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Diversity of Cold-Active Protease-Producing Bacteria from Arctic Terrestrial and Marine Environments Revealed by Enrichment Culture

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A new approach for enrichment culture was applied to obtain cold-active protease-producing bacteria for marine and terrestrial samples from Svalbard, Norway. The method was developed for the enrichment of bacteria by long-term incubation at low temperatures in semi-solid agar medium containing meat pieces as the main source of carbon and energy. ZoBell and 0.1× nutrient broth were added for marine and terrestrial microorganisms, respectively, to supply basal elements for growth. One to three types of colonies were observed from each enrichment culture, indicating that specific bacterial species were enriched during the experimental conditions. Among 89 bacterial isolates, protease activity was observed from 48 isolates in the screening media containing skim milk. Good growth was observed at 4°C and 10°C while none of the isolates could grow at 37°C. At low temperatures, enzyme activity was equal to or higher than activity at higher temperatures. Bacterial isolates were included in the genera *Pseudoalteromonas* (33 isolates), *Arthrobacter* (24 isolates), *Pseudomonas* (16 isolates), *Psychrobacter* (6 isolates), *Sphingobacterium* (6 isolates), *Flavobacterium* (2 isolates), *Sporosarcina* (1 isolate), and *Stenotrophomonas* (1 isolate). Protease activity was observed from *Pseudoalteromonas* (33 isolates), *Pseudomonas* (10 isolates), *Arthrobacter* (4 isolates), and *Flavobacterium* (1 isolate).

***Keywords*:** Arctic, enrichment, protease

Cold environments represent a large proportion of Earth's area, including the Arctic, the Antarctic, oceans, and mountain areas (Cowan *et al*., 2007). Numerous organisms, particularly bacteria, yeasts, microalgae, and fungi, have successfully colonized these environments. These organisms survive and grow well due to unique molecular and physiological adaptations despite the strong negative effect of low temperatures on biochemical reactions. These adaptations include increased structural flexibility of enzymes, unique lipid constituents of cell membranes, and rapid synthesis of cryoprotectants and cold-shock proteins (Gerday *et al*., 2000; Cowan *et al*., 2007). In polar environments, where the concentration of small and easily metabolizable molecules is very low because of the absence of woody plants and limited residence time of higher animals, extracellular enzymes secreted by cold-adapted microorganisms play crucial ecological roles in the cycling of organic matter (Staley and Herwig, 1993; Vazquez *et al*., 2004). Cold-active enzymes secreted by these microorganisms exhibit high catalytic efficiency at low and moderate temperatures, and are easily inactivated by a moderate increase in temperature (Gerday *et al*., 2000).

In addition to their ecological role in the natural environment, cold-adapted enzymes have applications in the agricultural, energy, food, medical, structural material, and textile industries. For example, cold-adapted cellulase can be used for biopolishing and stone-washing processes in the textile industry. β-Galactosidase is used at low temperatures in the milk industry to reduce the amount of lactose, which is responsible for severe induced intolerances in two thirds of the world's population (Gerday *et al*., 2000). Cold-adapted enzymes have a high specific activity at low and moderate temperatures and this property is economically beneficial due to energy savings. Cold-adapted enzymes accommodate a high level of stereo-specificity and minimize undesirable chemical reactions that can occur at higher temperatures. They are inactivated easily by a moderate increase in temperature. These enzymes are both innovative and invaluable (Russell, 1998; Gerday *et al*., 2000) and can be extremely useful in various applications. Among various kinds of industrial enzymes, proteases have the largest part of enzyme market; occupying approximately 60% (Dastager *et al*., 2008). They also have a diverse field of utility in detergent preparations, food industries, and meat and leather industries as well as in human and animal digestive aids (Gerday *et al*., 2000; Cavicchioli *et al*., 2002; Huston, 2008).

Protease-producing microbial strains have been obtained by the wide application of direct screening by clear zone formation on agar medium containing skim milk or casein (Sookkheo *et al*., 2000; Zeng *et al*., 2003; Lee *et al*., 2005; Zhou *et al*., 2005; Olivera *et al*., 2007; Dastager *et al*., 2008). Enrichment by subculturing in liquid medium was also applied for isolation of protease-producing bacteria (Dube *et al*., 2001). In this study, a new approach to enrich cold-active protease producing bacteria was devised and used as an

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application for marine and terrestrial samples from Svalbard, Norway. Analyses were also conducted on the physiological characteristics and diversity of enriched bacteria.

