Virgibacillus arcticus sp. nov., a moderately halophilic, endospore-forming bacterium from permafrost in the Canadian high Arctic

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A novel, moderately halophilic, endospore-forming bacterial strain, designated Hal 1^T, was isolated from a permafrost core collected from the Canadian high Arctic. The temperature for growth of strain Hal 1^T was 0–30 °C with no growth observed at either –5 or 37 °C (optimum growth at about 25 °C). Strain Hal 1^T was able to grow at NaCl concentrations of 0–20 % (w/v) and did not have an absolute NaCl requirement for growth; optimal growth was at 5 % (w/v) NaCl. The level of 16S rRNA gene sequence similarity between strain Hal 1^T and the type strains of *Virgibacillus carmonensis* and *Virgibacillus necropolis* was 98.2 %; values with respect to the type strains of other recognized *Virgibacillus* species were below 96.0 %. The DNA G + C content of strain Hal 1^T was 38.2 mol%. Levels of DNA–DNA relatedness between strain Hal 1^T and the type strains of *V. carmonensis* and *V. necropolis* were 14.0 and 21.0 %, respectively. The major fatty acid of strain Hal 1^T was anteiso-C_{15:0}, consistent with species of the genus *Virgibacillus*. The cell-wall peptidoglycan of strain Hal 1^T was type A1α and the major respiratory quinone was MK-7. On the basis of genotypic and physiological results, strain Hal 1^T (=DSM 19574^T=JCM 14839^T) is proposed as the type strain of a novel species of the genus *Virgibacillus*, namely *Virgibacillus arcticus* sp. nov.

At the time of writing, the genus *Virgibacillus* comprised 14 recognized species (An *et al.*, 2007; Chen *et al.*, 2008; Heyndrickx *et al.*, 1999; Heyrman *et al.*, 2003; Hua *et al.*, 2008; Lee *et al.*, 2006a; Quesada *et al.*, 2007; Yoon *et al.*, 2004b, 2005; Wang *et al.*, 2008). Members of the genus are Gram-positive, endospore-forming, motile rods and are taxonomically grouped within the order *Bacillales* (Garrity *et al.*, 2007). As part of the microbial description of permafrost from the Canadian high Arctic (Steven *et al.*, 2007), a number of halophilic media were tested in an attempt to isolate halophilic organisms identified in 16S rRNA gene clone libraries. As a consequence a novel bacterial strain, Hal 1^T, was isolated from permafrost dated to be approximately 5000–7000 years old (Steven *et al.*, 2007) at a depth of 9 m on quarter-strength halophiles moderate (¼ HM) medium adapted from Ventosa *et al.* (1982). Briefly, ¼ HM medium was adapted by adding one-quarter (w/v) proteose-peptone, yeast extract and

glucose. The final composition of ¼ HM medium comprised (per litre): 178 g NaCl, 2 g KCl, 1 g MgSO₄·7H₂O, 0.36 g CaCl₂·2H₂O, 0.23 g NaBr, 0.06 g NaHCO₃, 0.01 g FeCl₃, 1.25 g proteose-peptone, 2.5 g yeast extract and 0.25 g glucose, adjusted to about pH 7.0. Approximately 5 g of aseptically collected (Juck *et al.*, 2005) permafrost (Steven *et al.*, 2007) was added to 50 ml of ¼ HM medium and incubated with shaking at 37, 23 and 5 °C. Growth was noted after 2 weeks incubation at 23 °C and after 1 month at 5 °C. An aliquot of the enrichments was plated on ¼ HM plates, and single colonies were selected. Strain Hal 1^T was the dominant colony type from both the 23 and the 5 °C enrichments as verified by sequencing a partial region of the 16S rRNA gene [100 % sequence similarity identity by using primers 27F and 758R; see Steven *et al.* (2007) for PCR conditions and primer sequences]. DNA was isolated from a single colony of strain Hal 1^T and the almost-complete 16S rRNA gene was PCR amplified and sequenced as outlined by Steven *et al.* (2008). In brief, DNA was isolated by using the Gram-positive protocol of the DNeasy Tissue kit (Qiagen) and the 16S rRNA gene was PCR amplified and sequenced by using primers 27F and 1492R (Lane, 1991).

