The Journal of Microbiology (2011) Vol. 49, No. 3, pp. 355-362
DOI 10.1007/s12275-011-0232-0
Copyright © 2011, The Microbiological Society of Korea

Cultured Bacterial Diversity and Human Impact on Alpine Glacier Cryoconite

Yung Mi Lee[1], So-Yeon Kim[2], Jia Jung[2], Eun Hye Kim[1], Kyeung Hee Cho[1], Franz Schinner[3], Rosa Margesin[3], Soon Gyu Hong[1], and Hong Kum Lee[1*]

[1]*Division of Polar Life Sciences, Korea Polar Research Institute, Songdo Techno Park, Incheon 406-840, Republic of Korea*
[2]*Korean Minjok Leadership Academy, Gangwon-do 225-823, Republic of Korea*
[3]*Institute of Microbiology, University of Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria*

(Received June 29, 2010 / Accepted January 11, 2011)

The anthropogenic effect on the microbial communities in alpine glacier cryoconites was investigated by cultivation and physiological characterization of bacteria from six cryoconite samples taken at sites with different amounts of human impact. Two hundred and forty seven bacterial isolates were included in *Actinobacteria* (9%, particularly *Arthrobacter*), *Bacteroidetes* (14%, particularly *Olleya*), *Firmicutes* (0.8%), *Alphaproteobacteria* (2%), *Betaproteobacteria* (16%, particularly *Janthinobacterium*), and *Gammaproteobacteria* (59%, particularly *Pseudomonas*). Among them, isolates of *Arthrobacter* were detected only in samples from sites with no human impact, while isolates affiliated with *Enterobacteriaceae* were detected only in samples from sites with strong human impact. Bacterial isolates included in *Actinobacteria* and *Bacteroidetes* were frequently isolated from pristine sites and showed low maximum growth temperature and enzyme secretion. Bacterial isolates included in *Gammaproteobacteria* were more frequently isolated from sites with stronger human impact and showed high maximum growth temperature and enzyme secretion. Ecotypic differences were not evident among isolates of *Janthinobacterium lividum*, *Pseudomonas fluorescens*, and *Pseudomonas veronii*, which were frequently isolated from sites with different degrees of anthropogenic effect.

***Keywords*:** bacterial diversity, cryoconite, human impact

Cryoconite holes are vertical cylindrical melt holes in the glacier surface, which are produced when glacier surfaces with deposition of wind-borne debris are heated by solar radiation (Wharton *et al*., 1985). The debris in the cryoconite hole is a mixture of inorganic and organic particulates. Most of the organic carbons contained in the cryoconite holes originate allochthonously rather than by autochthonous primary production by autophototrophic bacteria or algae (Stibal *et al*., 2008). Cryoconite holes are regarded to be an important glacier habitat for microbial life, as sediments from cryoconite holes are characterized by lower pH values, finer texture, higher water content, and higher concentration of nutrients than samples from supraglacial moraines and kames, and support higher levels of microbial life (Stibal *et al*., 2006). Cryoconite holes have been known to support active microbial communities including bacteria, microalgae, fungi, and metazoans (Mueller *et al.*, 2001; Takeuchi *et al.*, 2001; Margesin *et al*., 2002; Säwström *et al.*, 2002; Christner *et al.*, 2003; Stibal *et al.*, 2006). Maintaining a very low temperature with ice at the bottom, cryoconite holes are habitats harboring cold- adapted microbial life and cold-active enzymes (Margesin *et al*., 2002, 2003b, 2005, 2007; Christner *et al*., 2003).

With the advancement of molecular techniques, cultivation-independent approaches for describing microbial diversity have opened up new perspectives for microbial ecology and have been widely used in research on microbial communities (Bai *et al*., 2006). Although cultivation-independent approaches avoid the limitations of traditional culture-based methods, by which approximately 1% of the environmental bacteria can be cultured by general laboratory practices (Kirk *et al*., 2004; Bai *et al*., 2006), the culture-based approach applied in this study has benefits over molecular approaches in some respects. It is not possible to determine and compare the physiological characteristics of samples using a molecular approach. Further, the characterization of culturable microorganisms can provide information on the ecological roles of at least some members of the microbial community, and thus may augment knowledge of community structure derived from direct molecular approaches (Jiang *et al*., 2006). In addition, a culture-based approach is required to assemble a collection of micoorganisms on which to conduct biochemical, genetic and physiological experiments, and within which to probe inter- and intra-species interactions (Jiang *et al*., 2006).

