Clostridium tagluense sp. nov., a psychrotolerant, anaerobic, spore-forming bacterium from permafrost

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A strictly anaerobic, Gram-positive, psychrotolerant, endospore-forming bacterium (strain A121^T) was isolated from a permafrost sample collected in the Canadian High Arctic. Phylogenetic analysis of the 16S rRNA gene sequence of strain A121^T showed its affiliation with the group of psychrophilic and psychrotolerant members of cluster I of the genus *Clostridium*, *Clostridium bowmanii* DSM 14206^T being the closest relative (sequence similarity 98.5%). Levels of DNA–DNA relatedness between strain A121^T and the type strains of phylogenetically related species ranged from 33 to 52%. Strain A121^T grew in PY broth at temperatures between 4 and 28 °C (optimum 15–20 °C), at pH 6.0–8.0 (optimum pH 6.5–7.2) and in NaCl concentrations of 0–10.0% (optimum 0–2.0%). The strain utilized a narrow range of carbohydrates as sources of carbon and energy, including glucose, fructose, trehalose, maltose and starch; it also hydrolysed gelatin. Predominant fatty acids were C_{16:1} cis9, C_{16:1} cis9 DMA, C_{16:0} and C_{14:0}. The DNA G+C content was 31.5 mol%. On the basis of its overall genotypic and phenotypic characteristics, strain A121^T is classified within a novel species of the genus *Clostridium*, *Clostridium tagluense* sp. nov. The type strain is A121^T (=VKM B-2369^T =DSM 17763^T).

The genus *Clostridium*, one of the largest genera of the prokaryotes, currently includes more than 170 species of anaerobic, spore-forming, Gram-positive bacteria that are unable to carry out dissimilatory reduction of sulfate. More than 70 species fall within cluster I, now regarded as the genus *Clostridium sensu stricto*, as defined on the basis of 16S rRNA gene sequence similarity (Hippe *et al.*, 1992; Collins *et al.*, 1994; Garrity *et al.*, 2004; Wiegel *et al.*, 2006). Although most of the species included in cluster I have been isolated from temperate environments, a series of psychrophilic and psychrotolerant species have been also described: *Clostridium estertheticum* subsp. *estertheticum* (Collins *et al.*, 1992; Spring *et al.*, 2003), *C. estertheticum*

subsp. *laramiense* (Kalchayanand *et al.*, 1993; Spring *et al.*, 2003), *Clostridium algidicarnis* (Lawson *et al.*, 1994) and *Clostridium frigidicarnis* (Broda *et al.*, 1999) from vacuum-packed meat, *Clostridium vincentii* from low-salinity pond sediments of the MacMurdo ice shelf in Antarctica (Mountfort *et al.*, 1997), four species isolated from an Antarctic microbial mat (*Clostridium frigoris*, *C. lacusfryxellense*, *C. bowmanii* and *C. psychrophilum*) (Spring *et al.*, 2003) and ‘*Clostridium algorithilum*’, isolated from a water brine in Siberian permafrost (Shcherbakova *et al.*, 2005). Phylogenetic data from previous studies (Spring *et al.*, 2003; Shcherbakova *et al.*, 2005; Gilichinsky *et al.*, 2005) showed that all of these species, with the exception of *C. algidicarnis*, *C. frigidicarnis* and *C. vincentii*, form an isolated branch within cluster I of the genus *Clostridium*. In this study, we describe a novel psychrotolerant bacterium of the genus *Clostridium* (strain A121^T) that was isolated from a permafrost core in the Canadian High Arctic. Based on phylogenetic analysis of its 16S rRNA gene sequence, DNA–DNA hybridization data and phenotypic character-

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A121^T is DQ296031.

Plots showing the effects of temperature and NaCl concentration on growth of strain A121^T, a comparison of the fatty acid profiles of strain A121^T and related type strains and an extended 16S rRNA gene sequence-based neighbour-joining tree are available as supplementary material with the online version of this paper.

istics, this isolate is shown to be closely related to the above-mentioned psychrotolerant and psychrophilic *Clostridium* species.

