becton Dickinson). Phase-contrast microscopy revealed that cells of strain Eur1 9.5^T were non-motile rods with ellipsoidal spores formed in large terminal sporangia (Fig. 1a). Microscopic examinations of Gram-stained cells of strain Eur1 9.5^T revealed Gram-positive cell walls; older cultures occasionally appeared to be Gram-negative. Rod-shaped vegetative cells were 3–3.5 μ m in length and approximately 0.5 μ m in width. Spores were approximately 1 μ m in width and 2 μ m in length based on estimates from transmission electron microscopy (TEM) images (Fig. 1b); TEM was undertaken on cells grown on R2A medium. Cells, ultrathin sections and copper TEM grids were prepared as described by Vali *et al.* (2004). The grids were stained with 4 % aqueous uranyl acetate and

Abbreviation: TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Eur1 9.5^T is DQ444975.

A table detailing the cellular fatty acid composition of strain Eur1 9.5^T is available as supplementary material with the online version of this paper.

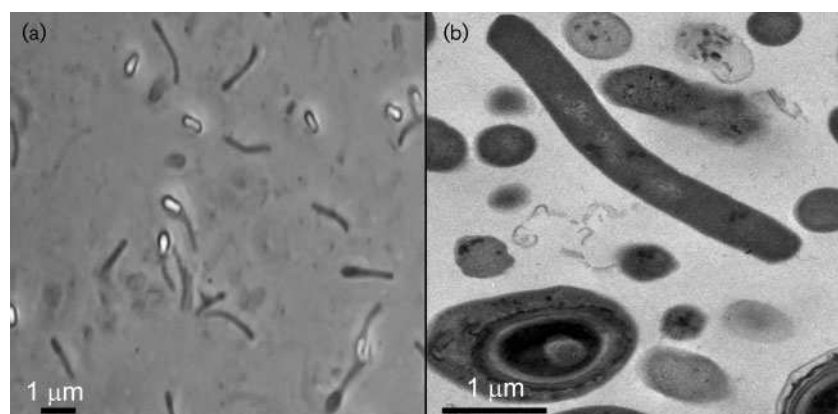


Fig. 1. Micrographs of cells of strain Eur1 9.5^T. (a) Phase-contrast micrograph showing vegetative cells and cells with large terminal sporangia; (b) TEM comparison between a vegetative cell and a spore (bottom left). Bars, 1 µm.

Reynold lead citrate and observed with a JEOL JEM-2000FX TEM at 80 kV.

Chromosomal DNA was prepared from cells of strain Eur1 9.5^T by using the Gram-positive protocol of the DNeasy Tissue kit (Qiagen) and was used as a template to amplify the nearly full-length 16S rRNA gene by using primers 27F and 1492R (Lane, 1991). The PCR mixtures and conditions were described by Steven *et al.* (2007). Sequencing of the PCR product resulted in a sequence of 1407 bp that was used for phylogenetic comparisons. Alignment of the 16S rRNA gene sequence of strain Eur1 9.5^T with BLAST (Altschul *et al.*, 1990) and RDP-II release 9 Classifier software (Cole *et al.*, 2007) indicated that strain Eur1 9.5^T formed a monophyletic clade affiliated with the order *Bacillales* (data not shown). The closest relative to strain Eur1 9.5^T in the GenBank database was an uncharacterized member of the *Bacillales* (Gsoil 1105; 94% 16S rRNA gene sequence similarity) and the closest matching recognized relative was the type strain of *Alicyclobacillus contaminans* (87% sequence similarity; Goto *et al.*, 2007). Phylogenetic relationships of some of the 16S rRNA gene sequences that were most closely related to strain Eur1 9.5^T were determined by aligning 16S rRNA gene sequences with the CLUSTAL W program and phylogenetic inference packages in MacVector 7.2 (Accelrys). Evolutionary distances between sequences were estimated by using the Jukes–Cantor model (Jukes & Cantor, 1969) and a phylogenetic tree was constructed according to neighbour-joining methods (Fig. 2). Bootstrap analysis (1000 iterations) was used to assess the robustness of the resulting tree. A second tree constructed according to the Tamura–Nei distance estimation (Tamura & Nei, 1993) and neighbour-joining methods revealed the same topology as the tree in Fig. 2, verifying the phylogenetic position of the 16S rRNA gene sequence of strain Eur1 9.5^T.