Microbial communities are highly sensitive to environmental changes and respond rapidly to changing environmental conditions or anthropogenic stress (Webster and Negri, 2006). Although a number of studies on the relationship between the microbial community composition and human impacts or environmental gradients have been reported for various natural environments, including a stream-groundwater exchange zone, soils, caves, sediments, fresh water, coastal water, and biofilms (Øvreås *et al*., 1998; Paerl, 1998; Dorigo *et al*., 2002; Hancock, 2002; Margesin *et al.*, 2003a; Powell *et al*., 2003; Nocker *et al*., 2004; Webster and Negri, 2006; Ikner *et al*., 2007), studies on the influence of human impact on microbial community composition of cryoconites have not been reported. In this study, the influence of human impact on the microbial com-

* For correspondence. E-mail: hklee@kopri.re.kr; Tel: +82-32-260-6001; Fax: +82-32-260-6301

Table 1. Samples used in this study

Sample No.	Sampling date	Locality (Austria)	Altitude a.s.l.	Parent rocks	Human impact
Cr4	2006-08-23	Pasterze - Großglockner, Hohe Tauern	2200 m	silicate	0 (absent)
Cr6	2006-08-25	Bankerferner - Wurmkogel, Ötztaler Alps	2820 m	silicate	0 (absent)
Cr7	2006-08-25	Pitztaler Jöchl Ferner, Ötztaler Alps	2875 m	silicate	0 (absent)
Cr9	2006-08-28	Tiefenbachferner - Ötztaler Alps	2900 m	silicate	1 (weak)
Cr5	2006-08-23	Zugspitze - Zugspitz glacier	2620 m	carbonate	2 (strong)
Cr3	2006-06-29	Eisgratferner - Stubaier glacier	2900 m	silicate	3 (very strong)

munities of cryoconite holes from an alpine area was investigated by comparing diversity and physiological characteristics of cultured bacterial isolates from samples of six cryoconite holes with different frequencies of human visits. Growth temperature and hydrolytic enzyme secretion of microorganisms were studied based on the assumption that frequent human visits may increase the opportunity to introduce microorganisms of human origin and to enrich populations of microorganisms utilizing exogenous proteins and lipids.

Materials and Methods

Study site and sample collection

Cryoconite samples were collected from mountains in the Austrian Alps during June and August 2006 and transported to the laboratory at the University of Innsbruck as quickly as possible. Samples (0.9 ml) were placed into cryo-vials with 0.9 ml of 20% glycerol and transported to the laboratory at the Korea Polar Research Institute in an ice-box packed with dry-ice. They were preserved at -80°C until used. The degree of human impact on the sampling site was designated as 0 (none), 1 (weak), 2 (strong), and 3 (very strong) according to the frequency of human visits to the area of the sampling site. The altitude ranged between 2200 and 2900 m above sea level and the parent rock of the glacier was mostly silicate except for one sample with carbonate rock (Table 1).

Bacterial cultures

For cultivation of bacterial isolates, samples were serially diluted up to 10^{-4} in sterilized distilled water and 100 μl of the diluted sample suspension were spread on five kinds of medium: nutrient agar (Difco, USA), 1/10 diluted nutrient agar (0.1NA), R2A (Difco, USA), 1/10 diluted R2A (0.1R2A), and YM agar (Difco) and incubated at 10°C and 20°C for 7 days. Then, three to four colonies from each of the various similar colony types, considering size, shape, elevation, margin, color, surface texture, and opacity, were selected and transferred to fresh media. The subculturing was repeated twice or more until pure isolates were obtained. Pure cultures of bacterial isolates were preserved at -80°C in 20% glycerol.

Physiological characterization

Cell suspensions were prepared by adding a half-full loop (5 mm diameter) of cells from agar plates to 500 μl of distilled water, with vigorous shaking using a vortex mixer. They were diluted 40 fold with sterilized distilled water and transferred to 96 well plates for replica plating. To examine the growth response to temperature, the suspension was inoculated onto solid media by replica plating with a 96 pin replicator (VP-408B, V&P Scientific, USA) and incubated at 4°C, 10°C, 15°C, 20°C, 25°C, 30°C, and 37°C for 3 days. Growth was evaluated by scoring as follows: 0, no growth; 1, the diameter of the colony was smaller than 4 mm and translucent; 2, the diameter of the colony was smaller than 4 mm and dense, or between 4 and 8 mm and translucent; 3, the diameter of the colony was between 4 and 8 mm, and dense; 4, the diameter of the colony was bigger than 8 mm. The secretion of protease and lipase was examined by replica plating onto 0.1 NA plates supplemented with 1% skim milk (Difco) or 1% tributyrate (Sigma, USA), respectively. The plates were incubated for 3 days for protease secretion, and for 7 days for lipase secretion at 10°C and 20°C. Enzyme secretion was scored as follows: 0, no clear zone; 1, faint clear zone; 2, clear zone was evident and width of clear zone was smaller than the radius of the colony; 3, width of clear zone was bigger than the radius and smaller than the diameter of the colony; 4, width of clear zone was bigger than the diameter of the colony.

Phylogenetic analysis of bacterial isolates

Bacterial strains were identified by sequence similarity and phylogenetic analysis of 16S rRNA gene sequences. The 16S rRNA gene was amplified from a single colony of pure cultures with two 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). PCR was carried out with 25 μl reaction mixtures containing 1× PCR reaction buffer, 200 μM of dNTPs, 0.2 μM of each primer, a single colony as a template and 1 unit of *Taq* DNA polymerase (In-Sung Science, Korea). The PCR procedure included an initial denaturing step at 95°C for 5 min and 30 cycles of amplification (95°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec) and a final extension step at 72°C for 5 min. PCR products were purified using the AccuPrep PCR Purification kit (Bioneer, Korea) and sequenced with the same primers used for PCR amplification. The sequence of the 16S rRNA gene was compared with that of type strains available in the EzTaxon database (Chun *et al*., 2007) to find closely related species and choose reference sequences for phylogenetic analyses. Phylogenetic trees were reconstructed by the neighbor-joining method (Saitou and Nei, 1987) based on the distance matrix generated according to the Kimura's two-parameter model (Kimura, 1980) using PHYLIP ver. 3.69 (Felsenstein, 2009). The confidence level of the tree topology was evaluated by bootstrap analysis using 1,000 sequence replications. Species affiliation of a bacterial isolate was determined when the isolate formed a monophyletic group with reference species and had 99% or higher similarity. Sequences were submitted to NCBI GenBank under the accession numbers HQ824836-HQ825082.