Materials and Methods

Enrichment of cold-active protease-producing bacteria

Enrichment media were prepared by adding 3-4 pieces of 5 mm cubic dimensions of pork in 20 ml aliquots of semi-solid ZoBell media (0.5 g peptone, 0.1 g yeast extract, 0.01 $FePO_4$, 6 g agar, 750 ml sea water, 250 ml DDW) for marine samples or 0.1× NA media [0.8 g nutrient broth (Difco), 6 g agar, 1,000 ml DDW] for terrestrial samples. The media were then sterilized by autoclave in 50 ml transparent plastic bottles (Figs. 1A and B). Twenty four terrestrial samples including soil, snow, ice, fresh water, plant debris, animal excrement, and lichens, and twenty two marine samples including biofilm, marine animals, sea water, and sediments were taken from Ny-Ålesund, Svalbard. Inoculation of approximately 0.1 g of solid samples and 100 µl of liquid samples in the enrichment media was completed at the sampling site. Samples were then brought to the laboratory and incubated at 4°C for 6 months.

Isolation of enriched bacteria

From the enrichment culture, inoculums were spread on the ZoBell or 0.1× NA media supplemented with 1% skim milk for marine or terrestrial samples, respectively, and incubated at 15°C. Selections of morphologically distinct colonies with or without hydrolytic zones were made and further purified by repeated streaking on the same medium. The pure cultures of bacterial isolates were preserved in 20% glycerol at -80°C.

Examination of temperature effects on growth and enzyme activity

Revival of bacterial strains from glycerol stock was achieved by inoculation on ZoBell or NA media and incubation at 15°C. Bacterial cells were suspended in saline solution (0.85% NaCl) and dispensed in 96-well microplate for replica plating. The cells were inoculated on ZoBell or NA media and incubated at 4, 10, 15, 20, 25, 30, and 37°C for three days to examine temperature effects on growth. The amount of growth was scored as a '4' when a dense colony was formed with the colony diameter >8 mm, a '3' when a dense colony was formed with the colony diameter between 4 and 8 mm, a '2' when a loose colony was formed with the colony diameter >4 mm or a dense colony was formed with the colony diameter <4 mm, a '1' when a loose colony was formed with the colony diameter <4 mm, and a '0' when no growth was observed.

The cells were inoculated on ZoBell or 0.1× NA supplemented with 1% skim milk and incubated at 4°C, 10°C, and 20°C for a week (Fig. 1C) to examine temperature effects on protease production and activity. Protease activity was scored as a '4' when the width of clear zone is bigger than colony diameter, a '3' when the width of clear zone was between colony radius and diameter, a '2' when clear zone is evident but width of clear zone was smaller than colony radius, a '1' when faint clear zone was formed, and a '0' when no clear zone was formed.

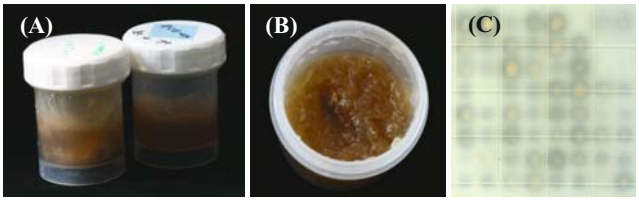

Fig. 1. (A), (B) Enrichment media incubated for 6 months after sample inoculation. (C) The formation of hydrolytic zones on the ZoBell agar plate supplemented with 1% skim milk after incubation at 4°C for 7 days.

Identification of bacteria

The 16S rRNA gene was amplified from a single colony with universal primers, 27F [5′-AGA GTT TGA TCM TGG CTC AG-3′] and 1492R [5′-GGT TAC CTT GTT ACG ACT T-3′], as described by Lane (1991) and Lee *et al*. (2006). PCR products were purified using LaboPass™ PCR Purification kit (COSMO, Korea) and directly sequenced using primers described by Lane (1991). Initially, an almost full length sequence of the 16S rRNA gene was compared with the sequences of type strains available in EzTaxon database (Chun *et al*., 2007) to determine taxonomic affiliation. Identity of bacterial isolates was determined based on the sequence similarity and phylogenetic relationships reconstructed by a neighbor-joining analysis (Saitou and Nei, 1987) based on a distance matrix calculated by Kimura's 2-parameter evolutionary model (Kimura, 1980). This analysis included sequences of type strains of closely related species.

Results

Isolation of enriched colonies

In most cases, one to three types of colonies were observed when the enriched semi-solid agar was spread on the solid media. This indicated that specific bacterial species were enriched during the experimental conditions. In total, forty two bacterial colonies were isolated from twenty two marine samples and forty seven colonies were isolated from twenty four terrestrial samples. As shown in Table 1, out of all the samples, thirty seven marine colonies and fourteen terrestrial colonies formed clear zones on the screening media.

