Celerinatantimonas yamalensis sp. nov., a cold-adapted diazotrophic bacterium from a cold permafrost brine

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A facultatively anaerobic nitrogen-fixing bacterium, strain C7T, was isolated from a permafrost cryopeg on the Yamal Peninsula, Russia. Comparative analysis of 16S rRNA gene sequences revealed that this bacterium was closely related to Celerinatantimonas diazotrophica S-G2-2T with a similarity of 95.5%. Strain C7T differed from Celerinatantimonas diazotrophica in its ability to hydrolyse gelatin and inability to use D-mannose, melibiose, L-rhamnose, myo-inositol, lactose, lactulose, D-mannitol, trehalose, DL-lactate, glycogen or L-proline as sole carbon sources. In addition, strain C7T grew over a temperature range of 0–34 °C with optimum growth at 18–22 °C. The whole-cell fatty acid profile included C16:0, C16:1ω7, C18:1ω7, C17 cyclo and summed feature 2 [comprising C12:0 aldehyde and/or unknown fatty acid 10.913 (MIDI designation) and/or iso-C16:1/C14:0 3-OH]. The DNA G+C content was 44.7 mol%. Strain C7T is thus considered to represent a novel species, for which the name Celerinatantimonas yamalensis sp. nov. is proposed. The type strain is C7T (=VKM B-2511T = DSM 21888T).

The order Alteromonadales in the class Gammaproteobacteria comprises nine families and 35 genera (www.bacterio.cict.fr) embracing a large group of heterotrophic, polar-flagellated, Gram-negative rod-shaped bacteria that are mainly non-fermentative aerobes and inhabit marine ecosystems. Recently, facultatively anaerobic N2-fixing mesophilic bacteria isolated from the roots of grasses native to the Atlantic Ocean coast of North America were described and assigned to the novel genus Celerinatantimonas as Celerinatantimonas diazotrophica (Cramer et al., 2011). C. diazotrophica was reported to differ from most members of Alteromonadales in the inability to reduce nitrate and produce cytochrome oxidase (Cramer et al., 2011; Bowman & McMeekin, 2005). Available data on the distribution of free-living diazotrophic micro-organisms in permanently cold ecosystems are limited to the marine cyanobacterial communities (e.g. Church et al., 2005; Severin et al., 2010), while nitrogen-fixing micro-organisms from permafrost have not been characterized in detail so far.

NaCl water brine lenses (cryopegs) have been found in the Arctic at a depth of a few tens of metres in the permafrost aged 10 000–200 000 years. They are characterized by sub-zero temperatures (from −11 to −2 °C) and a salinity level ranging from 60 to 300 g l−1 (Gilichinsky et al., 2005). These unique aquatic systems of marine origin are thought not to undergo external influence over geological timescales. Several psychrophilic and halophilic bacteria, both aerobes and anaerobes, have been isolated from Arctic cryopegs and described as representing novel species of the genera Clostridium, Desulfovibrio and Psychrobacter (Gilichinsky et al., 2005; Shcherbakova et al., 2005; Bakermans et al., 2006; Pecheritsyna et al., 2012). A novel facultatively anaerobic psychrotolerant bacterium from Arctic cryopegs, which is able to fix nitrogen, is the focus of the present study.

Abbreviations: MCL, maximum composite likelihood; ME, minimum-evolution; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and nifH gene sequences of strain C7T are FJ039852 and JF701923, respectively.