The 16S rRNA gene sequence of strain Hal 1^T showed highest similarity (98.2 % over 1350 bp) to both

Abbreviation: RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Hal 1^T is EF675742.

RAPD results from comparisons of strain Hal 1^T and the type strains of recognized *Virgibacillus* species are available as supplementary material with the online version of this paper.

Virgibacillus necropolis LMG 19488^T (AJ315056) and *Virgibacillus carmonensis* LMG 20964^T (AJ316302); levels of 16S rRNA gene sequence similarity between strain Hal 1^T and the type strains of other recognized *Virgibacillus* species were below 96 %, with closest matches to *Virgibacillus halodenitrificans* DSM 10037^T and *Virgibacillus marismortui* 123^T (95.9 % over 1350 bp). *V. necropolis* LMG 19488^T and *V. carmonensis* LMG 20964^T were obtained for subsequent comparisons, including API test strips, DNA–DNA hybridization, hydrolysis of a range of compounds, carbon utilization, pH growth range, antibiotic sensitivity and genotypic testing. The phylogenetic tree presented in Fig. 1 was based on an analysis of 1484 bp of aligned 16S rRNA gene sequences and indicates that strain Hal 1^T formed a separate branch with 100 % bootstrap support within the genus *Virgibacillus*. The tree was produced with the MacVector 7.0 software package (Oxford Molecular Ltd) by using the neighbour-joining method (Saitou & Nei, 1987) with Jukes–Cantor modelling (Jukes & Cantor, 1969). Similar topologies were also obtained by using the Tamura–Nei model (Tamura & Nei, 1993) and UPMGA (unweighted pair-group method with arithmetic mean) distance estimation (results not shown). As a consequence, it was decided to characterize strain Hal 1^T fully as it may represent a novel ancient lineage of *Virgibacillus* that has been trapped in permafrost for 5000–7000 years. Justification for the complete characterization of strain Hal 1^T was also corroborated by preliminary

results showing low temperature growth range and high salt tolerance as described in further detail below.

Table 1 provides a comparison of morphological and physiological traits between strain Hal 1^T and the type strains of recognized *Virgibacillus* species. Unless otherwise noted, strain Hal 1^T was routinely maintained and physiological tests were undertaken by using marine agar 2216 (MA; Difco), and cell morphology and motility were observed by using phase-contrast microscopy with a Nikon Eclipse E600 microscope. Hydrolysis of casein was tested by using growth on milk agar (Bacto) plates supplemented with skimmed milk power (15 g l⁻¹); methodologies are described below for the remainder of the experiments.

The colony colour of strain Hal 1^T was light pink, distinctly different from that of both *V. carmonensis* and *V. necropolis*. Strain Hal 1^T grew at 0–30 °C, with optimum growth at about 25 °C; no growth was observed at 37 or –5 °C. Therefore, strain Hal 1^T has a lower reported temperature range for growth than all recognized species of the genus *Virgibacillus* (Table 1). Strain Hal 1^T also grew under anaerobic conditions, as determined by using an anaerobic chamber with an AnaeroGen sachet and appropriate anaerobic indicator (OXOID). Salt requirements for growth were tested in marine broth (Difco) and MA. Optimal growth was observed in the presence of 5 % NaCl and weak growth was observed in media lacking NaCl and in the presence of 20 % NaCl; no growth was

Fig. 1. Neighbour-joining phylogenetic tree showing the taxonomic position of strain Hal 1^T based on 16S rRNA gene sequences. Values at nodes represent bootstrap percentages based on 1000 resamplings. The tree was rooted with *Escherichia coli* ATCC 11775^T (accession no. X80725; not shown). Bar, 0.02 changes per nucleotide position.

