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Article

Phylogenetic Analysis of Culturable Arctic Bacteria

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Abstract : We isolated and identified culturable Arctic bacteria that had inhabited soils around the Korean Arctic Research Station Dasan located at Ny-Alsund, Svalbard, Norway (79°N, 12°E). The collected soils were diluted in distilled water; the diluted soil-water was spread on 3 M petri-films at Dasan Station. The petri-films were transported to the laboratory at KORDI, and cultured at 4°C. Colonies grown on the petri-films were subsequently cultured on nutrient agar plates at 4°C every 7 days. The pure colonies were inoculated into nutrient liquid media, genomic DNA was extracted, and phylogenetic analysis was performed on the basis of 16S rDNA sequences. A total of 227 strains of bacteria were isolated. Among them, 16S rDNA sequences of 185 strains were identical with those of known strains isolated in this study, and 42 strains were finally identified. Phylogenetic analysis using 16S rDNA indicated that the 30 strains belonged to *Pseudomonas*, 7 strains to *Arthrobacter*, two strains to *Flavobacterium*, and the remaining to *Achromobacter*, *Pedobacter*, and *Psychrobacter*. Among the 42 strains, 14 bacteria produced protease: they were 6 strains of *Pseudomonas*, 4 strains of *Arthrobacter*, an *Achromobacter* strain, 2 strains of *Flavobacterium*, and a *Pedobacter* strain. We expect these Arctic bacteria can be used for screening to develop new industrial enzymes that are active at low temperatures.

Key words : Arctic bacteria, *Pseudomonas*, *Arthrobacter*, *Flavobacterium*, *Achromobacter*, *Pedobacter*, *Psychrobacter*

1. Introduction

Cold habitats offer good sources of useful genes and novel natural products with activity at low temperatures (Cowan 1997). At low temperatures, the rate of enzymatic reactions, the fluidity of cellular membranes, and the affinity of uptake and transport systems decrease (Phadtare *et al.* 2000). Therefore, biomolecules of organisms living in cold habitats may show distinctive physical properties.

The Arctic is a representative cold habitat, which remains one of the least explored, studied and understood places on earth. The studies on Arctic bacterial diversity have been restricted in the marine environment (Knoblauch *et al.* 1999; Sahm *et al.* 1999; Ravenschlag *et al.* 2001a, 2001b). The study on the bacterial diversity of the Arctic region provides potential benefits by storing the new gene pool. Understanding bacterial diversity also offers new insights into the biological mechanisms of adaptation to and tolerance of cold environments.

Psychrophilic and psychrotolerant bacteria are defined by optimal growth at temperatures below 20°C and growth at temperatures as low as 0°C; psychrotolerant bacteria are distinguished by growth at temperatures above 20°C (Morita 1975). Enzymes produced by psychrophilic and psychrotolerant bacteria are remarkably stable after long-term storage and occasional freeze-thaw cycles (Irwin *et al.* 2001). Therefore, psychrophilic and psychrotolerant bacteria have focused on isolation of cold-active enzymes (Davail *et al.* 1994; Huston *et al.* 2000; Secades *et al.* 2001; Nakagawa *et al.* 2003). For example, cold-active serine alkaline protease was isolated from a psychrophilic *Pseudomonas* strain (Zeng *et al.* 2003). Protease is an

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important enzyme of the medical, environmental, food, and chemical industries, etc. (Storer 1991; James & Simpson 1996; Vermeij & Blok 1996).

In this study, we isolated, cultured and identified Arctic terrestrial bacteria that inhabit the area around the Korean Arctic Research Station Dasan located at Ny-Alsund, Svalbard, Norway. We also screened protease-producing Arctic bacteria, which are candidates for a source of cold-active protease.