Results

Bacterial cultures and physiological characteristics

Two hundred and forty seven bacterial isolates were obtained from samples taken at six cryoconite sites with different levels of human impact. Most of the isolates could grow between

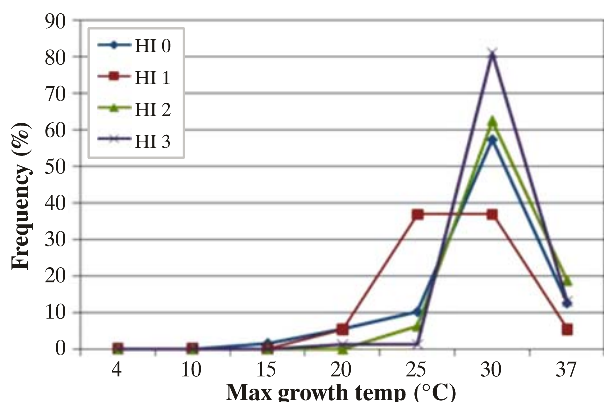

Fig. 1. Frequency of bacterial isolates with different maximum growth temperature for samples with different level of human impacts (HI).

4°C and 30°C, while growth at 37°C was observed only with 34 strains. Eighty nine isolates showed good growth (scores≥3) at 4°C and the number of isolates of good growth increased gradually up 20°C and then decreased to 37°C: 10°C-96; 15°C-106; 20°C-121; 25°C-96; 30°C-67; 37°C-18.

The distribution of maximum growth temperature showed similar patterns among samples from sites with different degrees of human impact except for one sample from a site with weak human impact (Fig. 1). The frequency of bacterial isolates increased with increasing temperature, peaked at 30°C and dropped at 37°C. However, differences among samples were also observed when the data were carefully examined. The frequency of bacterial isolates that could grow only below 20°C was 8% and 5% in samples from sites with no or weak human impact, compared to 0% and 1% in samples from sites with strong or very strong human impact, respectively. In contrast, the frequency of bacterial isolates that could grow at temperatures as high as 37°C was higher in samples from strongly impacted sites: 19% and 13% in samples from sites with strong or very strong human impact, compared to 13% and 5% in samples from sites with no or weak human impact, respectively.

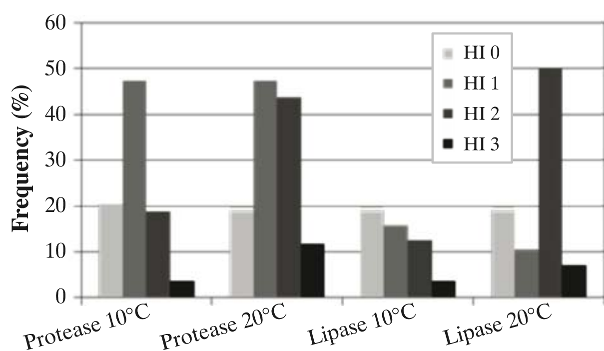

Fig. 2. Frequency of bacterial isolates without protease and lipase activities at 10°C and 20°C for samples with different level of human impacts (HI).

Among the 247 isolates, protease activity was observed for 206 isolates at 10°C and for 197 isolates at 20°C. Thirty four and 37 isolates showed high protease activity (scores≥3) at 10°C and 20°C, respectively. Lipase activity was observed for 215 isolates at 10°C and for 207 isolates at 20°C. Seventeen and 19 isolates showed high lipase activity (scores≥3) at 10°C and 20°C, respectively. Isolates with no protease activity were frequent in samples from sites with no or weak human impact and were rare in a sample from a site with very strong human impact (Fig. 2). Isolates with no lipase activity were very rare in samples from highly impacted sites, but the differences among samples were not as high as for protease activity. Scores of enzyme activity at 10°C and 20°C were compared for each isolate. In most cases, protease activity at the two temperatures seemed similar, but isolates with higher protease activity at 10°C than at 20°C were frequent from sites where human impact was strong (Fig. 3). In contrast, a correlation of lipase activity with human impact was not evident.