Growth of strain Eur1 9.5^T occurred over the temperature range 5–37 °C; no growth was observed at 42 °C and only slow growth was observed at 5 °C following 1 month of incubation. We propose that 5 °C represents the lower limit of growth for strain Eur1 9.5^T. Carbon source and electron donor studies were performed on basal media

[modified sulfur medium (per litre distilled water): 3.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.3 g (NH₄)₂SO₄, 0.25 g CaCl₂·2H₂O, 0.02 g FeCl₃·6H₂O, 15.0 g agar, adjusted to pH 7.0 with HCl; Atlas (1993)]. Carbon sources were added at a final concentration of 2.0 g l⁻¹. Acid production from carbohydrates was determined by using API 50CH test strips (bioMérieux) and carbon source utilization was also tested by using Biolog GP microplates. Strain Eur1 9.5^T formed white colonies on basal media with varying times of appearance depending on the carbon source or electron donor provided. It grew well on galactose, starch, tryptone, cellobiose, lactose, trehalose, mannitol, maltose, glucose and Casamino acids but showed relatively weak growth on glycerol, fructose, sodium lactate and yeast extract. Xylose did not support growth of strain Eur1 9.5^T. The novel strain did not grow in the presence of the electron donors NaNO₂ (1 and 5 mM) or cysteine hydrochloride (1 and 5 mM). Both Na₂SO₃ (5 mM) and Na₂S₂O₃ (5 and 20 mM) supported growth, indicating that strain Eur1 9.5^T was capable of facultative sulfur oxidation. No positive results were detected by using either API 50CH test strips or Biolog microplates, presumably due to the inability of strain Eur1 9.5^T to grow in liquid media.

To investigate tolerance to NaCl, modified sulfur medium supplemented with 2.0 g sodium thiosulfate l⁻¹ was prepared as above and NaCl concentrations were varied from 0 to 2% (w/v) at intervals of 0.5%. Eur1 9.5^T grew in media without added NaCl but did not grow at NaCl concentrations above 0.5% (w/v). The pH range for growth was determined on R2A plates adjusted prior to sterilization by directly adding 1 M HCl or NaOH. The pH was confirmed following autoclaving. Strain Eur1 9.5^T grew over the range pH 5.5–8.9; no growth was observed at pH 5.4 or 9.1. Antibiotic sensitivity was tested by the addition of sterile-filtered antibiotics (100 µg ml⁻¹) to both R2A medium or discs (BBL Sensi-Discs) on an R2A-grown lawn of cells of strain Eur1 9.5^T. Eur1 9.5^T did not grow with ampicillin, streptomycin, chloramphenicol, rifampicin or erythromycin added to the medium. With antibiotic discs, strain Eur1 9.5^T was sensitive to streptomycin (10 µg), chloramphenicol (30 µg) and tetracycline

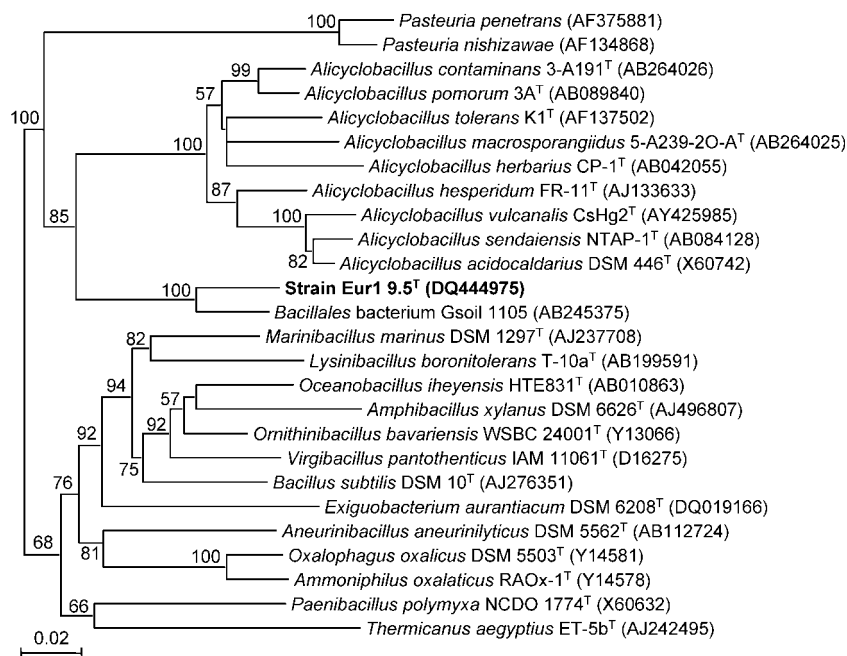


Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain Eur1 9.5^T. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at nodes. *Clostridium butyricum* ATCC 19398^T (GenBank accession no. AB075768) was used for outgroup rooting (not shown). Bar, 0.02 nucleotide substitutions per position.