2. Materials and methods

Sample collection

The sampling site is in the near-by area of Korean Arctic Research Station Dasan located at the Norwegian Polar Institute's Research Station in Ny-Alesund (78°55'N, 11°56'E), Svalbard, Norway (Kang *et al.* 2003). Soil samples were collected from the upper melted layer of soil with a 0.1 m depth using sterile 50 m*l* conical tubes on 5th-15th, August 2002. The samples collected from 6 different sites were sealed and transferred to Dasan station. Aliquote of 0.2 g of the collected soils were diluted in distilled water, the diluted soil-water was spread on the 3M petri-films of *E. coli* Count Plate, which were kept at 4°C for 1-7 days until transportation. The petri-films were transported to the laboratory at KORDI under cold conditions. The remaining soil samples were frozen at −20°C in conical tubes, transported in packages with dry ice and icepacks, and stored at −20°C.

Culture conditions

The petri-films were incubated at 4°C for 1 month; colonies formed on petri-films were subsequently cultured on nutrient agar plates (Difco 72063JD) at 4°C every 7 days. Colonies of distinct types on plates were streaked over and over again on fresh nutrient agar plates. The purified isolates were cultured in nutrient broth media at 25°C for 1 day, and stored at −80°C in a fresh medium that contained 15% (v/v) sterile glycerol.

DNA extraction and PCR amplification

Total genomic DNA was extracted using an AccuPrep genomic DNA Extraction kit (Bioneer, Korea) from 1 m*l* of isolates cultured in nutrient broth. From the genomic DNA nearly full-length 16S rDNA sequences were amplified by PCR using primers 27F(5'-AGA GTT TGA TCM TGG CTC AG-3') and 1522R(5'-AAG GAG GTT ATC CAN CCR CA-3'). The PCR mixture consisted of 5 μl of 10× PCR mix (final concentrations: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl pH 9.0), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μl of each primer, 1 μl of template DNA, and 2.5 units of *Taq* polymerase (TaKaRa, Japan) in a final volume of 50 μl. The PCR was performed in a thermal cycler (Biometra, Germany) using cycling conditions that consisted of an initial denaturation at 95°C for 5 min and then 30 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. A final extension was performed at 72°C for 7 minutes. The PCR products were analyzed by agarose gel electrophoresis, and purified with a Highpure PCR product Purification Kit (Roche, Germany).

Sequence analysis

The full sequences of the PCR products were analyzed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). The nucleotide sequence data were deposited in GenBank of the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov). Sequences of the 16S rDNA were submitted to an Advanced BLAST search program of the NCBI to identify sequences of closely related organisms. The related sequences were preliminarily aligned with the default settings of CLUSTAL W (Thompson *et al.* 1994), and complete sequence alignments were performed using PHYDIT (Chun 1995) and manual comparison. The phylogenetic analysis was performed with PHYLIP (Felsenstein, 1993), and phylogenetic trees were inferred using the neighbor-joining method (Saitou & Nei 1987).

3. Results and discussion

A total of 227 strains of bacteria were cultured. Among them, 16S rDNA sequences of 185 strains were identical with those of known strains isolated in this study, and 42 strains were finally identified (Table 1). Phylogenetic analysis using 16S rDNA indicated that the 30 strains belonged to *Pseudomonas*, 7 strains to *Arthrobacter*, two strains to *Flavobacterium*, and the remaining to *Achromobacter*, *Pedobacter*, and *Psychrobacter*. *Pseudomonas* and *Arthrobacter* were the dominant bacterial groups isolated in most of the tundra soil (Zhou *et al.* 1997). All of the 42 strains could grow above 20°C as well as at 4°C. Therefore, they seem to be psychrotolerant bacteria. For accurate characterization of the temperature response of the strains, we need to verify whether the optimal growth temperature is below 20°C or not.

Table 1. Culturable Artic bacteria identified on the basis of 16S rDNA sequences.

