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Isolation of facultatively anaerobic soil bacteria from Ny-Ålesund, Svalbard

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Abstract Anaerobic conditions in soil commonly occur even in upland environments. Physiological and biogeochemical properties of individual anaerobic bacteria, however, have been poorly understood due to difficulties in culture. This study aimed to isolate anaerobic bacteria in the Arctic tundra soil and to identify their physiological characteristics. Anaerobic culture and 16S rRNA gene sequence-based phylogenetic analysis showed that total 33 bacterial strains were affiliated with 15 species from the following 8 genera: *Bacillus*, *Carnobacterium*, *Clostridium, Paenibacillus*, and *Trichococcus* (Firmicutes), *Pseudomonas* and *Rahnella* (Gamma-proteobacteria), and *Cellulomonas* (Actinobacteria). All isolates were identified as facultatively anaerobic bacteria; this finding might be partially attributed to the characteristics of sampling sites, which temporarily developed anaerobic conditions because of the presence of stagnant melting snow. Six of the 33 bacterial strains were revived subsequently from glycerol stocks held −80 °C, and these were used for the physiological study: four isolates from Firmicutes, one isolate from Gamma-proteobacteria, and one isolate from Actinobacteria. Five isolates except KOPRI 80146 (*Bacillus* sp.) could grow at either 4 or 10 °C within a week. All six isolates showed cellulase or protease activities at 10 or 15 °C. Endospores were observed from four isolates belonging to Firmicutes. These physiological characteristics may contribute to the survival of these organisms at low temperatures and to their involvement in biogeochemical cycles in the tundra soil. These isolates may be used for further detailed studies for identifying their cold adaptation mechanisms and ecological roles in the Arctic.

Keywords Arctic tundra soil · Facultatively anaerobic bacteria · Actinobacteria · Firmicutes · Proteobacteria · Endospore

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Introduction

Soil environments become anaerobic when oxygen is exhausted, and anaerobic conditions frequently occur in soil such as water-saturated soil, heavy textured soil, or inside of aggregates (Tiedje et al. 1984; Cresser et al. 1993; Inglett et al. 2005). Oxygen can be depleted when plant roots are actively growing, or microorganisms are vigorously decomposing labile organic substrates (Brady and Weil 1998). Some Arctic tundra soils could temporarily become anaerobic resulting from increased temperature and melted snow in summer time (Sebacher et al. 1986). In these anaerobic soils, various anaerobic bacteria play key roles in several biogeochemical processes such as methane production or sulfate reduction (Tiedje et al. 1984).

In tundra soil, a considerable number of microorganisms have been detected despite low temperature. From Siberian and Spitsbergen tundra soil, about 10^9 microbial cells per gram of soil were detected (Kobabe et al. 2004; Hansen

et al. 2007). This number is comparable to the numerical number of microorganisms found in tropical and temperate soils, 10^8–10^9 cells per gram (Berg et al. 1998; Hengstmann et al. 1999; Sait et al. 2002; Khammar et al. 2009).

There have been many studies to determine microbial composition in polar regions through culture-dependent methods. For example, bacteria have been isolated from the Arctic tundra soils in Siberia, Svalbard, and the Canadian high Arctic, and the majority of the isolates belonged to the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Vishnivetskaya et al. 2006; Hansen et al. 2007; Steven et al. 2007). These bacterial isolates were cultured under the aerobic condition.

Information regarding anaerobic bacteria in tundra soils, however, remains poorly understood (Steven et al. 2009). Hansen et al. (2007) cultured bacteria from Spitsbergen tundra soil in the anaerobic condition and investigated the community composition through denaturant gradient gel electrophoresis (DGGE). They detected Actinobacteria, Bacteroidetes, Delta-proteobacteria, and Firmicutes; however, anaerobic bacteria were not isolated. Although there were some cases of isolated anaerobic bacteria such as *Methylosinus* and *Methylocystis* species by using methane enrichment culture from Canadian tundra soil (Pacheco-Oliver et al. 2002), the isolation of anaerobic bacteria is still very limited from the Arctic tundra soil.

Isolated bacteria may provide opportunities to study survival mechanisms or ecological roles in cold environments. For example, *Exiguobacterium sibiricum* 255-15 isolated from Arctic soil (Vishnivetskaya et al. 2000) showed the expression of specific proteins at near or below freezing temperature, which could be closely linked to the survival of *E. sibiricum* 255-15 at low temperature (Qiu et al. 2006). In addition, *E. sibiricum* 255-15 genes associated with carbohydrate metabolism, amino acid biosynthesis, cell wall structure, DNA replication, etc., were differentially expressed at low temperature (Rodrigues et al. 2008). These results showed how isolated bacteria could help us understand their survival mechanisms and ecological roles in cold environments through genome, transcriptome, and proteome analyses.