One supplementary table and three supplementary figures are available with the online version of this paper.
Cryopeg water samples (salinity 77 g l⁻¹) were obtained from the Yamas Peninsula area, Russia (69° 50’ N 159° 30’ E) during an expedition in 2006. The samples were kept at −20 °C for 2 months until analysis. The drilling system and subsequent procedure for recovering uncontaminated material were as described previously (Shi et al., 1997). An enrichment culture growing anaerobically under a nitrogen atmosphere at 15 °C was obtained using GPM medium (per litre tap water): 0.7 g K₂HPO₄, 0.7 g KH₂PO₄, 0.1 g MnSO₄, 5H₂O, 0.1 g MgSO₄. 7H₂O, 40.0 g NaCl, 0.5 g NH₄Cl, 1.0 g sodium ascorbate, 2.0 g glucose and 2.0 g peptone plus 10 ml trace element solution SL-10 (medium 320; DSMZ), pH 7.0–7.2. Pure cultures were isolated by serial dilution using 15 ml Hungate tubes with 10 ml of the same medium prepared anaerobically (Hungate, 1969) and under the same growth conditions. The culture purity was assessed by observing uniform cell types under a phase-contrast microscope. The material for incubation were single colonies developed on Bacto Marine Broth (BD Biosciences) or GPM medium supplemented with 15 g Difco agar l⁻¹ under 100 % nitrogen atmosphere at 15 °C. Colonies appearing 2 weeks after inoculation were circular with entire margin, smooth, convex, glistening and non-pigmented. As a result, two halophilic strains, C7ᵀ and C8, were obtained. The strains were grown and maintained at 18 °C in Bacto Marine Broth (BD Biosciences) or in mGPM medium modified from GPM to contain 80 g NaCl l⁻¹ and 5 g MgSO₄. 7H₂O l⁻¹ (pH 6.0–6.2). The strains showed 99.8 % 16S rRNA gene sequence similarity and exhibited identical morphological and growth characteristics under both anaerobic and aerobic conditions. Subsequent detailed study was performed with strain C7ᵀ. Celerinatantimonas diazotrophica S-G2-2ᵀ, used as a reference strain, was grown in DSMZ 514 medium.

For determination of the 16S rRNA gene sequence, genomic DNA was isolated by standard methods (Sambrook et al., 1989). The 16S rRNA gene was amplified using universal primers, F27 and 1492R (Lane, 1991). The PCR product was purified using a Wizard PCR Prep DNA Purification System. The sequencing reactions were performed using a CEQ Dye Terminator Cycle Sequencing kit according to the protocols provided by the manufacturer and analysed in a Beckman CoulterCEQ2000 XL automatic DNA sequencer. The NCBI GenBank BLAST utility (Altschul et al., 1997, Benson et al., 1998) was used to reveal the closest relatives of strain C7ᵀ. Nucleotide sequences were aligned in CLUSTAL_X (Chenna et al., 2003; Thompson et al., 1997), The TREECON (Van de Peer & De Wachter, 1997) and MEGA 4 (Tamura et al., 2007) software packages were used for phylogenetic analysis. The neighbour-joining (NJ) tree was constructed with the Jukes–Cantor model (Jukes & Cantor, 1969), and this tree was used as the base for the consensus tree. For the construction of the minimum-evolution (ME) tree, the Jukes–Cantor substitution model was also used, while the maximum composite likelihood (MCL) tree was created with the MCL model. A bootstrap analysis was performed according to the algorithm of the Kimura two-parameter model (Kimura, 1980). The percentages of replicate trees, where associated taxa are clustered together in the bootstrap test (from 1000 replicates), are shown next to the branches (Felsenstein, 1985).

Analysis of the 16S rRNA gene sequence (1433 bp) of strain C7ᵀ and relevant organisms showed that strain C7ᵀ was affiliated with the family Celerinatantimonadaceae with a high bootstrap support and had the highest sequence similarity (95.5 %) to C. diazotrophica S-G2-2ᵀ. The consensus phylogenetic tree indicated that these strains represent a separate lineage within the radiation of the order Alteromonadales (Fig. 1).

Cell morphology of strain C7ᵀ was examined using light and electron microscopy as described previously (Shcherbakova et al., 2005). Cells were rods (0.7–0.8 x 2–4 μm) with rounded ends, motile by single polar flagella (Fig. 2a) and occurring singly, in pairs or in short chains. A particular feature of the flagella was the presence of a sheath along their entire length (Fig. 2b). Electron microscopy of thin sections revealed a cell-wall structure with an outer membrane (Fig. 2c) typical of Gram-stain-negative bacteria as described by Dussault (1955). In addition, electron microscopy of thin sections revealed tubular structures on the cell surface and in the intercellular space as well as electron-dense inclusions of different size at the periphery of the cell cytoplasm (Fig. 2c). Spore formation was not observed.