Strain No.	Site	The closest species	Similarity (%)
5-4*	Moss of puddle	*Achromobacter ruhlandii*	97.68
7-1*	Soil of coast	*Arthrobacter polychromogenes*	99.44
21-1*	Vertical sediment of red river	*Arthrobacter polychromogenes*	99.60
41-1*	North coastal sediment	*Arthrobacter polychromogenes*	99.60
7-10*	Soil of coast	*Arthrobacter psychrolactophilus*	99.13
7-7	Soil of coast	*Arthrobacter sulfureus*	98.08
19-1	Soil of tundra	*Arthrobacter sulfureus*	98.08
23-5	Soil of mountain	*Arthrobacter sulfureus*	98.08
4-4*	Moss of puddle	*Flavobacterium hydatis*	98.95
4-6*	Moss of puddle	*Flavobacterium hydatis*	99.76
7-6*	Soil of coast	*Pedobacter cryoconitis*	99.44
52-5	Attached on a marine alga	*Psychrobacter glacincola*	98.42
25-11	Sediment of puddle	*Pseudomonas borealis* (D)	99.76
4-1	Moss of puddle	*Pseudomonas borealis* (D)	99.69
6-20	Soil of coast	*Pseudomonas borealis* (D)	99.71
21-19	Moss of tundra	*Pseudomonas corrugata* (E)	99.53
21-2*	Soil of mountain	*Pseudomonas corrugata* (E)	99.45
56-2	Soil of coast	*Pseudomonas corrugata* (E)	99.53
56-4	Soil of coast	*Pseudomonas corrugata* (E)	99.68
19-2	Soil of tundra	*Pseudomonas frederiksbergensis* (C)	99.76
19-5	Soil of tundra	*Pseudomonas frederiksbergensis* (C)	99.84
21-7*	Moss of tundra	*Pseudomonas frederiksbergensis* (C)	99.76
21-9*	Moss of tundra	*Pseudomonas frederiksbergensis* (C)	100
23-14	Soil of mountain	*Pseudomonas frederiksbergensis* (C)	98.98
26-8	Soil under polar icecaps	*Pseudomonas frederiksbergensis* (C)	98.97
4-13	Moss of puddle	*Pseudomonas frederiksbergensis* (C)	98.98
4-5	Moss of puddle	*Pseudomonas frederiksbergensis* (C)	98.98
5-5	Moss of puddle	*Pseudomonas frederiksbergensis* (C)	98.98
57-5	Soil of coast	*Pseudomonas frederiksbergensis* (C)	98.98
7-13	Soil of coast	*Pseudomonas frederiksbergensis* (C)	99.13
7-5	Soil of coast	*Pseudomonas frederiksbergensis* (C)	98.98
25-19	Sediment of puddle	*Pseudomonas mandelii* (F)	99.84
26-9	Soil under polar icecaps	*Pseudomonas mandelii* (F)	100
23-7	Soil of mountain	*Pseudomonas rhodesiae* (A)	99.29
23-9	Soil of mountain	*Pseudomonas rhodesiae* (A)	99.29
6-21*	Soil of coast	*Pseudomonas rhodesiae* (A)	99.21
6-17*	Soil of coast	Pseudomonas syringae	98.50
55-3	Moss of puddle	Pseudomonas taetrolens	98.58
55-5	Moss of puddle	Pseudomonas taetrolens	98.90
25-2	Sediment of puddle	Pseudomonas veroni	99.53
4-2*	Moss of puddle	*Pseudomonas veronii* (B)	99.21
6-11	Soil of coast	*Pseudomonas veronii* (B)	98.90

*protease-producing bacteria.

Pseudomonas species

Thirty strains showed a high similarity (more than 98%) with the *Pseudomonas* species that belongs to γ-Proteobacteria. The genus *Pseudomonas* is ubiquitous and diverse bacteria in nature (Spiers *et al.* 2000). They possess variable metabolic abilities that utilize a wide range of organic compounds with a significant ecological position in the carbon cycle, and they are also important as pathogens