Table 1. Differential characteristics between strain Hal 1^T and related *Virgibacillus* species

Taxa: 1, strain Hal 1^T; 2, *V. necropolis* (data from Heyrman *et al.*, 2003); 3, *V. carmonensis* (Heyrman *et al.*, 2003); 4, *V. halophilus* (An *et al.*, 2007); 5, *V. salexigens* (Garabito *et al.*, 1997; Heyrman *et al.*, 2003; Wainø *et al.*, 1999); 6, *V. marismortui* (Arahal *et al.*, 1999, 2000; Heyrman *et al.*, 2003); 7, *V. pantothenticus* (Heyndrickx *et al.*, 1999); 8, *V. proomii* (Heyndrickx *et al.*, 1999); 9, *V. halodenitrificans* (Yoon *et al.*, 2004b; Lee *et al.*, 2006a); 10, *V. koreensis* (Lee *et al.*, 2006a); 11, *V. dokdonensis* (Yoon *et al.*, 2005); 12, *V. olivae* (Quesada *et al.*, 2007); 13, *V. chiguensis* (Wang *et al.*, 2008); 14, *V. kekensis* (Chen *et al.*, 2008); 15, *V. salarius* (Hua *et al.*, 2008). Table adapted from An *et al.* (2007), Heyrman *et al.* (2003) and Wang *et al.* (2008). +, Positive; v, variable; w, weakly positive; –, negative; ND, not determined. All have MK-7 as the major menaquinone (not determined for *V. olivae*).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pigmentation	Light pink	–	Pink	Yellow	–	–	–	–	–	–	–	Yellow	–	–	–
Chains of cells	v	+	–	ND	+	+	+	+	+	–	ND	v	ND	–	+
Growth temperature (°C)	0–30	10–40	10–40	5–45	15–45	15–50	15–50	15–50	10–45	10–45	15–50	20–45	15–55	10–50	10–50
Spore shape*	E	E	E, S	E	E	E	E, S	E, S	E	E	E, S	E, S	E, S	E, S	E (S)
Spore location†	C, S	C, S, T	S	S	C, S, T	S, T	S, T	S, T	S, T	T	S, T	S, T	S, T	T	S, T
Anaerobic growth	+	–	–	–	–	–	+	+	+	+	+	–	+	–	–
Growth at:															
0.5 % NaCl	+	w	–	+	–	–	+	w	–	w	+	+	+	+	+
25 % NaCl	–	–	–	ND	w	+	–	–	w	–	–	ND	+	+	+
pH 10	–	–	–	+	+	–	–	–	–	–	ND	–	–	+	+
Nitrate reduction	+	+	+	+	–	+	v	–	+	–	–	+	+	+	–
Hydrolysis of:															
Casein	+	+	+	ND	+	+	+	+	+	ND	+	+	+	–	+
Gelatin	+	w	–	+	+	+	+	v	+	–	+	+	+	–	w
Acid production from:															
D-Glucose	w	w	–	+	w	+	–	+	+	w	+	–	+	+	+
D-Fructose	w	w	–	+	w	+	–	+	+	+	+	+	+	–	+
D-Mannose	w	w	–	+	w	+	–	+	+	–	+	–	+	–	+
Melibiose	+	–	–	–	ND	–	–	+	–	ND	–	ND	+	ND	–
Trehalose	+	w	–	+	–	–	+	+	+	w	–	ND	–	w	–
D-Mannitol	+	–	–	+	–	–	–	+	v (+)‡	–	–	–	w	w	–
Major (>10 %) fatty acids (listed from high to low concentration)	anteiso-C _{15:0}	anteiso-C _{15:0} , anteiso-C _{17:0}	anteiso-C _{15:0} , anteiso-C _{17:0}	anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{16:0}	iso-C _{15:0} , anteiso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0}	anteiso-C _{15:0} , anteiso-C _{17:0}	anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{15:0}	anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{16:0}	anteiso-C _{15:0} , iso-C _{16:0}	anteiso-C _{15:0} , iso-C _{17:0}	iso-C _{15:0} , anteiso-C _{17:0}	anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{15:0}	anteiso-C _{15:0} , iso-C _{14:0}	iso-C _{15:0} , anteiso-C _{15:0}
Major polar lipids§	PG, DPG, PLs	PG, DPG, PLs	PG, DPG, PE, PLs	ND	PG, DPG, PLs	PG, DPG, PLs	PG, DPG, PE, PLs	PG, DPG, PE, PLs	PG, DPG, PLs	PG, DPG, PLs	PG, DPG, PE, PLs	ND	PG, DPG, PE, PLs	PG, DPG, PLs	PG, DPG, PE, PLs
Cell-wall type	A1α	ND	ND	meso-DAP¶	meso-DAP	meso-DAP	meso-DAP	ND	meso-DAP	meso-DAP	meso-DAP	ND	meso-DAP	meso-DAP	meso-DAP
DNA G + C content (mol%)	38.2	37.3	38.9	42.6	36.3–39.5	39.0–42.8	36.9–38.3	36.8–37.0	38.0–39.0	41.0	36.7	33.4	37.3	41.8	37.3