The effects of temperature, salinity and pH on growth were tested in triplicate and confirmed by two additional transfers. Growth (turbidity) was assayed spectrophotometrically at 600 nm. Incubation times of 1–2 weeks were sufficient for optimal growth yields while up to 2 months was required for the maximal values. The effect of temperature on growth was examined at −2, 5, 10, 18, 24, 28, 37 and 42 °C in mGPM medium. Incubation at −2 °C was carried out in a cryobath filled with ethylene glycol. Growth ranges and optimal NaCl and Mg²⁺ concentrations were determined in the growth medium containing 0–20 % (w/v) NaCl and 0–10 % (w/v) MgSO₄. 7H₂O (at intervals of 1 %). The pH optimum for growth was determined using mGPM medium buffered with 10 mM MES and adjusted to pH 3.5–6, 10 mM HEPES for pH 6–8 and 10 mM CAPS for pH 8–10.

Strain C7ᵀ grew at 0–34 °C (optimally at 18–22 °C), showing properties of a facultative psychrophile according to the classification of Morita (1975). The strain required NaCl for growth; it grew with 2.0–12.0 % NaCl, with optimal NaCl concentration of 6.0–8.0 %. The bacterium tolerated 0.5–10.0 % MgSO₄. 7H₂O, showing optimal growth at 0.5 %. These data characterize the strain as a moderate halophile according to the parameters suggested by Oren (2006), growth being dependent on Mg²⁺ concentration in the culture medium. The pH growth range was 5.5–7.5, with optimum growth occurring at pH 6.0–6.2.
Phenotypic characteristics were determined following standard protocols (Smibert & Krieg, 1994). Strain C7T did not reduce nitrate, and did not produce H₂S or indole. It hydrolysed gelatin and Tween 80, but not agar, starch, casein or chitin. The formation of acid from sugars was assessed on solid mGMP medium (glucose and peptone were omitted) with 0.5 g yeast extract, supplemented with 1.0 % (w/v) test substrate. After incubation for 2 weeks, acid formation was determined by using bromcresol green pH test paper. Strain C7T required yeast extract (0.25–1.0 %, w/v) or peptone (0.25–1.0 % w/v) for growth. API ZYM strips (bioMérieux) were used according to the manufacturer's instructions to examine the enzyme activities of strain C7T. The suspension medium was supplemented with 8 % (w/v) NaCl. Positive reactions were scored in triplicate after incubation at 22 °C for 24 h. The utilization of carbohydrates, alcohols or amino acids as sole carbon sources by strains C7T and C. diazotrophica S-G2-2T was tested in mGMP medium in which glucose was omitted and amended with 0.2 % (w/v) of the respective carbohydrates, alcohols or sugars, or 0.1 % (w/v) of the respective amino acids at pH 6.2. All tests were performed in triplicate and confirmed by two transfers. The results are included in the species description and differences between strain C7T and C. diazotrophica S-G2-2T are highlighted in Table 1.

Non-gaseous fermentation products were analysed and quantified as described previously (Shcherbakova et al., 2005). Incubation of strain C7T with D-glucose at the optimal growth temperature (18 °C) showed that acetate dominated among the non-gaseous metabolic products under both anaerobic and aerobic conditions (Fig. S1 available in IJSEM Online). In addition, a small amount of ethanol (1 g l⁻¹) was produced by strain C7T during the fermentation of D-glucose.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the position of the novel strain C7T among members of the order Alteromonadales. The NJ method of Saitou & Nei (1987) was used to reconstruct the tree. The percentages of replicate trees, where associated taxa are clustered together in the bootstrap test (from 1000 replicates), are shown next to the branches (Felsenstein, 1988). Only values ≥50 % are shown. The bootstrap values shown at each node reflect analyses from three phylogenetic reconstruction methods listed in the order NJ/ME/MCL. Bar, 2 % estimated sequence divergence.
and 90.9 % of the total in strain C7T and C. diazotrophica S-G2-2T, respectively. The presence of hydroxy fatty acids in lipopolysaccharides of the cell wall is a prominent characteristic of Gram-negative bacteria in the class Gammaproteobacteria. C. diazotrophica S-G2-2T and strain C7T were both characterized by the presence of C14:0 3-OH in a feature comprising C12:0 aldehyde and/or unknown fatty acid 10.913 and iso-C16:1/C14:0 3-OH (10.28 and 12.54 %, respectively), while strain C7T contained additionally small amounts of C12:0 3-OH (0.1 %) and C15:0 3-OH (0.42 %). The strains also showed differences in some other minor components of their fatty acid profile (Table S1).