The isolation source (Quaternary-aged permafrost with present-day temperature of -6°C) was sampled from the upper horizon (18 m) of the core of the deep (451 m) borehole 92GSCTaglu in a gas-hydrate field located in the Mackenzie Delta, Northwest Territories, Canada ($69^{\circ}22' \text{N}$ $135^{\circ}00' \text{W}$). The recovered sample of ice-bonded sand of lake origin was kept frozen until laboratory analysis. Anaerobic strain A121^T was isolated from the sample and maintained using Hungate anaerobic techniques (Hungate, 1969). Both enrichment and pure cultures were grown in 15 ml Hungate roll tubes with 10 ml medium and 100% N_2 atmosphere. An enrichment culture was obtained using a medium of the following composition (g l^{-1}): KH_2PO_4 , 0.7; K_2HPO_4 , 0.7; NH_4Cl , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; NaCl , 1.0; sodium ascorbate, 1.0; glucose, 2.0; peptone, 2.0. One gram of the permafrost sample was suspended in 10 ml medium in Hungate roll tubes under anaerobic conditions and incubated at 6°C for 3 months up to a turbidity (OD_{600}) of 0.6. After incubation, the enrichment culture was transferred into a sterile medium of the same composition and serial tenfold dilutions were made. Aliquot from the highest dilutions were plated on Petri dishes of the same medium solidified with 20 g agar l^{-1} . The dishes were incubated in Oxoid anaerostats at 6°C for 1 month. As a result of serial transfers of single colonies from solid to liquid medium, a pure culture of a psychrotolerant anaerobic bacterium with a fermentative type of metabolism (designated strain A121^T) was isolated. The pure culture of strain A121^T was maintained and cultivated in PY broth of the following composition (g l^{-1}): peptone, 5.0; tryptone, 5.0; yeast extract, 10.0; salt solution, 10 ml. The salt solution contained (g l^{-1}): CaCl_2 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.48; K_2HPO_4 , 1.0; KH_2PO_4 , 1.0; NaHCO_3 , 10.0; NaCl , 2.0.

Motility and cell morphology were examined using an Axiostar Plus phase-contrast light microscope (Carl Zeiss) at a magnification of $\times 100$; cells for analysis were grown for 24–48 h in PY broth at 15°C . Electron microscopy of ultrathin sections for examination of cellular and spore ultrastructure was performed as described previously (Shcherbakova *et al.*, 2005). Growth rates were measured by OD_{600} using a Specol-221 spectrophotometer (Carl Zeiss); the incubation time was 48–72 h. The effect of pH on growth was measured at the optimal growth temperature of 15°C ; the pH of basal PY medium was adjusted by adding sterile 6 M solutions of HCl or NaOH. The influence of NaCl concentration on growth was examined at the optimal growth temperature (15°C) and pH (pH 6.5–7.2) in basal medium containing 0, 0.5, 1.0, 2.0, 5.0, 10.0 or 15.0% (w/v) NaCl. The viability of cells was tested by heating 24 h liquid cultures of strain A121^T at 37°C (for 24 h), at 50, 60, 70 and 80°C (for 10 min in a water bath) and by placing a culture in a freezer at -40°C for 2 months with subsequent thawing by increasing the

temperature up to 5°C over 30 min. After each treatment, the cultures were observed by microscopy and then transferred to fresh medium and incubated under optimal conditions.

Phenotypic tests to determine standard taxonomic characteristics such as Gram-staining and the presence of oxidase and catalase were performed following standard protocols (Gerhardt *et al.*, 1994). The substrate utilization pattern of strain A121^T was examined in mineral medium with 3 g yeast extract l^{-1} by adding sterile solutions of substrates with end concentrations of 3 g l^{-1} . The cultures were incubated for 3 days; growth was determined by OD_{600} and confirmed by two additional transfers. For the detection of nitrate-reducing and sulfate-reducing activities, the strain was cultivated in basal PY medium at the optimal growth temperature with addition of sterile solutions of NaNO_3 , $\text{Na}_2\text{S}_2\text{O}_3$, Na_2SO_3 and Na_2SO_4 (final concentration of each compound 2 g l^{-1}). Fermentation products (alcohols and fatty acids) formed in PY broth were analysed and quantified as described previously (Shcherbakova *et al.*, 2005). H_2 and CO_2 were analysed by GC according to Shtarkman *et al.* (1995). Lactate was determined with lactate dehydrogenase by a colorimetric method (Hohorst, 1970). Analysis of the cell-wall peptidoglycan was performed as described by Schleifer & Kandler (1972). The fatty acid pattern of strain A121^T was identified as described previously (Shcherbakova *et al.*, 2005).