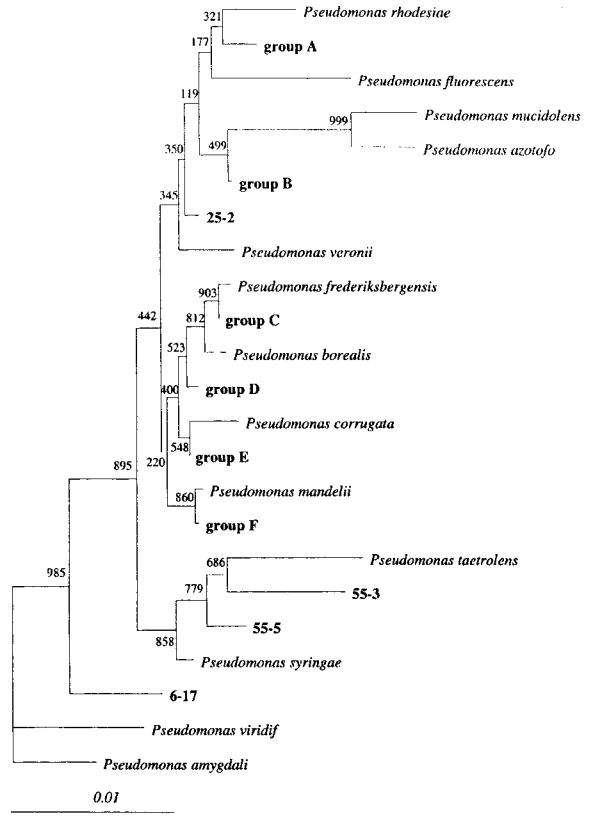

Fig. 1. Neighbor-joining tree of 16S rDNA sequences of Arctic bacteria belong to *Pseudomonas*. The sequences were collected from thirty Arctic bacteria isolated in this study and representative species selected from Ribosomal database (http://rdp.cme.msu.edu/html/). The sequences were aligned and used to construct a neighbor-joining tree based on the Kimura-2 parameter. The scale bar indicates the branch length that corresponds to 0.01 substitutions per position.

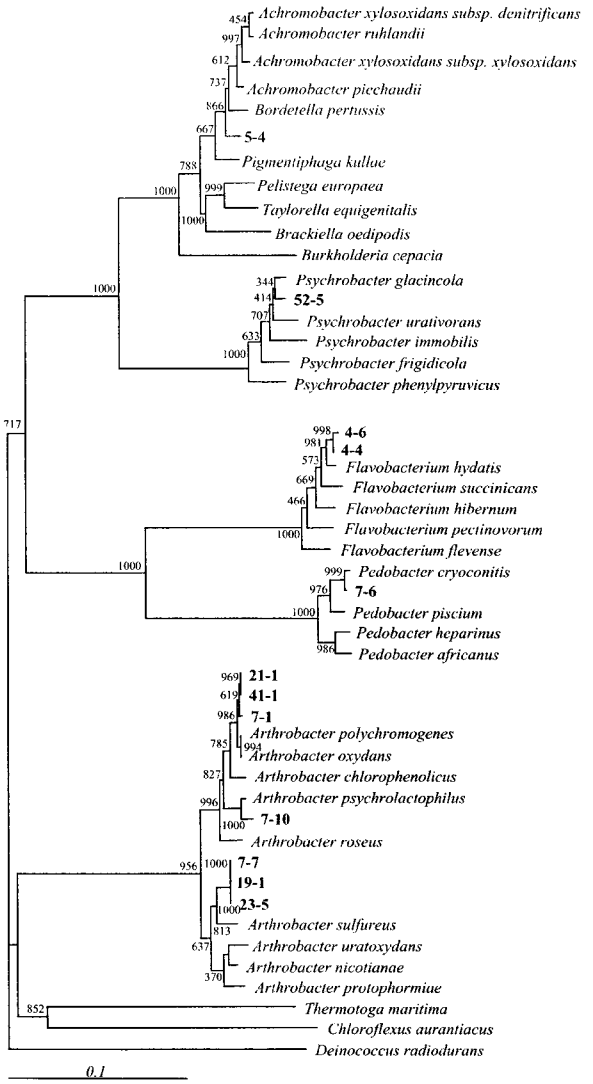

Fig. 2. Neighbor-joining tree of 16S rDNA sequences of Arctic bacteria belong to *Arthrobacter, Achromobacter, Flavobacterium* and *Pedobacter*. The sequences were collected from twelve Arctic bacteria isolated in this study and representative species selected from the Ribosomal database (http://rdp.cme.msu.edu/html/). The sequences were aligned and used to construct a neighbor-joining tree based on the Kimura-2 parameter. The scale bar indicates the branch length that corresponds to 0.1 substitutions per position.

of animals and plants (Yamamoto *et al.* 2000).