Diversity of bacterial isolates

The taxonomic affiliation of isolates was determined by a similarity search and phylogenetic analysis of 16S rDNA sequences with type strains of closely related species. Bacterial isolates

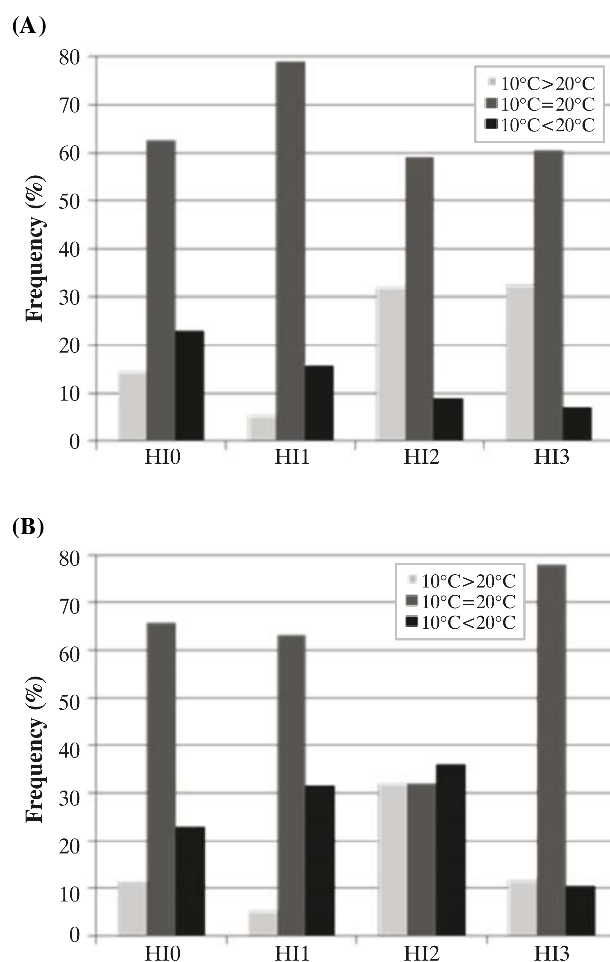

Fig. 3. Comparison of protease (A) and lipase (B) activities at 10°C and 20°C for samples with different level of human impacts (HI).