*E, Ellipsoidal; S, spherical.

†C, Central; S, subterminal; T, terminal.

‡Positive for the type strain.

§DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, Phosphatidylglycerol; PLs, unidentified phospholipids.

¶meso-DAP, meso-Diaminopimelic acid.

observed at 25 % NaCl. Strain Hal 1^T grew weakly without the addition of NaCl, as determined on minimal salts medium supplemented with yeast extract (50 mg l⁻¹) and glucose as sole carbon source (Greer *et al.*, 1990). The pH range for growth was determined in ¼ HM with the pH adjusted prior to autoclaving, with the pH confirmed following autoclaving. Strain Hal 1^T grew at pH 4.4–9.1; no growth was observed at pH 4.1 or 9.5. In comparison, *V. necropolis* LMG 19488^T and *V. carmonensis* LMG 20964^T grew at pH 5.7–9.5 with no growth observed for these two taxa at pH 5.0 or 10.1.

Strain Hal 1^T was catalase-positive, as determined by the addition of 3 % H₂O₂ to an active culture, with the production of bubbles indicating a positive reaction. Strain Hal 1^T was oxidase-negative, as determined by using a BD BBL DrySlide according to the manufacturer's instructions (Becton Dickinson). The API 20E and API Staph strip test systems were used according to the manufacturer's instructions (bioMérieux). The type strains of *V. necropolis* and *V. carmonensis* were used as controls in these tests. Strain Hal 1^T shared essentially the same API 20E profile as *V. carmonensis* with the exception of strong gelatinase activity. *V. necropolis* and *V. carmonensis* showed weak and no gelatinase activity, respectively, which is in accordance with the results reported by Heyrman *et al.* (2003). Various carbon substrates were screened in triplicate as described by Heyrman *et al.* (2003). Strain Hal 1^T utilized both D-glucose and sucrose as sole carbon sources, but no growth was observed on D-lactose, cellobiose, melibiose or trehalose. The profile of carbon source utilization for strain Hal 1^T differed from those of *V. carmonensis* and *V. necropolis* (Heyrman *et al.*, 2003). Antibiotic sensitivity was tested by the addition of antibiotics to a final concentration of 100 µg ml⁻¹ in MA medium. *V. carmonensis*, *V. necropolis* and strain Hal 1^T did not grow in the presence of ampicillin, chloramphenicol, rifampicin, erythromycin and streptomycin. Antibiotic sensitivity data for other species of *Virgibacillus* have been documented only for *Virgibacillus kekensis* and *Virgibacillus olivae* (Quesada *et al.*, 2007; Chen *et al.*, 2008). Similarities to strain Hal 1^T exist in that both *V. kekensis* and *V. olivae* were sensitive to ampicillin and rifampicin, *V. kekensis* was sensitive to chloroamphenicol (*V. olivae* was resistant) and *V. olivae* was sensitive to erythromycin (not tested for *V. kekensis*) (Quesada *et al.*, 2007; Chen *et al.*, 2008). In contrast, *V. kekensis* was resistant to streptomycin (Chen *et al.*, 2008) (not tested for *V. olivae*; Quesada *et al.*, 2007). However, it should be noted that a standardized single disc method (Bauer *et al.*, 1966) as used in the previous two studies was not utilized for the present study.