Out of the 30 *Pseudomonas* strains, 26 strains belong to six different groups (Table 1; Fig. 1). Even though the information of the full genome has been revealed for several *Pseudomonas* (Stover 2000; Nelson 2002), the classification of *Pseudomonas* strains is not fully established due to the lack of an accurate taxonomic system. Sequence analysis of 16S rDNA is frequently used for a taxonomic study (Moore *et al.* 1996). However, the degree of resolution obtained with 16S rDNA sequence analysis is not sufficiently discriminatory to permit resolution of intrageneric relationships because the rate of change in sequence of 16S rDNA is extremely low. Therefore, the Artic clones isolated in this study need further study to clarify their taxonomic status.

Even though the similarities are above 98%, group B, group D, 25-2, 55-3, 55-5, and 6-17 are candidates for new species.

Arthrobacter species

Seven strains showed high similarities (more than 98%)

with *Arthrobacter* species that belong to high G+C Gram-positive bacteria. *Arthrobacter* species were reported on from polar habitats such as Antarctica and Greenland (Osorio *et al.* 1999; Reddy *et al.* 2000, 2002). Three strains 7-1, 21-1, and 41-1 were closely aligned with *A. polychromogenes* and *A. oxydans* (Fig. 2). Despite the similarities, where values are above 97%, several new species including *A. oxydans* had been assigned on the