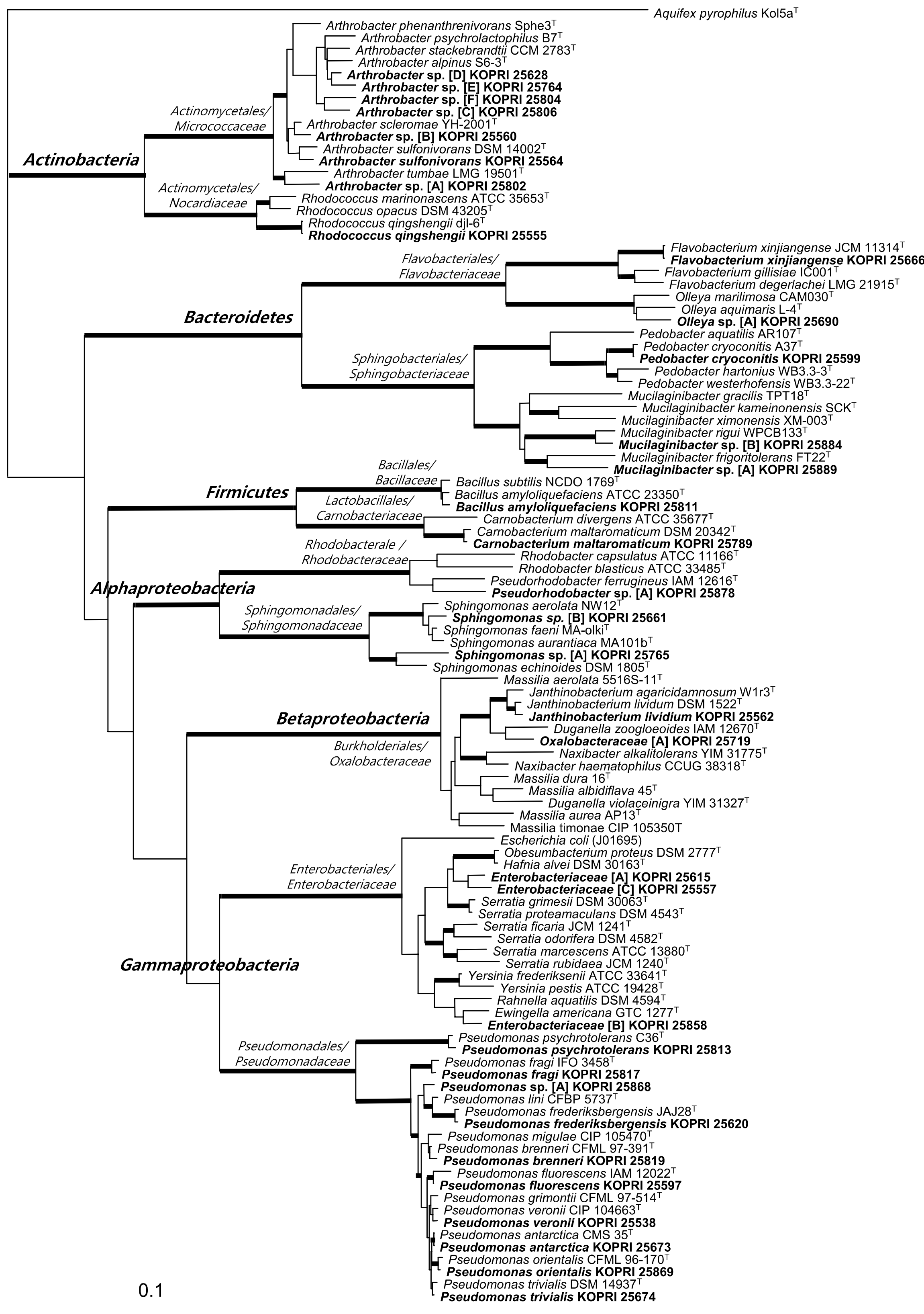

Fig. 4. Neighbor-joining tree of cryoconite isolates with closely related reference species based on the 16S rRNA gene sequences. Representative isolates for each phylotype are represented by bold letters. Branches supported by high bootstrap values (>70%) are represented by think lines.

were included in *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (Fig. 4 and Table 2). Seven phylotypes of *Arthrobacter* of the phylum *Actinobacteria* were isolated from pristine cryoconite sites with no human impact. In contrast, one isolate of *Rhodococcus qingshengii* in the same phylum was isolated from a site with high human impact. *Flavobacterium xinjiangense* of the phylum *Bacteroidetes* was usually isolated from sites with no human impact. Two phylotypes of *Mucilaginibacter* were isolated from sites with no or weak human impact. A phylotype of *Olleya* was isolated from sites with no or weak human impact. One isolate of *Pedobacter cryoconitis* was isolated from a site with very strong human impact. One isolate of *Bacillus amyloliquefaciens* of the phylum *Firmicutes* was isolated from a sample taken at a pristine site. A phylotype of *Carnobacterium* of the same phylum was also isolated from a sample taken at a site with no human impact. One isolate of *Pseudorhorobacter* of the class *Alphaproteobacteria* was iso-

Table 2. Bacterial isolates from cryoconite samples and their physiology

Species name	Number of isolates						Physiological characteristics				
	HI[a] 0			HI 1	HI 2	HI 3	Max. growth temp.[b] (°C)	Protease[d]		Lipase[d]	
	Cr4	Cr6	Cr7	Cr9	Cr5	Cr3		10°C	20°C	10°C	20°C
Actinobacteria		**20**		**0**	**1**	**0**					
Arthrobacter sulfonivorans		1					ND[c]	□	□	□	□
Arthrobacter sp. [A]		1					30	■	■	■	■
Arthrobacter sp. [B]		3					**25**/30	▨	■	▨	■
Arthrobacter sp. [C]		1	1				25	□	▨	■	■
Arthrobacter sp. [D]		3					20/25/30	■	▨	□	▨
Arthrobacter sp. [E]		9					15/**20**/30	▨	■	■	▨
Arthrobacter sp. [F]		1					25	■	■	■	■
Rhodococcus qingshengii					1		30	■	□	■	■
Bacteroidetes		**21**		**10**	**1**	**2**					
Flavobacterium xinjiangense	1		5		1		ND	□	□	□	□
Mucilaginibacter sp. [A]				1			37	■	■	□	■
Mucilaginibacter sp. [B]		1		1			ND	□	□	□	□
Olleya sp. [A]	4	9	1	8		1	20/**25**/30/37	▨	▨	■	■
Pedobacter cryoconitis						1	20	■	□	■	■
Firmicutes		**2**		**0**	**0**	**0**					
Bacillus amyloliquefaciens		1					ND	■	□	□	■
Carnobacterium maltaromaticum	1						37	■	■	■	■
Alphaproteobacteria		**1**		**2**	**0**	**2**					
Pseudorhodobacter sp. [A]			1				25	□	□	□	□
Sphingomonas sp. [A]				2		1	20/25/30	▨	▨	▨	▨
Sphingomonas sp. [B]						1	ND	□	■	■	■
Betaproteobacteria		**14**		**5**	**0**	**20**					
Janthinobacterium lividium	6	1	7	5		19	25/**30**/37	■	▨	■	■
Oxalobacteraceae [A]						1	30	■	■	■	□
Gammaproteobacteria		**69**		**2**	**14**	**61**					
Enterobacteriaceae [A]					2		30	■	■	■	□
Enterobacteriaceae [B]					1	1	37	▨	▨	▨	□
Enterobacteriaceae [C]					3		**30**/37	▨	▨	■	▨
Pseudomonas antarctica					2		25/30	■	■	■	■
Pseudomonas brenneri			3			6	**30**/37	■	■	■	■
Pseudomonas fluorescens	9	6	2			23	**30**/37	■	■	■	■
Pseudomonas fragi			1				30	■	□	□	■
Pseudomonas frederiksbergensis		7	1			1	**30**/37	■	■	■	■
Pseudomonas orientalis	1				1	1	30/37	■	▨	▨	▨
Pseudomonas psychrotolerans		1					30	■	■	■	■
Pseudomonas trivialis					1		30	■	■	■	■
Pseudomonas sp. [A]	2						30/37	■	■	■	■
Pseudomonas veronii	16	14	6	2	4	29	**30**/37	■	■	■	■