Temperature for growth and enzyme activity

Most of the bacterial isolates could grow at 4°C while none of the strains could grow at 37°C. Maximum temperature for growth was 20°C for twelve isolates, 25°C for twenty one isolates, and 30°C for fifty six isolates. Optimum growth temperature could not be clearly defined with the scoring method used in this study for the colony diameter and density on the agar media. However, in most cases, fairly good growth was observed at 4°C and 10°C (data not shown).

Protease activity was observed at 4°C, 10°C, and 20°C from forty five isolates, 10°C and 20°C from one isolate, 10°C from one isolate, and 20°C from one isolate. Enzyme activity scores at low temperatures were equal to or higher than high temperature in most cases, which was revealed by a higher average of enzyme activity scores (Figs. 2-4).

Diversity of bacterial isolates

It was revealed that eighty nine bacterial isolates were included in eight genera from the similarity search and phylogenetic analyses of 16S rDNA sequences with type strains of each genus (Table 1). The biggest number of isolates were included in the genus *Pseudoalteromonas* (33 isolates), followed by *Arthrobacter* (24 isolates), *Pseudomonas* (16

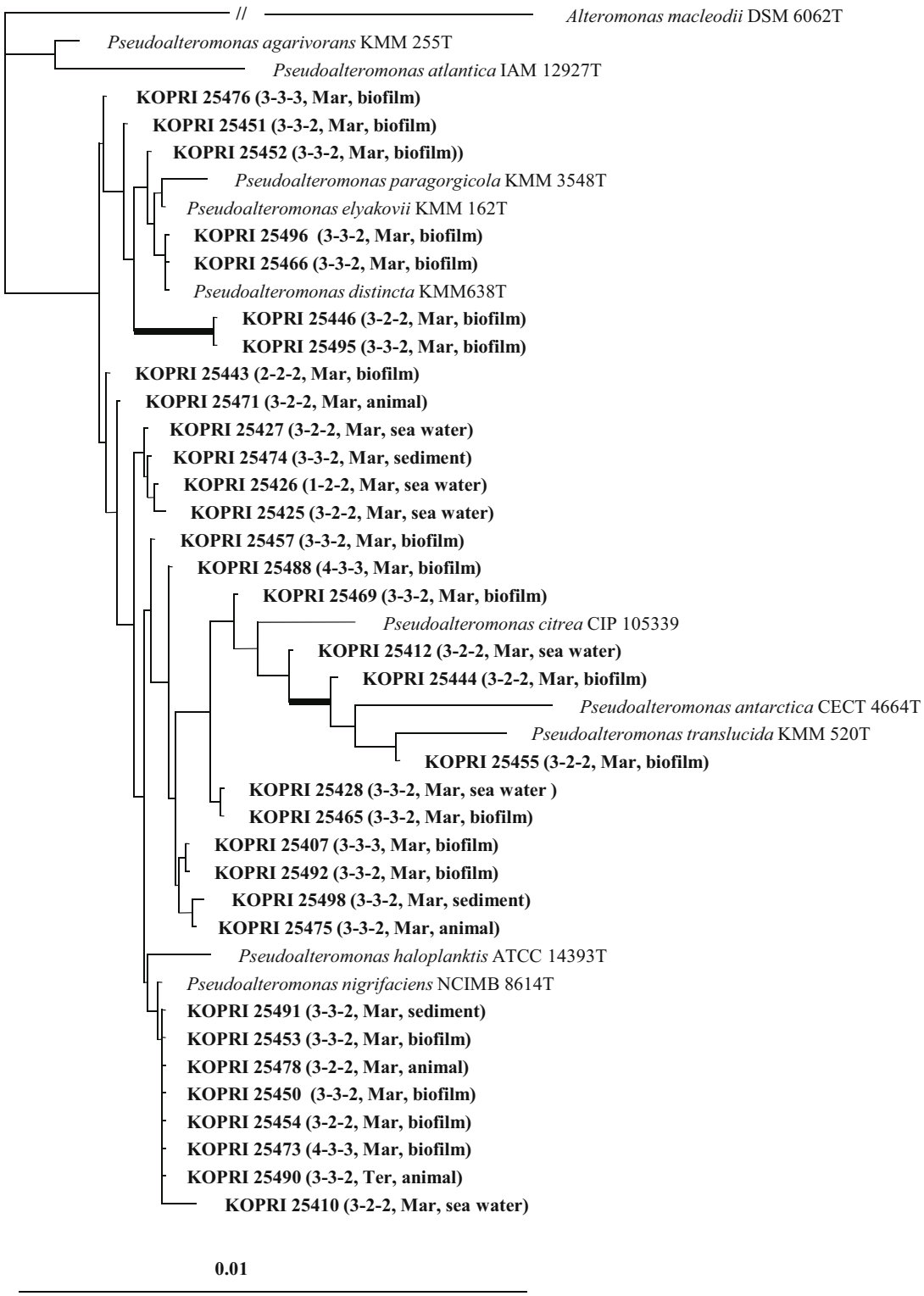

Fig. 2. Phylogeny of *Pseudoalteromonas* species reconstructed by neighbor-joining method. Branches supported by higher than 70% bootstrap values are represented by thick lines. Protease-producing isolates are indicated in bold. Scores for protease activity at 4, 10, and 20°C and sources for the isolates were represented in parentheses, where Ter and Mar indicate terrestrial and marine samples, respectively.

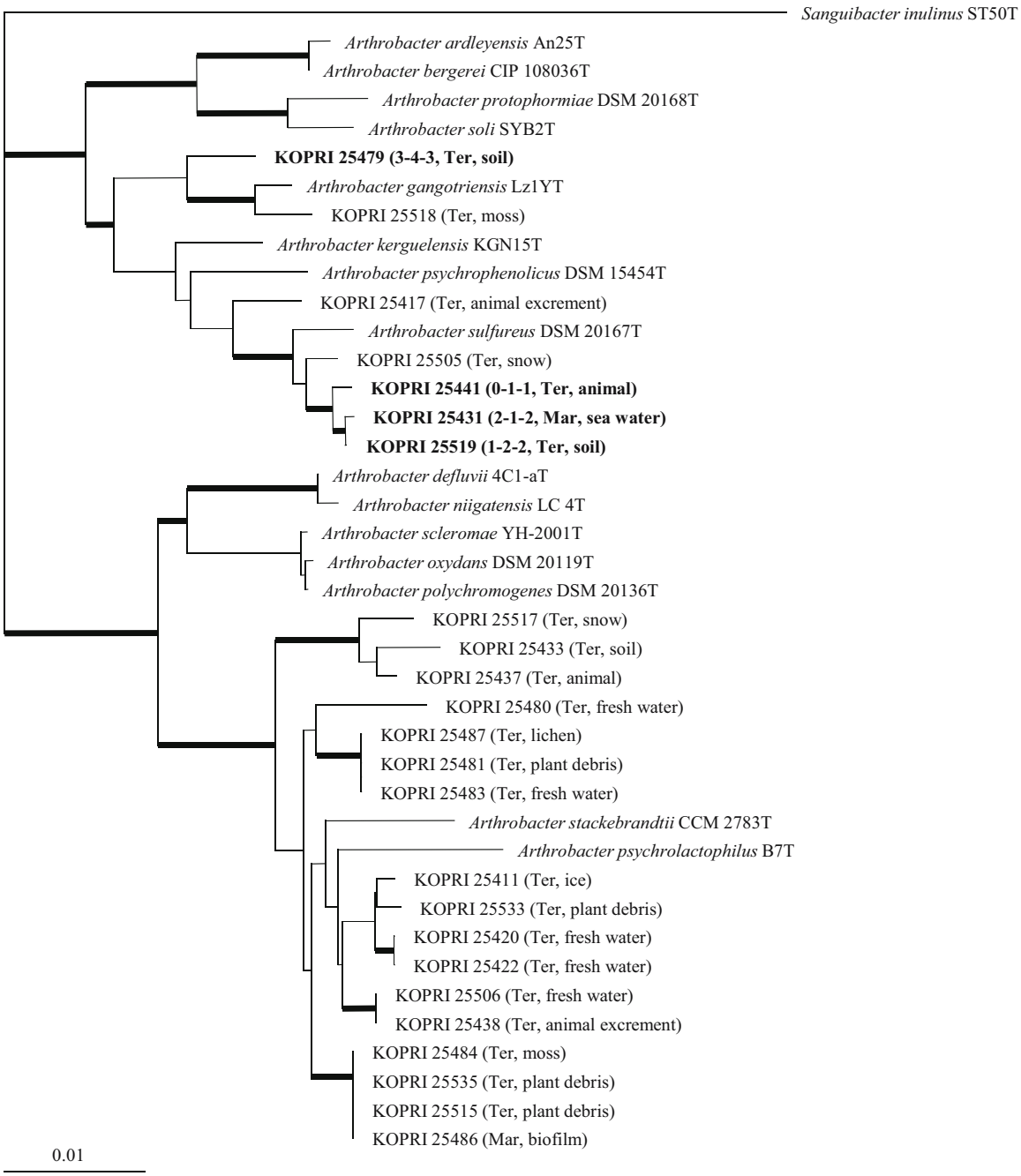

Fig. 3. Phylogeny of *Arthrobacter* species reconstructed by neighbor-joining method. Branches supported by higher than 70% bootstrap values are represented by thick lines. Protease-producing isolates are indicated in bold. Scores for protease activity at 4, 10, and 20°C and sources for the isolates are represented in parentheses, where Ter and Mar indicate terrestrial and marine samples, respectively.