The 16S rRNA gene was amplified by PCR using a Perkin Elmer model 2400 DNA thermal cycler. The gene was amplified in a 50 μl reaction mixture containing 2 μl (approx. 0.3 ng) template DNA, 1 μl (10 pmol) of each primer (Helicon) and a PCR kit (Fermentas) containing 5 μl PCR buffer with 25 mM MgCl_2 , 2 μl dNTP mixture (0.2 mM) and 1 μl *Taq* polymerase (5 U μl^{-1}). The universal prokaryotic primers 27F (5'-AGAGTTTGA-TCTGGCTCAG) and 1492r (5'-TACGGYTACCTT-GTTACGATT) were used. The PCR conditions were 94°C for 10 min for primary denaturation, followed by 30 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing) and 72°C for 120 s (chain extension) and a final 5 min chain-extension step at 72°C . The obtained PCR product was purified by using the Wizard PCR Preps DNA purification system (Promega) and sequenced. Sequencing reactions were performed using a CEQ dye terminator cycle sequencing kit (Beckman Coulter) and a CEQ2000 XL automatic DNA sequencer (Beckman Coulter), according to the protocols provided by the manufacturer. Reference sequences of the closest species were retrieved from GenBank (Benson *et al.*, 1999) by using tools of the NCBI BLAST and the Ribosomal Database Project II (Maidak *et al.*, 2001). Sequences were aligned and edited by using the sequence alignment editor BioEdit version 7.0.5.3 (Hall, 1999). The program TREECON W (Van de Peer & De Wachter, 1994) was applied for construction of a phylogenetic tree using the neighbour-joining method (Saitou & Nei, 1987) and Jukes–Cantor distance estimation (Jukes & Cantor, 1969); the topology of the phylogenetic

tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) based on 1000 replicates. The branching obtained was confirmed by high values of branching probability. The DNA G+C content was assessed by thermal denaturation of DNA using a Pye Unicam SP1800 spectrophotometer; DNA for this analysis was extracted by the method of Marmur (1961). DNA–DNA reassociation analysis was performed as described by

De Ley *et al.* (1970); genomic DNA for this analysis was extracted by the method of Sambrook *et al.* (1989).

Differential genotypic and phenotypic characteristics of strain A121^T are given in the species description and in Table 1, in comparison with phylogenetically close *Clostridium* species. Phenotypically, the isolate matched the description of the genus *Clostridium* perfectly: cells of

Table 1. Differential characteristics of strain A121^T and phylogenetically close *Clostridium* species

Strains: 1, A121^T; 2, '*C. algariphilum*' DSM 16153; 3, *C. bowmanii* DSM 14206^T; 4, *C. frigoris* DSM 14204^T; 5, *C. lacusfraxellense* DSM 14205^T; 6, *C. psychrophilum* DSM 14207^T; 7, *C. estertheticum* subsp. *laramiense* DSM 14864^T; 8, *C. estertheticum* subsp. *estertheticum* DSM 8809^T. Data for reference strains were taken from Shcherbakova *et al.* (2005) ('*C. algariphilum*' DSM 16153) and Spring *et al.* (2003) (remaining reference strains). All strains were positive for the utilization of glucose and fructose. Substrate utilization was determined by measuring the increase in OD₆₀₀: +, strong production of gas and acid; w, weak production; –, no significant gas or acid produced. ND, No data available.