Thus, we aimed to isolate and identify anaerobic bacteria from the tundra soil in this study. Furthermore, we tried to reveal their physiological characteristics by investigating temperature ranges for growth and by measuring selected enzyme activities.

Materials and methods

Study area and sample collection

The study was conducted near the Korean Arctic Research Station, Dasan, located in Ny-Ålesund, Svalbard, Norway (78°55′N, 11°56′E), in June 2009. The annual mean air temperature and precipitation of the sampling sites from 2000 to 2008 were −4.2 °C and 436 mm, respectively (retrieved on December 29, 2011, from http://retro.met.no). Generally, the ground is snow-covered between the end of September and the beginning of June, and the growing season is from June to August in Ny-Ålesund. All sampling sites were under stagnant melting snow and were composed of various soils (Table 1).

Soils were collected using a stainless core sampler, and 5 g subsamples were inoculated immediately into serum vials with 50 mL of EM1 culture medium (Balch et al. 1979; Mineral 1 (K_2HPO_4, 6 g per 1 L distilled water), 50 mL; Mineral 2 (KH_2PO_4, 6 g; $(NH_4)_2SO_4$, 6 g; NaCl, 12 g; $MgSO_4{\cdot}7H_2O$, 2.6 g; $CaCl_2{\cdot}2H_2O$, 0.16 g per 1 L distilled water), 50 mL; trace minerals (nitrilotriacetic acid, 1.5 g; $MgSO_4{\cdot}7H_2O$, 3.0 g; $MnSO_4{\cdot}2H_2O$, 0.5 g; NaCl, 1.0 g; $FeSO_4{\cdot}7H_2O$, 0.1 g; $CoSO_4$, 0.1 g; $CaCl_2{\cdot}2H_2O$, 0.1 g; $CuSO_4{\cdot}5H_2O$, 0.01 g; $AlK(SO_4)_2$, 0.01 g; H_3BO_3, 0.01 g; $Na_2MoO_4{\cdot}2H_2O$, 0.01 g per 1 L distilled water (pH to 7.0 with KOH), dissolve nitrilotriacetic acid with KOH to pH 6.5; then proceed to add minerals), 10 mL; trace

Table 1 Site description and soil sampling depth for each site

No. of site-core	Depth (cm)	Site description
1–1	1.5–4	Surface covered with 1.5 cm depth of moss layer. Below that, soil with black color and small pebbles were present. Sampling point is right next to snow melting area
2–1	2–4	Moss cover, water ponding
3–1	7–8	Lichen and organic crust cover
3–2	14–15	
3–3	18–19	
4–1	2–5	Moss cover, water ponding from snow melting
5–1	3–5	Wet soil
6–1	1.5–4	Moss cover, soil with brown color under snow melting water
7–1	1.5–3	Wet soil with brown color
8–1	1.5–4	Under snow melting water, sandy soil with light brown color
9–1	4–7	Sandy soil with light brown color
10–1	1–4	Organic crust and moss cover, dense clay soil with light brown color, small pebbles
10–2	4–7	
11–1	1–3	Organic crust and moss cover, dense clay soil with small pebbles, soil with mixed colors of light brown and black
11–2	3–6	
12–1	2–3	Near to a lake, surface 10 cm was a moss layer, soil with black color
13–1	1–4	Thick moss layer, sandy soil with gray color
13–2	4–8	
14–1	1–3	Thick moss layer, sandy soil with gray color

vitamins (biotin, 2 mg; folic acid, 2 mg; pyridoxine hydrochloride, 10 mg; thiamine hydrochloride, 5 mg; riboflavin, 5 mg; nicotinic acid, 5 mg; DL-calcium pantothenate, 5 mg; vitamin B_{12}, 0.1 mg; *p*-aminobenzoic acid, 5 mg; lipoic acid, 5 mg per 1 L distilled water), 10 mL; $FeSO_4 \cdot 7H_2O$, 0.002 g; $NaHCO_3$, 5.0 g; sodium acetate, 2.5 g; sodium formate, 2.5 g; yeast extract, 2.0 g; Trypticase, 2.0 g; L-cysteine hydrochloride·H_2O, 0.5 g; $Na_2S \cdot 9H_2O$, 0.5 g per 1 L distilled water. The air in the serum vials containing soil samples was exchanged with mixed gas (20 % CO_2: 80 % N_2), and the vials were stored at 4 °C for 2 months until further processing.