Respiratory quinones and polar lipids for strain C7T were determined following the procedures of Collins (1985) and Minnikin et al. (1979), respectively. Cells were grown in Bacto Marine Broth (BD Biosciences) at 18 °C (optimal for strain C7T) and harvested in the late exponential growth phase. The main respiratory quinone was ubiquinone Q-8 (98 %); a small amount of Q-7 (2 %) was also present. The polar lipids included phosphatidylethanolamine, phosphatidylglycerol, aminophospholipid, glycolipids and an unidentified phospholipid (Fig. S2). In contrast to the reference strain (Cramer et al., 2011), diphosphatidylglycerol and phosphatidylmonomethylethanolamine were not detected in polar lipids of strain C7T.

Isolation of DNA from the cells was carried out according to the method of Marmur (1961). Nucleotide content was assessed by thermal denaturation of DNA using a Pye Unicam SP1800 spectrophotometer. The DNA G+C content of strains C7T and C. diazotrophica S-G2-2T were 44.7 ± 0.5 and 41.8 ± 0.3 mol%, respectively (mean ± sd of 4 determinations). The DNA G+C content measured for C. diazotrophica S-G2-2T was close to the value (41.5 mol.%) determined by Cramer et al. (2011) using HPLC.

The ability of strain C7T to fix nitrogen was tested by the acetylene-reduction technique of Stewart et al. (1968). Briefly, the strain was grown anaerobically in Hungate’s tubes with nitrogen-free medium containing citrate as a sole carbon source to mid-exponential growth phase (in triplicate). Acetylene was injected into the anaerobic gas phase to a final concentration of 10 % (v/v) and the culture was further incubated at 18 °C on a rotary shaker (200 r.p.m.) for 48 h. The amount of ethylene produced was measured with a Cristall 5000.1 gas chromatograph equipped with a flame-ionization detector. An uninoculated tube was used to assay the level of ethylene contamination in the acetylene. Strain C7T grew in the nitrogen-free medium and was capable of N2 assimilation. Acetylene-reduction activity ranged from 600 to 750 nmol ethylene h⁻¹ (mg protein)⁻¹.

To support the diazotrophy and presence of the nifH gene in strain C7T, the partial nifH gene sequence was determined as described (Fedorov et al., 2008). Primers F1 (5′-TAYG-GIAARGGIGGIGIAARTC-3′) and nifH-3r (5′- TGGCCGCTASAKIGCCAT-3′) were used for amplification. An amplification product of the expected size (418 bp)

**Fig. 2.** Cell morphology of strain C7T: negative staining (a, b), thin section (c). TS, tubular structures; OM, outer membrane; N, nucleoid; CM, cytoplasmic membrane; F, flagellum; Sh, sheath. Bars, (a, b) 1 μm; (c) 0.5 μm.
was obtained, purified and sequenced. The sequence was compared with \textit{nifH} gene sequences retrieved from the GenBank database for the \textit{Gammaproteobacteria}. The maximum-likelihood tree was constructed using complete deletion, and the MCL model. The nucleotide sequences were aligned using \textit{CLUSTAL}\textsubscript{X} and imported into \textit{MEGA}, and maximum-likelihood phylogeny reconstructed as before. The percentages of replicate trees, where associated taxa are clustered together in the bootstrap test (from 1000 replicates), are shown next to the branches (Felsenstein, 1985). Evolutionary distances were computed using the Jukes–Cantor method (Jukes & Cantor, 1969). The sequence of the \textit{nifH} gene of strain C7\textsuperscript{T} was compared with those of known diazotrophs in the \textit{Gammaproteobacteria}. Analysis of \textit{nifH} genes showed that the sequences of \textit{C. diazotrophica} S-G2-2\textsuperscript{T} and strain C7\textsuperscript{T} clustered together (84.4\% similarity) and were distinctly separated from the \textit{nifH} gene sequences from other described diazotrophs (Fig. S3). The \textit{nifH} gene sequences from the \textit{C. diazotrophica} strains, on the other hand, were reported to be quite similar to each other (96.7–100\%) and formed a tight group (Cramer \textit{et al.}, 2011).