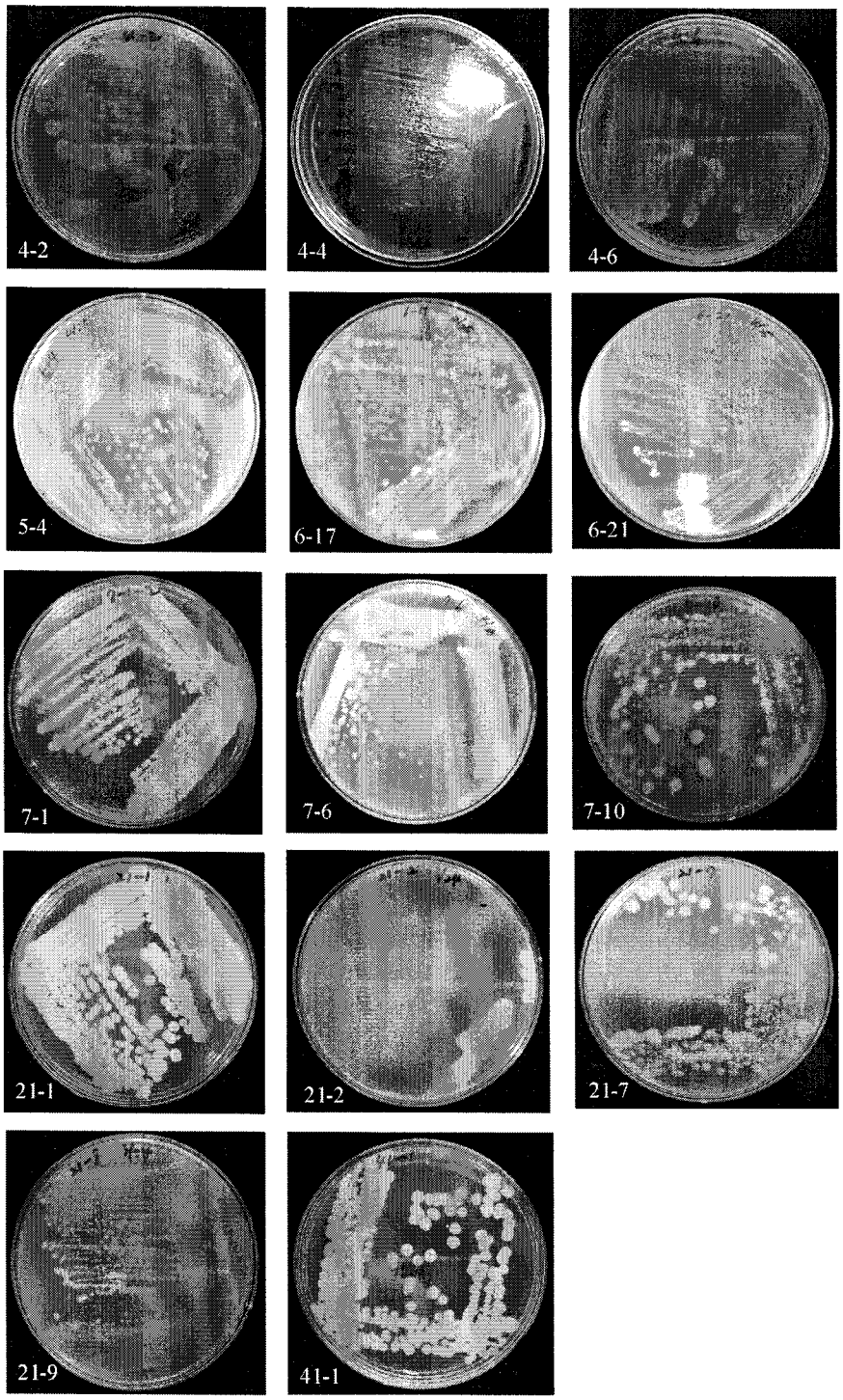

Fig. 3. Arctic bacteria showing protease activity.

basis of DNA-DNA relatedness or phenotypic difference in genus *Arthrobacter* (Wauters *et al.* 2000; Reddy *et al.* 2002). Therefore, the three strains are supposed to be a new species. The strain 7-10 seems to be *A. psychrolactophilus,* which is a psychrophilic bacterium. Three strains 7-7, 19-1 and 23-5 seem to be *A. sulfureus.*

Other species

The strain 5-4 showed the highest similarity to *Achromobacter ruhlandii,* which belongs to β-Proteobacteria. But the phylogenetic analysis shows that 5-4 formed an independent lineage outside the genus *Achromobacter* lineage; therefore, this strain is a candidate for a new genus. A strain 52-5 collected from a marine alga was closely aligned with *Psychrobacter glacincola* that belongs to γ-Proteobacteria. Several *Psychrobacter* species originated from marine habitats (Maruyama *et al.* 2000; Denner *et al.* 2001; Romanenko *et al.* 2002). Two strains 4-4 and 4-6 were aligned with *Flavobacterium hydatis,* which belongs to the Bacteroidetes species (Cytophaga-Flexibacter-Bacteroides group). Several *Flavobacterium* species have been reported in data collected in the Antarctica (McCammon *et al.* 1998; McCammon and Bowman 2000; Humphry *et al.* 2001). A strain 7-6 was closely aligned with *Pedobacter cryoconitis,* which belongs to the Bacteroidetes species (Cytophaga-Flexibacter-Bacteroides group). Genus *Pedobacter* was recently separated from *Sphingobacterium* (Steyn *et al.* 1998).

Among the 42 strains, 13 strains are candidates for new species or genera. Polar habitats including Arctic terrestrial habitat are good sources of new bacterial species and genera (Irgens *et al.* 1996; Bowman *et al.* 1997a, 1997b, 1997c, 1997d, 1998a, 1998b, 1998c; Gosink *et al.* 1998; Junge *et al.* 2002). For isolation of more diverse bacteria, we need more effective transportation methods than 3 M petri-films, which were used for convenience of transportation, which was restricted by volume. We also need to use various other culture media rather than nutrient media.

Protease-producing strains

Among the 42 strains, 14 bacteria showed protease activity (Fig. 3). They were 6 strains of *Pseudomonas* (4-2, 6-17, 6-21, 21-2, 21-7, 21-9); 4 strains of *Arthrobacter* (7-1, 7-10, 21-1, 41-1); an *Achromobacter* strain 5-4, two *Flavobactor* strains 4-4 and 4-6, and a *Pedobacter* strain 7-6. Further studies on protease-producing bacteria may search usable protease with specific activity.

They also have been focused on for the isolation of cold-active enzymes, which have biotechnological potential for novel applications, including food processing, additives in detergents, or in pharmaceutical medicine (Davail *et al.* 1994; Huston *et al.* 2000; Secades *et al.* 2001; Nakagawa *et al.* 2003; Zeng *et al.* 2003). Therefore, we expect these Arctic bacteria can be used for screening to develop new industrial enzymes that are active at low temperatures.

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