Culture and isolation of anaerobic bacteria

Soil samples in EM1 medium were vortex-mixed to facilitate separation of bacterial cells from soil particles and were left unattended for several hours at 4 °C until the particles sank to the bottom of the vials. The supernatants were diluted 100-fold with the identical medium, and 50 μL of the diluted subsamples were spread on Trypticase Soy Agar (TSA; Difco) medium and incubated anaerobically at 15 °C. Samples were handled inside the anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA), and anaerobic jars (Mitsubishi Gas Chemical Co., Japan) were used to maintain the anaerobic condition. After 10 days, colonies were selected based on differences in morphological characteristics. Each colony was subcultured more than 4 times to obtain a pure strain.

PCR amplification

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony using the boiling method (Englen and Kelley 2000). PCR was performed by using the crude extracts as the DNA templates, *Taq* DNA polymerase, and primers 27F (5′-AGAGTTTGATCMTGGCTC AG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991). PCR conditions for the amplification of bacterial 16S rRNA gene were maintained as described by Lane (1991). All PCR products were analyzed and checked by electrophoresis, and the PCR-amplified products were purified with exonuclease I (*Exo*I) and shrimp alkaline phosphatase (SAP) enzymes to eliminate nonincorporated dNTPs and primers.

Phylogenetic analysis

A nearly complete 16S rRNA gene sequence for each strain was obtained and compared with 16S rRNA gene sequences available in GenBank using the BLAST algorithm (Altschul et al. 1990) and EzTaxon server (Chun et al. 2007). The closest type strains were selected using EzTaxon database (Chun et al. 2007) to assign accurate taxonomic affiliations. For all sequences, chimeras were checked by using Chimera Slayer (Haas et al. 2011). The sequences of each strain were aligned with those of related taxa using *j*PHYDIT program (Jeon et al. 2005). Accurate multiple alignments were performed manually according to the 16S rRNA gene secondary structure information implemented in *j*PHYDIT program.

Phylogenetic trees were obtained by use of the neighbor-joining (Saitou and Nei 1987) and the maximum-parsimony (Fitch 1971) methods in MEGA 4 program (Tamura et al. 2007). An evolutionary distance matrix for the neighbor-joining method was generated according to the model of Kimura (1980). The robustness of tree topologies was assessed by bootstrap analyses based on 1,000 replications for the neighbor-joining and maximum-parsimony methods. The isolates that showed the same 16S rRNA gene sequence similarities from the same site or depth were regarded as identical strains.

Physiological characterization

Experiments to identify physiological characteristics were performed with six isolates revived from glycerol stocks at −80 °C. The six isolates were KOPRI 80146 (*Bacillus circulans*), KOPRI 80142 (*Carnobacterium viridans*), KOPRI 80191 (*Cellulomonas cellasea*), KOPRI 80157 (*Paenibacillus borealis*), KOPRI 80163 (*P. donghaensis*), and KOPRI 80167 (*P. macquariensis* subsp. *macquariensis*).

To examine the temperature range for growth, the six isolates were cultured at various temperatures (4, 10, 15, 20, 25, 30, and 37 °C) for a week. Among the tested temperatures, 4 °C was chosen as the lowest temperature based on the temperature range for growth of the closest species of the isolates (Table 2) and the lowest air temperature (about 3 °C) during summer at the sampling site. The growth of isolates was assessed based on colony formation on TSA.

The enzyme activities of the isolates were examined on agar plates under the aerobic condition. Substrates were supplemented to TSA medium; 2 % soluble starch (Sigma) for amylase, 1 % carboxymethylcellulose (Sigma) for cellulase, 1 % skim milk (Difco) for protease, 0.4 % colloidal chitin for chitinase, and 1 % tributyrate (Sigma) for lipase. The agar media were incubated for a week at the same temperatures as the growth test. The expressed enzymes activities were determined as clearing zones on agar plates within a week.

Endospores of the isolates were observed by using a staining method (Cappuccino and Sherman 1999). The cells preserved in the glycerol stock at −80 °C were suspended in Trypticase Soy Broth (TSB; Difco), exposed at 80 °C for 15 min using heating block, and spread on TSA medium.