(30 µg) but was resistant to penicillin (10 µg). Anaerobic growth was tested by using an anaerobic chamber with an AnaeroGen sachet and indicator (Oxoid), which indicated that strain Eur1 9.5^T was unable to grow in the absence of oxygen. Catalase activity was tested by the addition a few drops of 3 % H₂O₂ to a fresh plate of cells of strain Eur1 9.5^T and observing for the production of gas bubbles. Oxidase activity was tested by using a BD BBL DrySlide according to the manufacturer's instructions (Becton Dickinson). Strain Eur1 9.5^T was catalase- and oxidase-negative.

Several chemotaxonomic characteristics were determined for strain Eur1 9.5^T. The fatty acids of strain Eur1 9.5^T were prepared from R2A-grown cells according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990) at Keystone Laboratories, Edmonton, Canada. The major fatty acids (>10 % of the total) were iso-C_{15:0} (50.95 %) and C_{13:0} 3-OH and/or iso-C_{15:1} (26.53 %). The fatty acid profile of strain Eur1 9.5^T did not match any profiles in the MIDI aerobic bacteria library (RTSBA6). The full fatty acid profile of strain Eur1 9.5^T is given in Supplementary Table S1 available in IJSEM Online. Analysis of the respiratory quinones was carried out by the Identification Service and Dr Brian Tindall, DSMZ, Braunschweig, Germany, and the peptidoglycan structure was also determined at the DSMZ according to the technique of Rhuland *et al.* (1955). Strain Eur1 9.5^T contained menaquinone 7 (MK-7) as the predominant isoprenoid quinone and *meso*-diaminopimelic acid, characteristic of peptidoglycan type A1γ. The DNA G + C content was 53.1 mol% (mean of triplicate assays) based on the method described by Gonzalez & Saiz-Jimenez (2002).

Phylogenetic analysis of the 16S rRNA gene sequence revealed that strain Eur1 9.5^T was related to the genus *Alicyclobacillus* (Fig. 2). The majority of species in the genus *Alicyclobacillus* are characterized by acidophilic, moderately thermophilic growth and a lipid composition dominated by cyclic fatty acids, with the exception of a small number of species (see footnote to Table 1) that contain predominantly branched-chain fatty acids (Goto *et al.*, 2003, 2007). Strain Eur1 9.5^T was clearly distinguished from members of the genus *Alicyclobacillus* based on its relatively neutral pH range for growth, inability to grow at temperatures above 37 °C and predominance of the fatty acid iso-C_{15:0} in the lipid profile. A comparison of the phenotypic and chemotaxonomic characteristics between strain Eur1 9.5^T and related genera is presented in Table 1. Some of the characteristics of strain Eur1 9.5^T were surprising, given the extreme conditions of the permafrost environment. Permafrost is characterized by temperatures constantly below 0 °C (Eureka permafrost is at -16 °C), and it is hypothesized that active indigenous bacteria survive in thin, salty water veins that exist between soil particles (Steven *et al.*, 2006). The low salt tolerance (0–0.5 %) and relatively high growth temperature range (5–37 °C) of strain Eur1 9.5^T (compared with the permafrost *in situ* temperature of -16 °C) suggests that it was not an active member of the permafrost microbial community and probably survived as spores. Therefore, given the salinity, temperature and age of the permafrost (5000–7000 years) from which strain Eur1 9.5^T was isolated (Steven *et al.*, 2007), it is possible that cells of Eur1 9.5^T existed as frozen dormant survivors over geological time-scales. The environmental, physical and biological isolation imposed by the permafrost environment may explain the genetic diversity of strain Eur1 9.5^T from other character-

Table 1. Phenotypic and chemotaxonomic characteristics of genera related to strain Eur1 9.5^T

Data for related genera were taken from Goto *et al.* (2003) and Karavaiko *et al.* (2005) (*Alicyclobacillus*), Heyndrickx *et al.* (1996) and Shida *et al.* (1997) (*Paenibacillus*) and An *et al.* (2007) and Heyrman *et al.* (2003) (*Virgibacillus*). –, Negative for growth; v, variable; ND, no data available; *meso*-DAP, *meso*-diaminopimelic acid. All taxa have MK-7 as the major isoprenoid quinone.