Taken together, all the above data indicate that the diazotrophic strain C7\textsuperscript{T} from a cryopeg represents a novel species of the genus \textit{Celerinatantimonas} which, at the time of writing, includes a single described species, \textit{C. diazotrophica}. The name proposed for this organism is \textit{Celerinatantimonas yamalensis} sp. nov. This conclusion is based on the phylogeny and the moderate 16S rRNA gene sequence similarity (95.5\%) between strains C7\textsuperscript{T} and \textit{C. diazotrophica} S-G2-2\textsuperscript{T} as well as on distinct differences between the strains at the phenotypic level. A key feature of the novel species is the lack of menaquinone in the respiratory chain. The novel species also differs from \textit{C. diazotrophica} S-G2-2\textsuperscript{T} in growth temperature optimum and range, as well as in the ability to hydrolyse gelatin. The strains also showed differences in the range of substrates used as carbon sources (Table 1).

Biological nitrogen fixation is characteristic of specialized groups of prokaryotes possessing the enzyme nitrogenase which comprise both autotrophic and heterotrophic bacteria and archaea, and are widely distributed in various ecosystems (Dixon & Kahn, 2004). Marine heterotrophic nitrogen-fixing bacteria are represented by a taxonomically diverse group and include aerobes, microaerophiles, and facultative and strict anaerobes (Herbert, 1999). Under nitrogen-limited conditions in marine water, microbial nitrogen fixation represents a selective advantage for nitrogen-fixing bacteria (Capone \textit{et al.}, 2008). In this context, the finding of a cold-adapted N\textsubscript{2}-fixing bacterium in aquatic systems of marine origin (cryopegs) seems to be

\begin{table}
\centering
\caption{Differential characteristics between strain C7\textsuperscript{T} and \textit{Celerinatantimonas diazotrophica} S-G2-2\textsuperscript{T}}
\begin{tabular}{|l|l|l|}
\hline
Characteristic & C7\textsuperscript{T} & \textit{Celerinatantimonas diazotrophica} S-G2-2\textsuperscript{T} \\
\hline
Cell size (µm) & 0.7–0.8 × 2–4 & 0.7 × 1.5 \\
\hline
Growth NaCl range (% w/v) (optimum) & 2.0–12.0 (6.0–8.0) & 2.5–8.0 (7.0–7.5)\textsuperscript{*} \\
\hline
Temperature range (°C) (optimum) & 0–34 (18–22) & 17–49 (31)\textsuperscript{*} \\
\hline
pH range (optimum) & 4.0–8.5 (6.0) & 3.5–8.0 (6.0)\textsuperscript{*} \\
\hline
Catalase & – & + \\
\hline
Hydrolysis of gelatin & + & – \\
\hline
Acid production from: & & \\
\hline
\textit{D}-Mannose & – & + \\
\hline
\textit{L}-Arabinose & + & – \\
\hline
Raffinose & + & – \\
\hline
Utilization of carbon sources: & & \\
\hline
Raffinose, pyruvate, malate, xylan & + & – \\
\hline
\textit{D}-Mannose, melibiose, \textit{L}-rhamnose, \textit{myo-}
inositol, lactose, lactulose, \textit{D}-mannitol, 
trehalose, \textit{D}-lactate, \textit{L}-proline, glycogen & – & + \\
\hline
Enzyme activity: & & \\
\hline
\textit{N}-Acetyl-\textit{b}-glucosaminidase, \textit{a}-chymotrypsin, 
\textit{a}-galactosidase, \textit{b}-galactosidase, \textit{b}-glucosidase & – & +\textsuperscript{*} \\
\hline
Major cellular fatty acids (>20 \%) & \textit{C}_{16:0}, \textit{C}_{16:1}\textsubscript{v}/\textsubscript{c} & \textit{C}_{16:0}, \textit{C}_{16:1}\textsubscript{v}/\textsubscript{c} \textit{C}_{16:0}\textsubscript{v}/\textsubscript{c} \textit{C}_{16:1}\textsubscript{v}/\textsubscript{c} \\
\hline
Ubiquinones & \textit{Q}_{8} (98 \%); \textit{Q}_{7} (2 \%) & \textit{Q}_{8} (93 \%); \textit{Q}_{9} (5 \%); \textit{Q}_{7} (2 \%)\textsuperscript{*} \\
\hline
Menaquinones & & \textit{MK}_{8} (100 \%)\textsuperscript{*} \\
\hline
Major polar lipids & PE, PL, PG, APL, G & CL, PG, PE, PMME, APL\textsuperscript{*} \\
\hline
DNA G + C content (mol\%) & 44.7 & 41.8 (41.5\%) \\
\hline
\end{tabular}
\end{table}