Table 2 The type strains of the species closest to the facultatively anaerobic bacteria isolated in this study. This table showed the original sampling site, aerobicity, and temperature ranges for growth of the type strains

Strain no. (accession no.)[a]	No. of site-core	Type strains of the closest species[b]					
		Taxon (accession no.)[a]	Similarity (%)	Original isolation source	Anaerobic growth	Temperature range for growth (°C)	Optimal growth temperature (°C)
KOPRI 80146 (JN873180)	4–1	*Bacillus circulans* ATCC 4513^T (AY724690)	99.9	Sewage	F	20–42	30[c]
KOPRI 80142 (JN873180)	1–1	*Carnobacterium viridans* MPL-11^T (AF425608)	99.9	Refrigerated vacuum-packaged food	F	2–30	30[c]
KOPRI 80153 (JN873180)	7–1	″	99.9				
KOPRI 80155 (JN873180)	7–1	″	99.9				
KOPRI 80191 (JN873180)	12–1	*Cellulomonas cellasea* DSM 20118^T (X83804)	96.9	Soil	ND	ND	30[c]
KOPRI 80192 (JN873180)	13–2	″	97.6				
KOPRI 80182 (JN873180)	12–1	*C. amygdalinum* BR-10^T (AY353957)	99.3	Sludge bed-reactor treating potato starch waste water	An	20–60	45
KOPRI 80151 (JN873180)	6–1	*Clostridium gasigenes* DSM 12272^T (AF092548)	100	Incidences of 'blown-pack' spoilage of vacuum-packed chilled lamb	An	−1.5 to 26	20–22
KOPRI 80152 (JN873180)	6–1	″	100				
KOPRI 80157 (JN873180)	3–1	*Paenibacillus borealis* KK19^T (AJ011322)	99.2	Spruce forest humus in Finland	F	5–37	28
KOPRI 80145 (JN873180)	3–1	*P. donghaensis* JH8^T (EF079062)	97.9	Deep sea sediment of East Sea, Korea	F	4–30	20–25
KOPRI 80147 (JN873180)	3–2	″	97.9				
KOPRI 80156 (JN873180)	3–3	″	97.8				
KOPRI 80158 (JN873180)	4–1	″	97.9				
KOPRI 80163 (JN873180)	8–1	″	98.0				
KOPRI 80144 (JN873180)	2–1	*P. macquariensis* subsp. *macquariensis* NCTC 10419^T (X60625)	97.9	Macquarie Island	F	0–25	15–20
KOPRI 80167 (JN873180)	10–2	″	98.9				
KOPRI 80169 (JN873180)	10–1	″	97.8				
KOPRI 80172 (JN873180)	11–2	″	97.8				
KOPRI 80188 (JN873180)	6–1	*Pseudomonas extremaustralis* CT14-3^T (AJ583501)	98.0	Temporary pond in Antarctica	F	4–37	30
KOPRI 80189 (JN873180)	11–1	″	99.8				
KOPRI 80149 (JN873180)	5–1	*P. frederiksbergensis* JAJ28^T (AJ249382)	98.8	Coal gasification site in Frederiksberg, Denmark	Ae	4–30	30
KOPRI 80154 (JN873180)	7–1	*P. veronii* CIP 104663^T (AF064460)	99.9	Mineral water, France	Ae	4–36	28
KOPRI 80171 (JN873180)	10–1	*Rahnella aquatilis* DSM 4594^T (AJ233426)	99.3	Fresh water	F	4–37	30[c]

Table 2 continued

Strain no. (accession no.)[a]	No. of site-core	Type strains of the closest species[b]					
		Taxon (accession no.)[a]	Similarity (%)	Original isolation source	Anaerobic growth	Temperature range for growth (°C)	Optimal growth temperature (°C)
KOPRI 80173 (JN873180)	11–2	*Trichococcus palustris* DSM 9172^T (AJ296179)	99.0	Swamp, Russia	F	0–33	30[c]
KOPRI 80175 (JN873180)	11–1	″	99.0				
KOPRI 80179 (JN873180)	12–1	″	99.0				
KOPRI 80183 (JN873180)	13–2	″	99.0				
KOPRI 80185 (JN873180)	13–1	″	99.0				
KOPRI 80187 (JN873180)	14–1	″	99.0				
KOPRI 80176 (JN873180)	11–1	*T. pasteurii* KoTa2^T (X87150)	99.9	Septic pit, Germany	F	0–42	25–30
KOPRI 80159 (JN873180)	3–2	*T. patagoniensis* PMagG1^T (AF394926)	99.9	Guano of Magellanic penguins in Chilean Patagonia	F	–5 to 35	28–30
KOPRI 80165 (JN873180)	9–1	″	99.9				

ND No data available, *Ae* strictly aerobic, *F* facultatively anaerobic, *An* strictly anaerobic

[a] GenBank accession numbers

[b] Data from Jordan (1890), Marshall and Ohye (1966), Zhilina et al. (1995), Elomari et al. (1996), Kalbe et al. (1996), Andersen et al. (2000), Broda et al. (2000), Elo et al. (2001), Holley et al. (2002), Parshina et al. (2003), Pikuta et al. (2006), Choi et al. (2008), López et al. (2009)

[c] Data from DSMZ (retrieved from http://www.dsmz.de/)

Then, the medium was anaerobically incubated at 10 °C. After 3 weeks, harvested colonies were resuspended in distilled water and smeared on microscopic slide glass and heat-stained with malachite green (5 % wt/vol in distilled water), washed with distilled water, and counterstained with safranin-O (2.5 % wt/vol in ethanol). The samples were subsequently washed with distilled water, and cells were observed with the Olympus BX51 microscope.