[a] HI, human impact classified according to the frequency of human visits.
[b] Mode of maximum growth temperature for each phylotype is represented by boldface font. When the number of bacterial isolates is same for each temperature, the mode value was not presented.
[c] ND, not determined
[d] The frequency of bacterial isolates with enzyme activity is shown by symbols: □ (<20%), ▨ (20-80%), ■ (>80%).

lated from a pristine site and two phylotypes of *Sphingomonas* were isolated from sites with weak or very strong human impact. *Janthinobacterium lividum* of the *Betaproteobacteria* was one of the most frequently isolated species. It was isolated from sites with no, weak and very strong human impact. One isolate labeled as *Oxalobacteraceae* [A], which was isolated from a site with very high human impact, was related to *Janthinobacterium* and *Duganella*. Three phylotypes included in the *Enterobacteraceae* of *Gammaproteobacteria* were isolated from samples with strong or very strong human impact. Ten phylotypes of *Pseudomonas* in the *Gammaproteobacteria* were isolated from both pristine and human impacted sites. Among them *Pseudomonas veronii* was the most frequently isolated species.

Discussion

Ellis *et al*. (2003) suggested that the culturable portion of the bacterial community appeared to be more affected by heavy metal contamination than the community as a whole and that difference makes a culture-dependent approach useful for determining the impact of anthropogenic activity, which is primarily responsible for such contamination. In this study, five kinds of media with different nutrient compositions and two different incubation temperatures, 10°C and 20°C, were used to increase the recovery of a diverse collection of microbial species from samples taken at sites with different levels of human impact. Two hundred and forty seven bacterial isolates were affiliated with *Actinobacteria* (9%), *Bacteroidetes* (14%), *Firmicutes* (0.8%), *Alphaproteobacteria* (2%), *Betaproteobacteria* (16%), and *Gammaproteobacteria* (59%). The dominance of *Proteobacteria* in the samples is consistent with a previous report (Bai *et al*., 2006), which showed that isolates related to the alpha, beta and gamma subdivisions of *Proteobacteria* were the typical and dominant group isolated from cold environments by cultivation methods.

Maximum growth temperature of each isolate was investigated based on the hypothesis that higher levels of human activity may increase the opportunity to introduce bacterial species of human origin with higher maximum growth temperatures. Although the influence of human activities on the maximum growth temperature was not evident from the general distribution patterns (Fig. 1), the frequency of bacterial isolates that could grow only below 20°C was low, while the frequency of bacterial isolates that could grow as high as 37°C was high in samples from strongly impacted sites, which supports this idea. Ikner *et al*. (2007) mentioned animals and plants as a source of organic matter. Inputs from warm-blooded animals are another possible source of microorganisms with high growth temperature in cryoconites; however we did not detect any sign of animal activity around the cryoconite holes.

Two isolates that grow only below 15°C were included in *Arthrobacter* and nine isolates that grow only below 20°C were included in *Arthrobacter*, *Flavobacterium*, *Olleya*, *Pedobacter*, and *Sphingomonas* (Table 2). Most of them were isolated from cryoconite samples taken at pristine sites. They were classified in *Actinobacteria* (6 isolates), *Bacteroidetes* (4 isolates), and *Alphaproteobacteria* (1 isolate). Thirty one isolates that could grow at 37°C were included in *Carnobacterium*, *Enterobacteriaceae*, *Janthinobacterium*, *Olleya*, and *Pseudomonas* (Table 2). Of these, seventeen isolates were cultured from cryoconite sites with no or weak human impact, whereas fourteen isolates were obtained from sites with strong or very strong human impact. They were classified in *Bacteroidetes* (2 isolates), *Firmicutes* (1 isolate), *Betaproteobacteria* (3 isolates), *Gammaproteobacteria* (25 isolates). From these results, it appears that bacterial species with low maximum growth temperatures tend to occur at sites with low human impact and are usually included in *Actinobacteria* and *Bacteroidetes*, whereas those with a high maximum growth temperature show no correlation with the level of human impact and are usually included in *Gammaproteobacteria*.

The relationship between human impact and maximum growth temperature among isolates of the same phylotypes was examined for *Janthinobacterium lividium*, *Pseudomonas fluorescens*, and *Pseudomonas veronii*, which were frequently isolated from sites with different levels of human impact. Isolates that could grow at 37°C were obtained from sites with both low and high human impact. Moreover, isolates having different maximum growth temperatures were observed at similar frequency in all of the samples (data not shown). These results indicate that the relationship between human impact and maximum growth temperature cannot be explained by ecotypic variation of phylotypes.