Characteristic	Eur1 9.5 ^T	<i>Alicyclobacillus</i>	<i>Paenibacillus</i>	<i>Virgibacillus</i>
Anaerobic growth	—	—	v	v
Optimal growth temperature (°C)	25–30	35–60	28–37	20–35
pH range	5.5–8.9	1.5–5	7	5–10
NaCl tolerance (%)	0–0.5	0–4	0–5	0–25
DNA G + C content (mol%)	53.1	49–63	45–54	36–43
Peptidoglycan type	A1γ (<i>meso</i> -DAP)	ND	A1γ (<i>meso</i> -DAP)	<i>meso</i> -DAP
Major fatty acid(s)	iso-C _{15:0}	ω-cyclohexane-C _{17:0} *, ω-cyclohexane-C _{18:0} , ω-cyclohexane-C _{19:0}	anteiso-C _{15:0} , iso-C _{16:0}	anteiso-C _{15:0} , iso-C _{15:0} , anteiso-C _{17:0}

*With the exception of *A. pomorum*, which has iso-C_{15:0} as the predominant component (Goto *et al.*, 2003), and *A. macrosporangiidus* and *A. contaminans*, which contain iso-C_{15:0} and iso-C_{16:0} as major components (Goto *et al.*, 2007).

ized organisms. Understanding the diversity and survival of micro-organisms in permafrost will lead to a better understanding of the low-temperature limits for microbial life and long-term cell dormancy.

Based on the data presented here, strain Eur1 9.5^T is suggested to represent a novel species of a new genus, for which the name *Tumebacillus permanentifrigoris* gen. nov., sp. nov. is proposed.

Description of *Tumebacillus* gen. nov.

Tumebacillus [Tu'me.ba.cil.lus. L. adj. prefix *tume-* (as in *tumefacere* to make swollen) swollen, N.L. masc. n. *bacillus* small rod, N.L. masc. n. *Tumebacillus* swollen rod, referring to the large, swollen terminal sporangia observed during microscopy].

Cells are rod-shaped, form spores and stain Gram-positive. Growth is aerobic and occurs via chemolithoautotrophic (sulfur) and heterotrophic metabolism. NaCl is not a requirement for growth. The major cellular fatty acid is iso-C_{15:0}; contain minor amounts of iso-C_{15:1} H/C_{13:0} 3-OH and anteiso-C_{15:0}. The characteristic diamino acid in the cell wall is *meso*-diaminopimelic acid and the peptidoglycan type is A1γ. The predominant respiratory quinone is menaquinone 7 (MK-7). The phylogenetic position based on 16S rRNA gene sequence analysis is within the order *Bacillales*. The type species is *Tumebacillus permanentifrigoris*.

Description of *Tumebacillus permanentifrigoris* sp. nov.

Tumebacillus permanentifrigoris (per.ma.nen.ti.fri'go.ris. L. part. adj. *permanens* permanent; L. gen. neut. n. *frigoris* of/ from frost; N.L. gen. neut. n. *permanentifrigoris* from

permanent frost, permafrost, referring to the isolation of the type strain from permafrost).

Has the following characteristics in addition to those given for the genus above. Cells are non-motile, 3–3.5 × 0.5 μm in size, with large terminal endospores. Forms yellow colonies on R2A plates with no growth in liquid media. Utilizes galactose, starch, tryptone, cellobiose, lactose, trehalose, mannitol, maltose, glucose and Casamino acids as carbon sources. Sensitive to ampicillin, streptomycin, chloramphenicol, rifampicin, erythromycin and tetracycline, but resistant to penicillin. The temperature range for growth is 5–37 °C (optimal growth at 25–30 °C). Catalase- and oxidase-negative. The DNA G + C content of the type strain is 53.1 mol%. The full fatty acid profile is given in Supplementary Table S1.

The type strain, Eur1 9.5^T (=DSM 18773^T =JCM 14557^T), was isolated from a 9-m-deep permafrost sample from the Canadian high Arctic.

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