Endospores were also observed by transmission electron microscopy (TEM). Cells were grown in hungate tubes anaerobically within a week at 15 °C. Specimens for the endospore observation were prepared by the methods described in Lee et al. (2008) with some modification of centrifugation force (5,000×*g*) and time (5 min). The prepared samples were observed by transmission electron microscope (JEM1010, JEOL, Japan) operating at 80 kV at National Instrumentation Center for Environmental Management (NICEM) in Seoul National University.

Accession numbers for nucleotide sequences

All 16S rRNA gene sequences from the isolates were submitted to GenBank under accession numbers JN873148 to JN873180.

Results

Identification of the isolates and phylogenetic analysis of 16S rRNA gene sequences

A total of 33 bacteria were isolated through anaerobic culture from the Arctic tundra soil in this study (Table 2). The taxonomic affiliation based on similarity of 16S rRNA gene sequences indicated that the isolates were identified as 15 species from the following eight genera: *Bacillus*, *Carnobacterium*, *Clostridium, Paenibacillus*, and *Trichococcus* (Firmicutes), *Pseudomonas* and *Rahnella* (Gammaproteobacteria), and *Cellulomonas* (Actinobacteria) (Table 2; Fig. 1).

Phylogenetic analyses showed that some isolates might be new species. One of the isolates, KOPRI 80191, showed 96.9 % sequence similarity with the closest species *C. cellasea* DSM 20118^T and formed an independent branch from *C. cellasea* (Fig. 1). Two *Pseudomonas* strains, KOPRI 80149 and KOPRI 80188, may be new species candidates. Although they showed more than 98 % sequence similarity with their closest species (98.0 and 98.8 %), phylogenetic analyses indicated that the isolates KOPRI 80149 and KOPRI 80188 formed different

branches from their closest strains *Pseudomonas frederiksbergensis* JAJ28^T and *P. extremaustralis* CT14-3^T, respectively. Moreover, the bootstrap value supported that the KOPRI 80149 could be an independent branch (Fig. 1). The two potential new species candidates were unrepresented currently in GenBank/EMBL/DDBJ and EzTaxon server (retrieved on November 2, 2012, from http://www.ncbi.nlm.nih.gov/genbank/). However, more sensitive tools for genetic recognition of the isolates should be applied to identify these isolates as new species.

All isolates in this study were identified as facultatively anaerobic bacteria. We observed growth of the isolates under the aerobic conditions as well as anaerobic conditions. The isolates showed faster growth in the aerobic than anaerobic condition (data not shown). Ten type strains of the closest species for these isolates were also facultatively anaerobic (Table 2).

Physiological characterization of the six representative bacterial isolates

The isolates could grow at low temperature. *Carnobacterium viridans* KOPRI 80142 and *C. cellasea* KOPRI 80191 formed colonies at 4 °C within a day. *Paenibacillus borealis* KOPRI 80157, *P. donghaensis* KOPRI 80163, and *P. macquariensis* subsp. *macquariensis* KOPRI 80167 could grow at 10 °C (Table 3). Five isolates could not grow at 37 °C except *B. circulans* KOPRI 80146 that did not show any colony at 4 and 10 °C within a week.

In enzyme assay for the six selected isolates, all isolates showed cellulase or protease activities (Table 3). *Bacillus circulans* KOPRI 80146, *C. cellasea* KOPRI 80191, *Paenibacillus donghaensis* KOPRI 80163, and *P. macquariensis* subsp. *macquariensis* KOPRI 80167 degraded both cellulose and protein (Table 3). In particular, *Paenibacillus macquariensis* subsp. *macquariensis* KOPRI 80167 showed relatively very high enzyme activity on cellulose degradation compared to other isolates, forming a clear zone 2–3 times wider than the colony size of the isolate at 20–30 °C. *Cellulomonas cellasea* KOPRI 80191 showed the highest protease activities than the other isolates, and the clear zone was two times wider than the other isolates' clear zone size at 20–30 °C. *Carnobacterium viridans* KOPRI 80142 showed stronger protease activity than *C. cellasea* KOPRI 80191 at 10–20 °C. Although no isolates showed enzyme activities at 4 °C in this experiment, the isolates could degrade cellulose and/or protein substrates at 10 °C within a week (Table 3). The six isolates did not show amylase, chitinase, and lipase activities (data not shown).