isolates), *Psychrobacter* (6 isolates), *Sphingobacterium* (6 isolates), *Flavobacterium* (2 isolates), *Sporosarcina* (1 isolate), and *Stenotrophomonas* (1 isolate). Most of the *Pseudoalteromonas* isolates were from marine samples (Table 1). It was determined from the phylogenetic analysis that the isolates were phylogenetically very diverse and protease was produced by all of them on the screening media and, in most cases, with higher activity at low temperatures (Fig. 2). Most of the *Arthrobacter* isolates were isolated from terrestrial samples (Table 1). Among the twenty four isolates, protease activity was detected from four isolates, which are related to *Arthrobacter sulfureus* and *Arthrobacter gangotriensis*. Protease activity was not detected from seventeen isolates related to *Arthrobacter stackebrandtii* and *Arthrobacter psychrolactophilus*, two isolates related to *A. sulfureus*, and one isolate related to *A. gangotriensis* (Fig. 3). All of the *Pseudomonas* strains were isolated from terrestrial samples (Table 1). Distribution of ten protease-producing isolates occurred in five phylogenetic

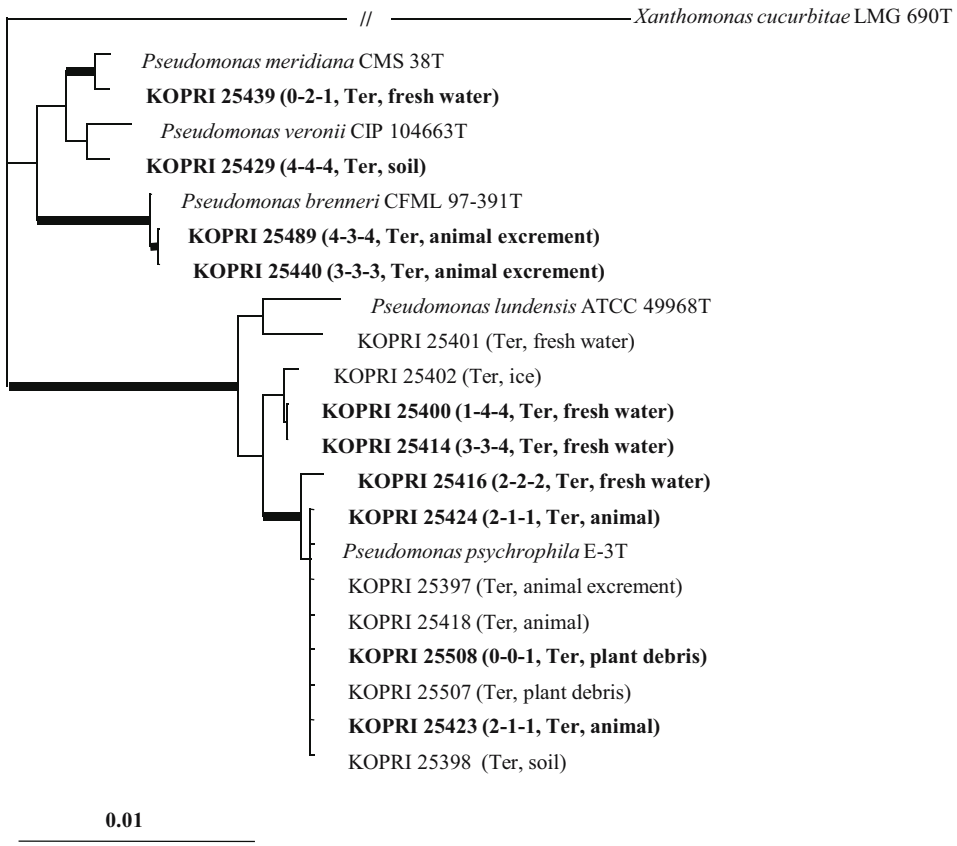

Fig. 4. Phylogeny of *Pseudomonas* species reconstructed by neighbor-joining method. Branches supported by higher than 70% bootstrap values are represented by thick lines. Protease-producing isolates are indicated in bold. Scores for protease activity at 4, 10, and 20°C and sources for the isolates are represented in parentheses, where Ter and Mar indicate terrestrial and marine samples, respectively.