\textsuperscript{*}Data from Cramer \textit{et al.} (2011).
important for understanding the processes relevant to the nitrogen cycle in permafrost ecosystems.

**Description of Celerinatantimonas yamalensis sp. nov.**

*Celerinatantimonas yamalensis* (ya.mal.en’sis. N.L. fem. adj. *yamalensis* belonging to the Yamal Peninsula, referring to the region where the type strain was isolated).

Cells are non-spore-forming rods with rounded ends, occurring singly, in pairs or in short chains, and are 0.7–0.8 × 2–4 μm in size. On Bacto Marine Broth, colonies are convex, glistening, non-pigmented, circular with an entire margin and smooth. The growth temperature ranges from 0 to 34 °C, with optimal growth temperature of 18–22 °C. Growth occurs at NaCl concentrations from 2.0 to 12.0 % with optimal NaCl concentration of 6.0–8.0 %, and at pH 5.5–7.5 (optimal 6.0–6.2). Facultative anaerobes, capable of fermentative metabolism. Negative for catalase and cytochrome oxidase. Negative for nitrate reduction and production of H2S and indole. Hydrolyses gelatin and Tween 80, but not agar, starch, chitin or casein. Methyl red test is positive, but Voges–Proskauer reaction is negative. Acid is produced from D-glucose, cellobiose, raffinose, L-arabinose and D-fructose, but not from mannose, maltose or lactose. Yeast extract (0.25–1.0 %) or peptone (0.25–1.0 %) are required for growth. Positive in the following enzyme APY ZYM tests: alkaline phosphatase, acid phosphatase, leucine arylamidase, esterase (C4), esterase lipase (C9) and naphthol-AS-BI-phosphohydrolase. Negative for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, z-galactosidase, β-galactosidase, β-glucuronidase, z-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, L-mannosidase, z-fucosidase, arginine deamidase, lysine carboxylase and ornithine carboxylase. Uses the following substrates as sole carbon source: D-glucose, raffinose, L-arabinose, D-fructose, maltose, lactose, sucrose, cellobiose, dulcitol, sorbitol, glycerol, succinic acid, pyruvate, citrate, D-glucurate, N-acetylglucosamine, Tween 40 and 80, D-glutamic acid and xylan. No growth occurs on glycogen, D-galactose, gentiobiose, lactulose, D-mannitol, D-mannose, melibiose, D-ribose, L-rhamnose, D-xylene, N-acetyl-D-galactosamine, adonitol, L-arabinol, L-sorbose, L-fucose, L-rhamnose, turanose, xylitol, myo-inositol, acetic acid, formic acid, phenylethylamine, 2,3-butanediol, malate, D-lactate, propionic acid, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, D-serine, L-serine, L-threonine, L-proline, L-tyrosine, L-valine, L-cysteine, L-lysine, methanol, butanol, ethanol, butyrate, caproate, heptanoate or fumarate. Ferments D-glucose, cellobiose, D-glucurate and N-acetylglucosamine, but not sucinate, fumarate, D-lactate, pyruvate or Tween 80. Predominant fatty acids are C16:0, C16:1ω7c, C18:1ω9t7, C17 cyclo and summed feature 2 [comprising C12:0 aldehyde and/or unknown fatty acid 10.913 (MIDI designation) and/or iso-C16:1ω3-C14:0 3-OH]. Major ubiquinone is Q-8.

The type strain, **C7** (=DSM 21888 = VKM B-2511), was isolated from a permafrost water brine on the Yamal Peninsula, northern Siberia, Russian Federation. The DNA G+C content of the type strain is 44.7 mol%.

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