Among the six isolates, four strains belonging to Firmicutes formed endospores (Fig. 2) of which morphology were central and/or terminal: *B. circulans* KOPRI 80146, *P. borealis* KOPRI 80157, *P. donghaensis* KOPRI 80163, and *P. macquariensis* subsp. *macquariensis* KOPRI 80167.

Discussion

Facultatively anaerobic bacteria of 15 species were isolated from Arctic tundra soil. The anaerobic bacteria belonged to the phyla Firmicutes, Gamma-proteobacteria, or Actinobacteria (Fig. 2). These phyla also have been mainly cultured in tundra soils under the aerobic condition as well (Vishnivetskaya et al. 2006; Hansen et al. 2007; Steven et al. 2007) and have been identified as the major groups of bacteria in permafrost through culture-independent methods (Yergeau et al. 2010; Campbell et al. 2010).

Among 15 isolated species, eight have been reported from polar environments. Six species have been reported from tundra soils: *B. circulans, C. cellasea*, *P. borealis*, *P. donghaensis*, *P. frederiksbergensis,* and *P. veronii* (Lee et al. 2004; Hansen et al. 2007; Steven et al. 2008). Two species were isolated from Antarctic areas: *P. macquariensis* subsp. *macquariensis* from sub-Antarctic soil, and *Pseudomonas extremaustralis* from an Antarctic pond (Marshall and Ohye 1966; López et al. 2009). However, the other seven species (*C. viridans*, *Clostridium amygdalinum*, *C. gasigenes*, *Rahnella aquatilis*, *Trichococcus palustris*, *T. pasteurii,* and *T. patagoniensis*) have not been isolated from tundra soils within our literature review.

All isolates in this study were identified as facultatively anaerobic bacteria. Among the 15 type strains of the closest species, ten strains are also facultatively anaerobic bacteria (Table 2). There could be several reasons why we could not isolate strictly anaerobic bacteria. First, some sampling sites might be temporarily exposed to the aerobic condition. All sampling sites were under stagnant melting snow at the time of sampling, but upper soil could become aerobic due to soil water draining and evapotranspiration later. It was observed that the water table fell below 50 cm during summer season at a point which was close to the sampling area around the Korean Arctic Research Station. Sampling in a deep profile would be necessary to isolate strictly anaerobic bacteria. Second, repeated sub-culture to obtain pure strains might disturb consortia of strictly anaerobic bacteria. Previous reports showed that anaerobic bacteria formed consortia which were essential for survival (Kato and Watanabe 2010).

Among 15 isolated species, some isolates may have a syntrophic relationship with methanogens. The closest species, *C. amygdalinum* BR-10^T and *C. gasigenes* DSM 12272^T, of the isolates KOPRI 80151, KOPRI 80152, and KOPRI 80182 have been reported to produce hydrogen