lineages. The relationship between protease-producing ability and phylogenetics was not evident as closely related isolates showed different protease activity (Fig. 4). Six isolates from marine biofilm and animal samples of the genus *Psychrobacter* were related to *Psychrobacter cryohalolentis* and *Psychrobacter cibarius*. Protease activity was not detected from either of these isolates (data not shown). Six isolates of *Sphingobacterium* were divided into two monophyletic lineages and each of the lineages contained three isolates from terrestrial soil and fresh water, respectively. The lineage of fresh water isolates was related to *Sphingobacterium faecium.* A sister relationship with a group composed of *Sphingobacterium anhuiense*, *Sphigobacterium kitahiroshimense*, *S. faecium*, and fresh water isolates was formed with the lineage of terrestrial soil samples. Protease activity was not detected from these isolates (data not shown). High protease activity was detected in one of the two *Flavobacterium* isolates from terrestrial soil, which is related to *Flavobacterium hydatis.* No protease activity was detected in the other *Flavobacterium* isolate from the soil sample, which is related to *Flavobacterium pectinovorum*. Protease activity was not detected from the isolate of *Sporosarcina*. The isolate was related to *Sporosarcina psychrophila* (data not shown). An isolate of the genus *Stenotrohomonas* from the terrestrial soil sample also did not exhibit protease activity. The isolate was related to *Stenotrophomonas humi*, *Stenotrophomonas nitritireducens*, *Stenotrophomonas terrae*, and *Pseudomonas pictorum*.

Discussion

A new approach to enrich cold-active protease-producing microorganisms was tested with terrestrial and marine samples from the Arctic in this study. Formulation of this method was based on the assumption that microorganisms producing cold-active protease will survive at low temperatures after long time incubation when protein was supplied as a main source of energy and carbon. ZoBell and 0.1× nutrient broth were added for marine and terrestrial microorganisms, respectively, to supply basal elements for growth. Semi-solid agar medium was used by adding 0.6% agar to the medium to suppress growth of microorganisms dependent on hydrolyzed nutrients by protease-producing microorganisms by reducing diffusion rate of hydrolyzed protein. Results of bacterial enrichment, in which only one to three types of bacterial colonies were recovered from each enrichment culture, imply that finite species of bacteria were enriched by the process tested in this study. In addition, the implications of very good growth and high protease activity at low temperatures are that enrichment was carried out to enhance bacterial populations that are well adapted to the test condition, which is characterized by low temperatures and protein as a main nutrient.

Although the bacterial isolates from the enrichment culture

Table 1. Bacterial isolates from Arctic marine and terrestrial samples

Genus	Samples	Number of isolates	Number of protease-secreting isolates
Arthrobacter	**Marine**	**2**	**1**
(*Actinobacteria*)	Biofilm	1	0
	Sea water	1	1
	Terrestrial	**22**	**3**
	Animal	2	1
	Animal excrement	2	0
	Fresh water	5	0
	Ice	1	0
	Lichen	1	0
	Moss	2	0
	Plant debris	4	0
	Snow	2	0
	Soil	3	2
Flavobacterium	**Terrestrial**	**2**	**1**
(*Bacteroidetes*)	Soil	2	1
Pseudoalteromonas	**Marine**	**33**	**33**
(*Gammaproteobacteria*)	Animal	4	4
	Biofilm	20	20
	Sea water	6	6
	Sediment	3	3
Pseudomonas	**Terrestrial**	**16**	**10**
(*Gammaproteobacteria*)	Animal	3	2
	Animal excrement	3	2
	Fresh water	5	4
	Ice	1	0
	Plant debris	2	1
	Soil	2	1
Psychrobacter	**Marine**	**6**	**0**
(*Gammaproteobacteria*)	Animal	1	0
	Biofilm	5	0
Sphingobacterium	**Terrestrial**	**6**	**0**
(*Bacteroidetes*)	Fresh water	3	0
	Soil	3	0
Sporosarcina	**Marine**	**1**	**0**
(*Firmicus*)	Sea water	1	0
Stenotrophomonas	**Terrestrial**	**1**	**0**
(*Gammaproteobacteria*)	Soil	1	0
Total		89	48