Hydrolytic enzyme activities were investigated based on the assumption that human activities may increase exogeneous proteins and lipids in the cryoconite holes, and microorganisms adapted to the increased level of macromolecules would be more frequently detected at sites with stronger human impact. In this study, most of the bacteria isolated from a highly impacted site showed protease and lipase activities (Fig. 2). However, the relationship between enzyme activities and human impact could not be generalized for all of the samples. The loose relationship may partly be explained by the small number of sites sampled for each level of human impact and the uneven number of bacterial isolates from each sample. On the other hand, the variation may partly be explained by different bacterial communities among sites with a similar level of human impact. As an example, among three samples from sites with no human impact, the Cr6 sample had many isolates included in *Actinobacteria* as compared to the other samples (Table 2). As bacterial isolates included in *Actinobacteria*, *Bacteroidetes*, and *Alphaproteobacteria* showed a low frequency of enzyme activities as compared to those included in *Firmicutes*, *Betaproteobacteria*, and *Gammaproteobacteria*, frequent isolation of *Arthrobacter* spp. from the Cr6 sample might have affected the frequency of enzyme activities in samples from sites with no human impact. The very low frequency of protease activity in Cr9, a sample from a site with weak human impact, can be explained by frequent isolation of *Olleya* sp. The high frequency of protease activity in Cr3, a sample from a site with very strong human impact, can be explained by frequent isolation of *J. lividum* and *Pseudomonas* spp.

Previous studies on the correlation between the composition of a microbial community and human impact reported that anthropogenic impacts may influence the composition of microbial communities (Saul *et al*., 2005; Webster and Negri, 2006; Ikner *et al*., 2007; Labbé *et al*., 2007). In a study on the impact of tourism on culturable microbial diversity in a limestone cave, it was reported that diversity decreased as

human impact increased and the abundance of phyla differed according to the degree of human impact. *Proteobacteria* were most abundant in the high impact areas, while *Firmicutes* predominated in the low and medium impact areas (Ikner *et al*., 2007). Saul *et al*. (2005) reported that the composition of bacterial cultures obtained from around Scott Base, Antarctica, differed according to the hydrocarbon concentration. *Actinobacteria* (39%), *Cytophaga*/*Flavobacterium*/*Bacteroidetes* (CFB) (18%), *Proteobacteria* (22%), and Low G+C Gram-positive bacteria (12%) predominated in the control samples, whereas isolates affiliated with *Actinobacteria* (26%) and *Proteobacteria* (65%) dominated in hydrocarbon-contaminated soil. Although the correlation was not clear, a predominace of *Proteobacteria* in samples from the more impacted areas was observed in the current study: 66% from sites with no human impact, 48% from the site with weak human impact, 100% for the site with strong, and 98% for the site with very strong human impact. In contrast, the proportion of *Actinobacteria* decreased; 16%, 0%, 6%, and 0% for no to very high degree of human impact. Webster and Negri (2006) reported that microbial diversity and abundance in biofilms established on artificial surfaces was affected by the degree of human impact and the dominance of bacterial groups across three differentially contaminated Antarctic sites was as follows: *Alphaproteobacteria*, *Gammaproteobacteria* and CFB at the least impacted site, green sulfur and sulfate reducing bacteria near the semi-impacted site and *Planctomycetales* and sulfate reducing bacteria near the highly impacted site. Although studies of cultured microbial diversity may not reflect *in situ* microbial communities directly, the current study found that *Actinobacteria* and *Bacteroidetes* were enriched in cryoconite holes with no or weak human impact and some taxonomic groups in *Gammaproteobacteria* were enriched in samples from strongly impacted sites.

The different isolation frequency of each taxonomic group seems to explain different physiological characteristics such as maximum growth temperature and enzyme secretion. These results can be explained partly by increased introduction of bacterial species with high growth temperature due to frequent human visits and partly by adaptation of bacterial species with various physiological characteristics to various environmental conditions resulting from human activities.

Culture-dependent approaches were applied to determine and compare the physiological characteristics of isolates. Although culture-dependent approaches for microbial community analysis are often criticized for their selectivity and inherent 'culturability' problems (Ellis *et al*., 2003), diversity and physiological characterization of microbial communities in cryoconites based on culture-based methods does provide basic information on how microbial diversity in an alpine glacier is affected by human activities.. Further studies on the physiology of microorganisms and investigation of environmental conditions combined with culture-independent microbial diversity studies may provide a better understanding of the anthropogenic environmental changes and concomitant microbial adaptations in cryoconite holes.

Acknowledgements

This research was supported by Korea Polar Research Institute (Grant PE06050).

References

Bai, Y., D. Yang, J. Wang, S. Xu, X. Wang, and L. An. 2006. Phylogenetic diversity of culturable bacteria from alpine permafrost in the Tianshan Mountains, northwestern China. *Res. Microbiol.* 157, 741-751.

Christner, B.C., B.H. Kvitko, II., and J.N. Reeve. 2003. Molecular identification of bacteria and eukarya inhabiting an Antarctic cryoconite hole. *Extremophiles* 7, 177-183.

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.

Dorigo, U., A. Bérard, and J.F. Humbert. 2002. Comparison of eukaryotic phytobenthic community composition in a polluted river by partial 18S rRNA gene cloning and sequencing. *Microb. Ecol.* 44, 372-380.

Ellis, R.J., P. Morgan, A.J. Weightman, and J.C. Fry. 2003. Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Appl. Environ. Microbiol.* 69, 3223-3230.

Felsenstein, J. 2009. PHYLIP (Phylogeny Inference Package) version 3.69. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, Washington, USA.

Hancock, P.J. 2002. Human impacts on the stream-groundwater exchange zone. *Environ. Manag.* 29, 763-781.