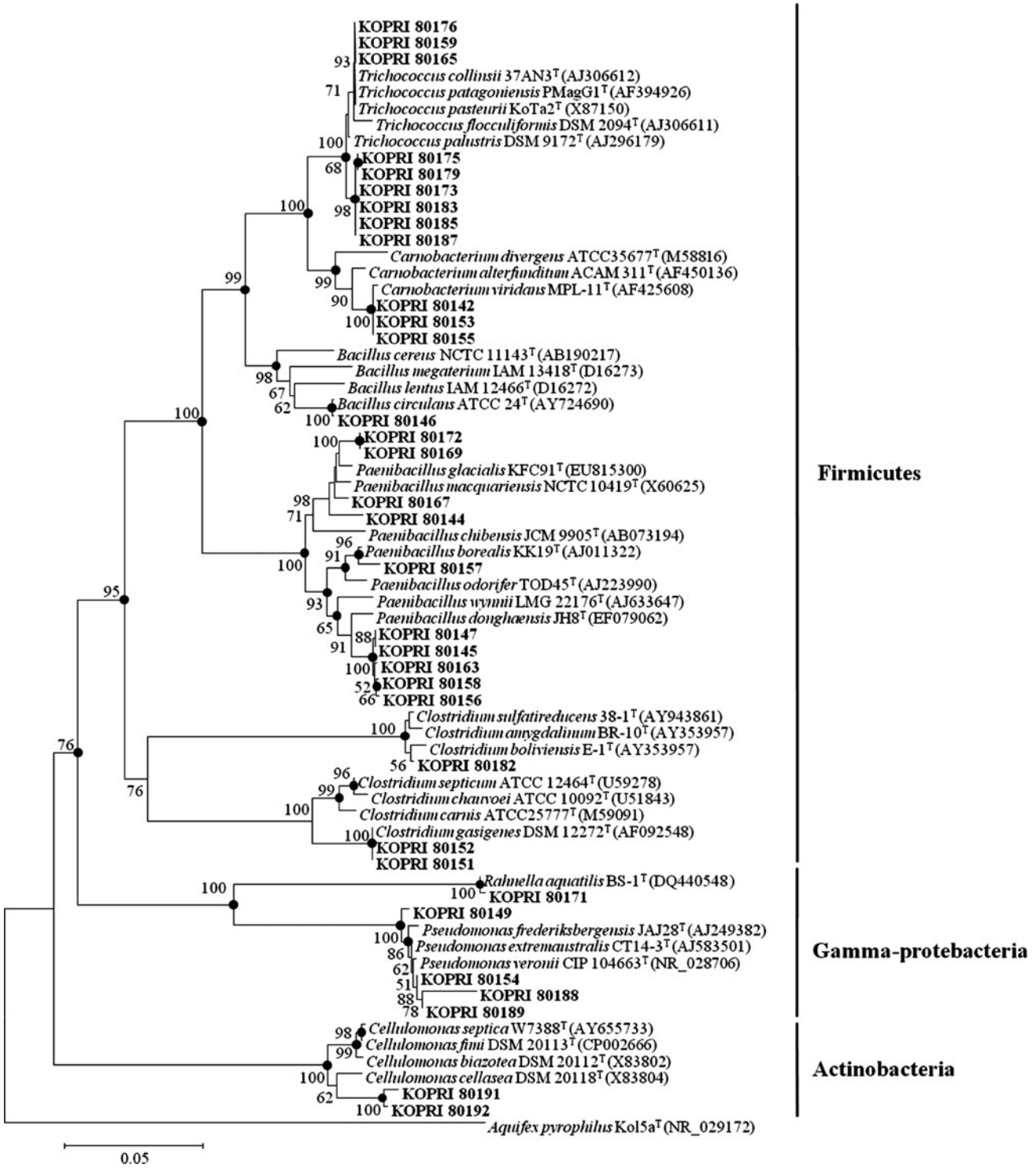

Fig. 1 Neighbor-joining tree showing the phylogenetic positions between the isolates and their closest taxa on the basis of 16S rRNA gene sequences. Bootstrap values ≥50 % (1,000 replicates) are indicated at the nodes. *Solid circles* indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm (not shown). The tree was rooted by using *Aquifex pyrophilus* Kol5a^T (NR_029172) as an outgroup. *Bar*, 0.05 substitutions per nucleotide position

during the fermentation of organic substrates (Broda et al. 2000; Jayasinghearachchi et al. 2010), and hydrogen can be used as a substrate for methanogenesis by methanogen.

Four isolates produced endospores (Fig. 2), and this endospore-forming ability under the extreme environment might be one of the ways to survive at low temperature.

Paenibacillus borealis, *P. donghaensis*, and *P. macquariensis* subsp. *macquariensis* which are the closest species to the isolates were reported to grow at low temperatures (−1.5 to +5 °C) and to form endospores (Marshall and Ohye 1966; Broda et al. 2000; Elo et al. 2001; Choi et al. 2008). It was also reported that *Bacillus* formed endospores (Nicholson 2002). In the case of *P. extremaustralis*, it did not form endospores but could grow at low temperature with a help of biopolymer polyhydroxybutyrate (PHB) which resists oxidative stress following cold (Tribelli and López 2011). *P. frederiksbergensis* could produce alkanes-degrading enzyme, alkane hydrolase, at low temperature; thus, this would contribute to psychrophilic characteristics (Abdel-Megeed 2011). These mechanisms of biopolymer or enzyme production by the isolates should be further investigated.