were classified into eight genera, most of the protease-producing isolates were included, with high phylogenetic diversity, in *Pseudoalteromonas* and *Pseudomonas*. 55% of total isolates and 90% of protease-producing isolates were accounted for by the two genera. The two genera have been reported as main sources of protease-producing bacteria from the marine environment (Chen *et al*., 2003; Zeng *et al*., 2003; Lee *et al*., 2005; Zhou *et al*., 2005; Olivera *et al*., 2007). Including the two genera mentioned above, *Psychrobacter* and *Stenotrophomonas*, *Gammaproteobacteria* recovered in this study have been reported as a major bacterial group from cold environments including sea ice, glacier, and marine sediments (Groudieva *et al.*, 2004; Olivera *et al.*, 2007; Salwan *et al.*, 2010). It was revealed that many of the bacterial isolates from cold environments had higher or equivalent hydrolytic enzyme activities at low temperatures than at high temperatures in these studies (protease scores in Figs. 2-4). *Arthrobacter* and *Flavobacterium* included some of the protease-producing isolates. These protease-producing isolates have been isolated from various environments including soil, sediment, biofilm, volcanic rocks, glacier, and sea water (Zhu *et al.*, 2003; Chen *et al*., 2005; Heyman *et al.*, 2005; Kageyama *et al*., 2008; Ding *et al.*, 2009; Fu *et al.*, 2010; Yoon *et al.*, 2010). There were twenty four isolates of *Arthrobacter* obtained in this study and isolated from various marine and terrestrial samples including biofilm, sea water, ice, soil, fresh water, and several kinds of biological materials (Table 1). It is suggested that diverse *Arthrobacter* species are distributed in many different natural environments from these results and previous studies.

No protease activity was detected from the isolates included in *Psychrobacter*, *Sphingobacterium*, *Sporosarcina*, and *Stenotrophomonas.* Activity was only detected from several isolates of *Arthrobacter*. These were unexpected results as they were isolated from enrichment culture with protein as major carbon and energy sources. No protease activity detection from enriched bacterial isolates could be explained by failure of protease activity detection on the screening media because only skim milk was tested as a protease substrate. Otherwise, it can be explained by bacterial growth dependent on the hydrolyzed nutrient by other microorganisms. Further studies using various substrates to detect protease activity, co-cultivation experiments, or cultivation of pure culture with meat as sole carbon and energy sources are required for clarification.

Providing protein as main sources of energy and nutrients in the semi-solid agar medium, enrichment of cold-active protease-producing bacteria was quite successful by long-term incubation at low temperatures in this study. This strategy can be applied to the isolation of bacteria with lipase, chitinase, cellulase, hemicellulase or other hydrolyzing enzyme activities by modification of substrates and incubation temperature.

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References

Cavicchioli, R., K.S. Siddiqui, D. Andrews, and K.R. Sowers. 2002. Low-temperature extremophiles and their applications. *Curr. Opin. Biotechnol.* 13, 253-261.

Chen, M., X. Xiao, P. Wang, X. Zeng, and F. Wang. 2005. *Arthrobacter ardleyensis* sp. nov., isolated from Antarctic lake sediment and deep-sea sediment. *Arch. Microbiol*. 183, 301-305.

Chen, X.L., Y.Z. Zhang, P.J. Gao, and X.W. Luan. 2003. Two different proteases produced by a deep-sea psychrotrophic bacterial strain, *Pseudoalteromonas* sp. SM9913. *Mar. Biol.* 143, 989-993.

Chun, J., J.H. Lee, Y. Jung, M. Kim, S. Kim, B.K. Kim, and Y.W. Lim. 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int. J. Syst. Evol. Microbiol.* 57, 2259-2261.

Cowan, D.A., A. Casanueva, and W. Stafford. 2007. Ecology and biodiversity of cold-adapted microorganisms, pp. 119-132. *In* C. Gerday and N. Glansdorff (eds.), Physiology and biochemistry of extremophiles. American Society for Microbiology, Washington, D.C., USA.

Dastager, S.G., A. Dayanand, W.J. Li, C.J. Kim, J.C. Lee, D.J. Park, X.P. Tian, and Q.S. Raziuddin. 2008. Proteolytic activity from an alkali-thermotolerant *Streptomyces gulbargensis* sp. nov. *Curr. Microbiol.* 57, 638-642.

Ding, L., T. Hirose, and A. Yokota. 2009. Four novel *Arthrobacter* species isolated from filtration substrate. *Int. J. Syst. Evol. Microbiol.* 59, 856-862.

Dube, S., L. Singh, and S.I. Alam. 2001. Proteolytic anaerobic bacteria from lake sediments of Antarctica. *Enz. Microb. Technol.* 28, 114-121.

Fu, Y., X. Tang, Q. Lai, C. Zhang, H. Zhong, W. Li, Y. Liu, L. Chen, F. Sun, and Z. Shao. 2010. *Flavobacterium beibuense* sp. nov., isolated from marine sediment. *Int. J. Syst. Evol. Microbiol.* Article in press.