Characteristic	1	2	3	4	5	6	7	8
Spore shape*	S	S	S	S	S	S	E	E
Spore location†	ST	C	T–ST	T	T–ST	T–ST	ST–T	ST–C
Temperature optimum (°C)	15	5–6	12–16	5–7	8–12	4	15	6–8
pH optimum	6.5–7.2	6.5–7.2	6.6–7.2	6.8–7.2	6.5–7.1	6.5–7.0	6.5	6.5–7.2
Utilization of:								
Arabinose	–	+	–	+	–	+	+ (–) ^{a‡}	+
Cellobiose	–	+	–	+	+	+	+ (–) ^a	w (+) ^b
Galactose	–	+	+	+	+	–	+	+
Inositol	–	+	–	w	+	–	+	+
Lactose	–	+	–	+	+	–	–	–
Maltose	+	+	+	+	–	+	+	+
Mannitol	–	+	–	–	+	–	+	+
Mannose	–	+	+	+	–	+	+	+
Melezitose	–	+	–	+	+	–	–	–
Melibiose	–	+	–	+	+	–	+	+
Raffinose	–	+	–	+	+	–	+	+
Rhamnose	–	–	–	+	–	–	+	+
Sucrose	–	+	+	+	+	+	+	+
Xylose	–	+	+	+	+	+	+ (–) ^a	+
Ribose	–	+	+	w	+	–	–	–
Starch	+	–	–	+	+	–	+	w (+) ^b
Salicin	–	+	+	+	+	–	+	+
Trehalose	+	+	+	+	+	+	–	–
Xylan	–	+	ND	ND	ND	ND	ND	ND
Hydrolysis of:								
Starch	–	–	–	–	–	–	+	w (+) ^b
Gelatin	+	–	–	–	–	–	–	–
Fermentation products§	A, B, v, f, 2	B, L, a, f, 2	B, A, f, 2, l, 4	B, f, l, a, 2	B, F, a, l, 2	L, 2, 4, b	B, 4, l, a, f, 2	B, A, f, l, 4, 2
DNA G+C content (mol%)	31.5	31.4	32.0	31.9	32.1	31.8	32.4	33.9
Genetic comparison with strain A121 ^T								
DNA–DNA hybridization (%)	(100)	48	35	33	35	52	ND	ND
16S rRNA gene sequence similarity (%)	(100)	98.2	98.5	98.2	98.1	94.4	98.2	98.0

*E, Ellipsoidal; S, spherical.

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

‡Data in parentheses differ from results obtained by Spring *et al.* (2003) and were taken from: a, Kalchayanand *et al.* (1993); b, Collins *et al.* (1992).

§a, Acetic acid; b, butyric acid; f, formic acid; v, valeric acid; l, lactic acid; 2, ethanol; 4, butanol. The sequence of products listed reflects decreasing concentration; capitals indicate concentrations above 10 mM. Fermentation products produced only in small amounts (<1 mM) were not considered.

the strain were Gram-positive rods that formed spherical endospores (Fig. 1), and the strain was obligately anaerobic and unable to carry out dissimilatory reduction of sulfate. According to the classification of Morita (1975), the strain was psychrotolerant: growth occurred at temperatures of

Fig. 1. Micrographs of cells of strain A121^T. (a) Phase-contrast micrograph (bar, 10 µm); (b, c), ultrathin sections of actively growing cells (bars, 1 µm); (d) ultrathin section of spore (bar, 1 µm). cw, Cell wall; cm, cytoplasmic membrane; cap, capsule; C, core; K, cortex; IM, inner membrane; OM, outer membrane; CT, spore coat.

4–28 °C (optimum 15–20 °C), but did not grow at 37 °C. Very slow growth was observed at –2 °C after 2 months of incubation. The strain grew at pH 6.0–8.0 (optimum pH 6.5–7.2) and in NaCl concentrations between 0 and 12–14 % (w/v) (optimum 0–2.0 %). The doubling time of strain A121^T as defined by doubling of OD₆₀₀ in the exponential phase of growth was 12.7 h under optimal growth conditions. Plots demonstrating the influence of temperature and NaCl concentration on growth of strain A121^T are available as Supplementary Fig. S1 in IJSEM Online. Cells of the strain did not survive heating at 37 °C or above, but survived freezing at –40 °C for 2 months; spore formation was not observed after heating, but single spores were found after freeze-thaw treatment. These features of cell viability and spore formation seem to correspond to the nature of the isolation source and the psychrotolerant temperature pattern of the isolate. Strain A121^T did not exhibit any nitrate-reducing or sulfate-reducing activity. In comparison with related clostridial species, the isolate utilized a much narrower range of carbohydrates: only glucose, fructose, maltose, trehalose and starch supported growth. In contrast to related clostridial species, strain A121^T hydrolysed gelatin. The major fermentation products after 10 days of incubation were acetate (33.3 mM), butyrate (11.3 mM), isobutyrate (6.3 mM) and valerate (3.8 mM). Additionally, small amounts of formate and ethanol were produced (<1 mM); lactate was not detected. H₂ (10 %) and CO₂ (12 %) accumulated in the gas phase after incubation. The fatty acid patterns of strain A121^T and related strains are available in Supplementary Table S1. Comparison with patterns of related members of the genus *Clostridium* revealed certain common features: domination of mono-unsaturated omega-7-*cis*-hexadecene acid (C_{16:1 cis9}; 18.8 %) and hexadecene aldehyde (C_{16:1 cis9} DMA; 22 %) and unsaturated palmitic acid (C_{16:0}; 15 %) and myristic acid (C_{14:0}; 10.4 %). The only exception is '*C. algariphilum*' DSM 16153, which demonstrated somewhat different features (Supplementary Table S1).