Table 3 Temperature ranges for growth and for cellulase and protease activities of the isolates after aerobic culture during a week

Strain no.	Temperature range (°C)		
	Growth	Cellulase activity	Protease activity
B. circulans KOPRI 80146	15–37	25–37	15–37
C. viridans KOPRI 80142	4–30	–	10–30
C. cellasea KOPRI 80191	4–30	10–30	15–30
P. borealis KOPRI 80157	10–30	15–30	–
P. donghaensis KOPRI 80163	10–30	15–30	25–30
P. macquariensis subsp. *macquariensis* KOPRI 80167	10–30	10–30	15–25

None of the isolates showed the activities of amylase, chitinase, and lipase

The isolates showed enzyme activity at low temperature. *Carnobacterium viridians* KOPRI 80142, *C. cellasea* KOPRI 80191, and *P. macquariensis* subsp. *macquariensis* KOPRI 80167 could degrade protein or cellulose at 10 °C. *C. cellasea* could degrade cellulose (Yoon et al. 2008), and *P. borealis* and *P. donghaensis* could degrade xylan and pectin, respectively (Elo et al. 2001; Choi et al. 2008). Arctic tundra soil is characterized as a large accumulation of macromolecules due to slow decomposition rates (Dai et al. 2002). Most of the sampling sites in the present study were also covered with moss and/or lichen, and some sites exhibited a thick moss layer (Table 1). Therefore, the isolates may contribute to biogeochemical cycling through organic matter mineralization at low temperature.

In conclusion, we identified culturable facultatively anaerobic bacteria in the Arctic tundra soil and checked the abilities of growth and enzyme activities at low temperature. Endospores observed in *Bacillus* and *Paenibacillus* might be one of the survival mechanisms in cold environments. Active enzyme activities at low temperature could play a role of organic matter mineralization in the Arctic tundra soil. Using these cultured bacteria, further

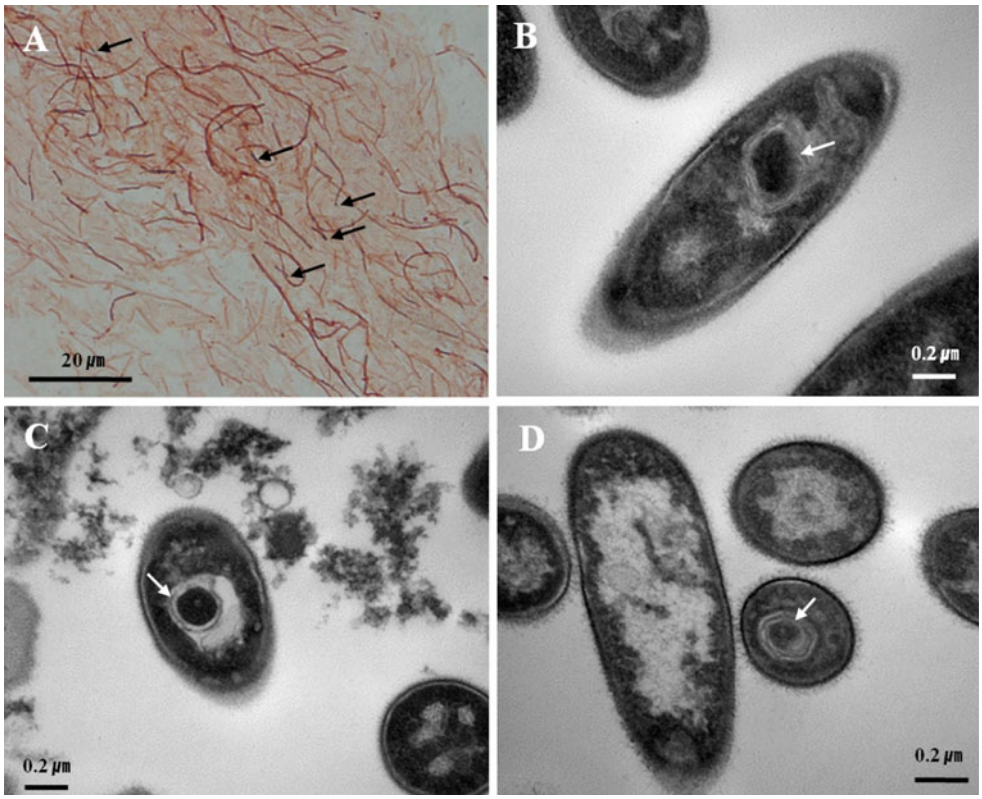

Fig. 2 The images of endospores of the isolates by microscopy after staining (**a**) and by transmission electron microscopy (TEM) (**b**, **c** and **d**). **a** *Bacillus circulans* KOPRI 80146. Spores (*black arrows*) are green and bacterial cell bodies are pink, **b** *Paenibacillus borealis* KOPRI 80157, **c** *P. donghaensis* KOPRI 80163, **d** *P. macquariensis* subsp. *macquariensis* KOPRI 80167. The *black arrow* in **b**, **c**, and **d** indicates endospore

detailed studies on physiological and biochemical characteristics would be possible in the near future, so that we can understand their adaptation and survival mechanisms as well as ecological roles in the Arctic.

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