Fatty acids of strain Hal 1^T were prepared from cells grown on MA for 48 h at room temperature and were identified by using the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990) at Keystone Laboratories, Edmonton, Canada. Fatty acids of strain Hal 1^T comprising more than 1 % of the total fatty acid content were iso-C_{14:0} (5.84 %), iso-C_{15:0} (1.78 %),

anteiso-C_{15:0} (80.93 %), iso-C_{16:0} (2.93 %), C_{16:0} (1.56 %) and anteiso-C_{17:0} (2.78 %). The fatty acid profile of strain Hal 1^T was similar to those of the type strains of all recognized species of the genus *Virgibacillus* (see Table 1), in that strain Hal 1^T shared the same dominant fatty acids and also possessed anteiso-C_{15:0} as the major fatty acid. Specifically, the anteiso-C_{15:0} content of the type strains of both *V. carmonensis* and *V. necropolis* (grown on MA) was also high, 65.54 ± 1.4 and 71.5 % (Heyrman *et al.*, 2003), respectively; the corresponding value for the type strains of all other *Virgibacillus* species is in the range 30.5–52.9 % (data from An *et al.*, 2007; Chen *et al.*, 2008).

Analyses of the respiratory quinones, peptidoglycan and polar lipids were carried out by the identification service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and Dr B. J. Tindall (DSMZ). Strain Hal 1^T contained MK-7 as the major respiratory quinone (100 %), which fits the description of the genus *Virgibacillus* (Heyrman *et al.*, 2003; and see Table 1). The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and two unidentified phospholipids, which is also typical for the genus *Virgibacillus* (Table 1). Strain Hal 1^T did not contain any diaminopimelic acid isomers, with dinitrophenylation revealing that lysine was the only N-terminal amino acid. Therefore, it was concluded that the peptidoglycan type of strain Hal 1^T was A1α (L-Lys direct, type A11 according to the DSMZ catalogue of strains). The presence of peptidoglycan type A1α is typically characteristic of genera of the *Bacillus* rRNA group 2 (Ash *et al.*, 1991; Rheims *et al.*, 1999; Stackebrandt *et al.*, 1987) within the *Bacillales*; for example, the recently described *Marinibacillus* (Yoon *et al.*, 2004a) and *Jeotgabacillus* (Yoon *et al.*, 2001); however, peptidoglycan type A1α has also been detected for *Bacillus seohaeanensis* (Lee *et al.*, 2006b) and the genera *Sedimentibacter* within the *Clostridiales* (Breitenstein *et al.*, 2002). Although the presence of cell-wall peptidoglycan type A1α differs from recognized *Virgibacillus* species (Table 1), we recommend that strain Hal 1^T is included within the genus *Virgibacillus* based on the results of other genotypic and phenotypic tests. As more representatives of *Virgibacillus* are identified and characterized, the peptidoglycan types characteristic of this genus may be better resolved.

DNA–DNA hybridization experiments between strain Hal 1^T and the type strains of *V. necropolis* and *V. carmonensis* were undertaken by the DSMZ. DNA was isolated by using a French pressure cell and was purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977), and DNA–DNA hybridization was carried out as detailed by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983). The level of DNA–DNA relatedness between strain Hal 1^T and the type strains of *V. necropolis* and *V. carmonensis* was 21.0 and 14.0 %, respectively. This was corroborated by genome-based comparisons between strain Hal 1^T and the type strains of *V. necropolis* and *V. carmonensis* by using randomly amplified polymorphic DNA (RAPD). RAPD analysis was undertaken by using

two 9-nt random primers with the following sequences: primer 0944-10, 5'-AGAAGGCCG; primer 0940-12, 5'-ACGCGCCCT (Miguez *et al.*, 1997). The RAPD results are shown in Supplementary Fig. S1 and indicate obvious genotypic differences between strain Hal 1^T and the type strains of *V. carmonensis* and *V. necropolis*. The G+C content of strain Hal 1^T was 38.2 mol%, as identified by triplicate assays via the fluorimetric melting temperature-based method of Gonzalez & Saiz-Jimenez (2002). All previously characterized type strains of species of the genus *Virgibacillus* have a DNA G+C content in the range 36.3–42.8 mol% (Table 1 and references therein); the DNA G+C content of strain Hal 1^T is thus within the range reported for members of the genus *Virgibacillus*.