Phylogenetic analysis of the 16S rRNA gene sequence (1454 nt) of strain A121^T (Fig. 2 and Supplementary Fig. S2) shows that the isolate is closely related to the group of psychrotolerant and psychrophilic species that form an isolated branch within cluster I of the genus *Clostridium*; *C. bowmanii* DSM 14206^T was the closest match, with a sequence identity of 98.5 %. The 16S rRNA gene sequence identity between the isolate and strains of related species was 98–98.5 % (Table 1) with the exception of *C. psychrophilum* A-1/C-an/I^T, which showed lower identity (96.4 %); similarity with more distant species, not included in this branch, was less than 96 %. These sequence identity data agree with the range of 92–99 % that was suggested as a range of similarity for members of cluster I, or *Clostridium sensu stricto* (Wiegel *et al.*, 2006). The DNA G + C content of strain A121^T (31.5 mol%) was very close to reported values for related clostridial species (Table 1); however, DNA–DNA reassociation between strain A121^T

Fig. 2. Position of strain A121^T among closely related species of cluster I of the genus *Clostridium*, based on 16S rRNA gene sequence phylogenetic analysis. GenBank accession numbers are shown in parentheses. The tree was built with the neighbour-joining method (Saitou & Nei, 1987) by using Jukes–Cantor distance estimation (Jukes & Cantor, 1969). Bootstrap percentages (from 1000 bootstrap resamplings for each node) are shown at branching points. Bar, 0.02 estimated substitutions per nucleotide position. A version of this tree incorporating an extended selection of reference sequences is available as Supplementary Fig. S2.

and strains of these species was much lower than the conventional 70 % threshold (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), thus indicating that the isolate represents a distinct species (Table 1).

On the basis of the data presented, isolate A121^T should be classified as a member of a novel species of the genus *Clostridium*, for which the name *Clostridium tagluense* sp. nov. is proposed.

Description of *Clostridium tagluense* sp. nov.

Clostridium tagluense (tag.lu.en'se. N.L. neut. adj. *tagluense* after the Taglu gas-hydrate field in the Northwest Territories of Canada, the source of isolation of the type strain).

Cells are Gram-positive, motile rods with rounded ends (1.0–1.2 × 3–10 µm), occurring singly or in pairs. Endospores are spherical, located in a subterminal position. Colonies on PY agar are round, convex, cream-coloured and 1–2 mm in diameter. Obligate anaerobe; catalase- and oxidase-negative. Nitrate and sulfate are not reduced. The temperature optimum for growth is 15–20 °C; the lower growth limit is 4 °C and the upper growth limit is 28 °C. No growth at 37 °C. Grows at pH 6.0–8.0 (optimum pH 6.5–7.2) and in NaCl concentrations between 0 and 12–14 % (w/v) (optimum 0–2.0 %). Under optimal conditions, the doubling time is 12.7 h. Fermentable compounds include fumarate, malate, tryptase, betaine, choline, glucose, maltose, fructose, trehalose and starch. Xylose, xylan, arabinose, cellobiose, lactose, mannose, sucrose, ribose, raffinose, rhamnose, melibiose, melezitose, inositol, mannitol and salicin do not support growth. Gelatin is hydrolysed, but starch is not. Major

fermentation products formed in PY broth are acetate, butyrate, valerate, H₂ and CO₂. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. Major cellular fatty acids are C_{16:1} *cis*9 DMA, C_{16:1} *cis*9, C_{16:0} and C_{14:0}. The DNA G+C content of the type strain is 31.5 mol%.

The type strain, A121^T (=VKM B-2369^T =DSM 17763^T), was isolated from a permafrost sample from the core of the borehole 92GSCTaglu in the Mackenzie Delta, Northwest Territories, Canada.

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