Ikner, L.A., R.S. Toomey, G. Nolan, J.W. Neilson, B.M. Pryor, and R.M. Maier. 2007. Culturable microbial diversity and the impact of tourism in Kartchner Caverns, Arizona. *Microb. Ecol.* 53, 30-42.

Jiang, H.L., S.T.L. Tay, A.M. Maszenan, and J.H. Tay. 2006. Physiological traits of bacterial strains isolated from phenol-degrading aerobic granules. *FEMS Microbiol. Ecol.* 57, 182-191.

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

Kirk, J.L., L.A. Beaudette, M. Hart, P. Moutoglis, J.N. Klironomos, H. Lee, and J.T. Trevors. 2004. Methods of studying soil microbial diversity. *J. Microbiol. Methods* 58, 169-188.

Labbé, D., R. Margesin, F. Schinner, L.G. Whyte, and C.W. Greer. 2007. Comparative phylogenetic analysis of microbial communities in pristine and hydrocarbon-contaminated Alpine soils. *FEMS Microbiol. Ecol.* 59, 466-475.

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 Press, New York, NY, USA.

Margesin, R., H. Dieplinger, J. Hofmann, B. Sarg, and H. Lindner. 2005. A cold-active extracellular metalloprotease from *Pedobacter cryoconitis*: production and properties. *Res. Microbiol.* 156, 499-505.

Margesin, R., P.A. Fonteyne, F. Schinner, and J.P. Sampaio. 2007. *Rhodotorula psychrophila* sp. nov., *Rhodotorula psychrophenolica* sp. nov. and *Rhodotorula glacialis* sp. nov., novel psychrophilic basidiomycetous yeast species isolated from alpine environments. *Int. J. Syst. Evol. Microbiol.* 57, 2179-2184.

Margesin, R., D. Labbé, F. Schinner, C.W. Greer, and L.G. Whyte. 2003a. Charcterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Appl. Environ. Microbiol.* 69, 3085-3092.

Margesin, R., C. Spröer, P. Schumann, and F. Schinner. 2003b. *Pedobacter cryoconitis* sp. nov., a facultative psychrophile from alpine glacier cryoconite. *Int. J. Syst. Evol. Microbiol.* 53, 1291-1296.

Margesin, R., G. Zacke, and F. Schinner. 2002. Characterization of heterotrophic microorganisms in alpine glacier cryoconite. *Arct. Antarct. Alp. Res.* 34, 88-93.

Mueller, D.R., W.F. Vincent, W.H. Pollard, and C.H. Fritsen. 2001. Glacial cryoconite ecosystems: a bipolar comparison of algal communities and habitats. *Nova Hedwigia* 123, 173-197.

Nocker, A., J.E. Lepo, and R.A. Snyder. 2004. Influence of an oyster reef on development of the microbial heterotrophic community of an estuarine biofilm. *Appl*. *Environ*. *Microbiol*. 70, 6834-6845.

Øvreås, L., S. Jensen, F.L. Daae, and V. Torsvik. 1998. Microbial community changes in a perturbed agricultural soil investigated by molecular and physiological approaches. *Appl*. *Environ*. *Microbiol*. 64, 2739-2742.

Paerl, H.W. 1998. Structure and function of anthropogenically altered microbial communities in coastal waters. *Curr*. *Opin*. *Microbiol*. 1, 296-302.

Powell, S.M., J.P. Bowman, I. Snape, and J.S. Stark. 2003. Microbial community variation in pristine and polluted nearshore Antarctic sediments. *FEMS Microb*. *Ecol*. 45, 135-145.

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

Saul, D.J., J.M. Aislabie, C.E. Brown, L. Harris, and J.M. Foght. 2005. Hydrocarbon contamination changes the bacterial diversity of soil from around Scott Base, Antarctica. *FEMS Microbiol*. *Ecol*. 53, 141-155.

Säwström, C., P. Mumford, W. Marshall, A. Hodson, and J. Laybourn-Parry. 2002. The microbial communities and primary productivity of cryoconite holes in an Arctic glacier (Svalbard 79°N). *Pol*. *Biol*. 25, 591-596.

Stibal, M., M. Šabacká, and K. Kaštovská. 2006. Microbial communities on glacier surfaces in Svalbard: impact of physical and chemical properties on abundance and structure of cyanobacteria and algae. *Microb*. *Ecol*. 52, 644-654.

Stibal, M., M. Tranter, L.G. Benning, and J. Řehák. 2008. Microbial primary production on an Arctic glacier is insignificant in comparison with allochthonous organic carbon input. *Environ*. *Microbiol*. 10, 2172-2178.

Takeuchi, N., S. Kohshima, and K. Seko. 2001. Structure, formation, and darkening process of albedo-reducing material (cryoconite) on a Himalayan glacier: a granular algal mat growing on the glacier. *Arct*. *Antarct*. *Alp*. *Res.* 33, 115-122.

Webster, N.S. and A.P. Negri. 2006. Site-specific variation in Antarctic marine biofilms established on artificial surfaces. *Environ*. *Microbiol*. 8, 1177-1190.

Wharton, R.A., Jr., C.P. McKay, G.M. Simmons, Jr., and B.C. Parker. 1985. Cryoconite holes on glaciers. *BioScience* 35, 499-503.