In summary, the morphology, 16S rRNA gene sequence, fatty acid profile and DNA G+C content of strain Hal 1^T are characteristic of the genus *Virgibacillus*. However, physiological and genotypic results encompassing gelatinase activity, carbon substrate utilization, growth temperature, anaerobic growth, pH and temperature growth range, DNA–DNA relatedness and RAPD profiling indicate that strain Hal 1^T represents a species of *Virgibacillus* separate from its closest phylogenetic relatives, namely *V. carmonensis* and *V. necropolis*. Therefore, we suggest that strain Hal 1^T represents a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus arcticus* sp. nov. is proposed.

As strain Hal 1^T is not able to grow at subzero temperatures, it may not be considered to be an active member of the permafrost microbial community. However, given that it is moderately halophilic and endospore-forming, it is highly likely that it survived an extremely long period of time within the permafrost environment. This adds to current evidence that members of this bacterial genus are capable of surviving and being revived from extremely long periods of dormancy (Satterfield *et al.*, 2005).

Description of *Virgibacillus arcticus* sp. nov.

Virgibacillus arcticus (arc'ti.cus. L. masc. adj. *arcticus* northern, Arctic, the place of origin of the type strain).

Gram-positive, motile rods (0.5–0.8 × 2–5 µm) occurring as single, paired or rarely as short chains of cells. Colonies are 2 mm in diameter after 1 week of incubation on ¼ HM at room temperature. Forms elliptical endospores, located centrally or subterminally. Capable of anaerobic growth. Growth occurs at 0–30 °C, but no growth is observed at –5 or 37 °C. Growth occurs from 0 to 20% NaCl (optimum, 5% NaCl); no growth occurs at 25% NaCl. Grows between pH 4.4 and 9.1 (optimum, about pH 7.0). Growth occurs on D-glucose and sucrose, but not on D-lactose, cellobiose, melibiose or trehalose as sole carbon source. Catalase-positive and oxidase-negative. Positive for hydrolysis of casein. In API 20E tests, positive for gelatinase and nitrate reduction, but negative for ONPG, arginine dihydrolase, lysine decarboxylase,

ornithine decarboxylase, citrate utilization, H₂S production, urease, tryptophan deaminase, indole and the Voges–Proskauer reaction. Acid is not produced from inositol, D-sorbitol, L-rhamnose, sucrose, amygdalin or L-arabinose; acid is produced weakly from D-glucose, D-mannitol and melibiose. In API Staph tests, acid is produced from trehalose, D-mannitol, xylitol and melibiose; acid is produced weakly from D-glucose, D-fructose, D-mannose, maltose and lactose. Negative for production of alkaline phosphatase and acetyl-methyl-carbinol, and acid production from raffinose, xylose and sucrose. Acid is produced weakly from methyl α-D-glucoside. Negative for *N*-acetylglucosamine, arginine dihydrolase and urease. Sensitive to ampicillin, chloramphenicol, rifampicin, erythromycin and streptomycin. Major fatty acids (>1%) of the type strain are iso-C_{14:0}, iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, C_{16:0} and anteiso-C_{17:0}. The peptidoglycan and respiratory quinone types are A1α and MK-7, respectively. Major polar lipids comprise diphosphatidylglycerol, phosphatidylglycerol and two unidentified phospholipids. The DNA G+C content of the type strain is 38.2 mol%.

The type strain, Hal 1^T (=DSM 19574^T=JCM 14839^T), was isolated from a permafrost core of 9 m depth collected near Eureka, Ellesmere Island, in the Canadian high Arctic.

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