Gerday, C., M. Aittaleb, M. Bentahir, J.P. Chessa, P. Claverie, T. Collins, S. D'Amico, and *et al*. 2000. Cold-adapted enzymes: from fundamentals to biotechnology. *Trends Biotechnol.* 18, 103-107.

Groudieva, T., M. Kambourova, H. Yusef, M. Royter, R. Grote, H. Trinks, and G. Antranikian. 2004. Diversity and cold-active hydrolytic enzymes of culturable bacteria associated with Arctic sea ice, Spitzbergen. *Extremophiles* 8, 475-488.

Heyman, J., J. Verbeeren, P. Schumann, J. Swing, and P. De Vos. 2005. Six novel Arthrobacter species isolated from deteriorated mural paintings. *Int. J. Syst. Evol. Microbiol*. 55, 1457-1464.

Huston, A.L. 2008. Biotechnological aspects of cold-adapted enzymes, pp. 347-363. *In* C. Gerday and N. Glansdorff (eds.), Psychrophiles: from biodiversity to biotechnology. Springer-Verlag, Berlin, Heidelberg, Germany.

Kageyama, A., K. Morisaki, S. Omura, and Y. Takahashi. 2008. *Arthrobacter oryzae* sp. nov. and *Arthrobacter humicola* sp. nov. *Int. J. Syst. Evol. Microbiol.* 58, 53-56.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16, 111-120.

Lane, D.J. 1991. 16S/23S rRNA sequencing, pp. 115-175. *In* E. Stackebrandt and M. Goodfellow (eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons Inc., New York, N.Y., USA.

Lee, Y.K., H.J. Jung, and H.K. Lee. 2006. Marine bacteria associated with the Korean brown alga, *Undaria pinnatifida*. *J. Microbiol.* 44, 694-698.

Lee, Y.K., K.C. Sung, J.H. Yim, K.J. Park, H. Chung, and H.K. Lee. 2005. Isolation of protease-producing Arctic marine bacteria. *Ocean Pol. Res.* 27, 215-219.

Olivera, N.L., C. Sequeiros, and M.L. Nievas. 2007. Diversity and enzyme properties of protease-producing bacteria isolated from sub-Antarctic sediments of Isla de Los Estados, Argentina. *Extremophiles* 11, 517-526.

Russell, N.J. 1998. Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications. *Adv. Biochem. Eng. Biotechnol.* 61, 1-21.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406-425.

Salwan, R., A. Gulati, and R.C. Kasana. 2010. Phylogenetic diversity of alkaline protease-producing psychrotrophic bacteria from glacier and cold environments of Lahaul and Spiti, India. *J. Basic Microbiol*. 50, 1-10.

Sookkheo, B., S. Sinchaikul, S. Phutrakul, and S.T. Chen. 2000. Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS33. *Protein Expr. Purif.* 20, 142-151.

Staley, J.T. and R.P. Herwig. 1993. Degradation of particulate organic material in the Antarctic, pp. 241-264. *In* E.I. Friedmann (ed.), Antarctic Microbiology. Wiley-Liss Inc., New York, N.Y., USA.

Vazquez, S.C., S.H. Coria, and W.P. Mac Cormack. 2004. Extracellular proteases from eight psychrotolerant antarctic strains. *Microbiol. Res.* 159, 157-166.

Yoon, J.H., S. Park, S.J. Kang, S.J. Oh, S.C. Myung, and W. Kim. 2010. *Flavobacterium ponti* sp. nov., isolated from seawater. *Int. J. Syst. Evol. Microbiol.* Article in press.

Zeng, R., R. Zhang, J. Zhao, and N. Lin. 2003. Cold-active serine alkaline protease from the psychrophilic bacterium *Pseudomonas* strain DY-A: enzyme purification and characterization. *Extremophiles* 4, 335-337.

Zhou, M.Y., X.L. Chen, H.L. Zhao, H.Y. Dang, X.W. Luan, X.Y. Zhang, H.L. He, B.C. Zhou, and Y.Z. Zhang. 2005. Diversity of both the cultivable protease-producing bacteria and their extracellular proteases in the sediments of the South China Sea. *Microb. Ecol.* 58, 582-590.

Zhu, F., S. Wang, and P. Zhou. 2003. *Flavobacterium xinjiangense* sp. nov. and *Flavobacterium omnivorum* sp. nov., novel psychrophiles from the China No. 1 glacier. *Int. J. Syst. Evol. Microbiol.* 53, 853-857.