Vol. 27(2):205-214 | Ocean and Polar Research | June 2005

Article

Genomic DNA Extracted from Ancient Antarctic Glacier Ice for Molecular Analyses on the Indigenous Microbial Communities

SangHoon Lee[1]*, Kay Bidle[2], Paul Falkowski[2], and David Marchant[3]

[1]*Korea Polar Research Institute, KORDI*
Ansan P.O. Box 29, Seoul 425-600, Korea
[2]*Institute of Marine and Coastal Science, Rutgers University*
New Brunswick, NJ 08901, USA
[3]*Department of Earth Sciences, Boston University*
Boston, MA 02215, USA

Abstract : From ancient Antarctic glacier ice, we extracted total genomic DNA that was suitable for prokaryotic 16S rDNA gene cloning and sequencing, and bacterial artificial chromosome (BAC) library and end-sequencing. The ice samples were from the Dry Valley region. Age dating by $^{40}Ar/^{39}Ar$ analysis on the volcanic ashes deposited *in situ* indicated the ice samples are minimum 100,000-300,000 yr (sample DLE) and 8 million years (sample EME) old. Further assay proved the ice survived freeze-thaw cycles or other re-working processes. EME, which was from a small lobe of the basal Taylor glacier, is the oldest known ice on Earth. Microorganisms, preserved frozen in glacier ice and isolated from the rest of the world over a geological time scale, can provide valuable data or insight for the diversity, distribution, survival strategy, and evolutionary relationships to the extant relatives. From the 16S gene cloning study, we detected no PCR amplicons with *Archaea*-specific primers, however we found many phylotypes belonging to *Bacteria* divisions, such as *Actinobacteria, Acidobacteria, Proteobacteria* (α, β, and γ), *Firmicutes*, and *Cytophaga-Flavobacterium-Bacteroid.* BAC cloning and sequencing revealed protein codings highly identical to phenylacetic acid degradation protein paaA, chromosome segregation ATPases, or cold shock protein B of present day bacteria. Throughput sequencing of the BAC clones is underway. Viable and culturable cells were recovered from the DLE sample, and characterized by their 16S rDNA sequences. Further investigation on the survivorship and functional genes from the past should help unveil the evolution of life on Earth, or elsewhere, if any.

Key words : Microbial diversity, Antarctic bacteria, Glacial microbiology, Ancient genes

1. Introduction

Prokaryotes possess diverse and versatile metabolic capacities, which enable them to occupy almost every niche that one can imagine on Earth, including the extremely dry, sub-zero environments in the Antarctic. Microorganisms preserved frozen in glacier are the past history of life on this planet. Bacterial cells entombed in ice over a geological time scale are of our particular interest, since they are likely to have been isolated from the rest of the world under the frozen condition for that long period. If we could revive the frozen cells, or retrieve the genetic material from the cells preserved in the ice, they will provide valuable data or insight for the diversity, distribution, survival strategy, and evolutionary relationships to the extant relatives. They might also provide clues as to the origin and dispersal of the ice-trapped biota, or the paleoclimatic conditions at the time of the ice formation.

Recent studies reported phylogenetic diversities of ancient bacterial cells preserved in ice for 10^5-10^6 years (Christner *et al.* 2003, 2001; Miteva *et al.* 2004; Priscu *et*

*Corresponding author. E-mail : hoon@imcs.rutgers.edu
shlee@kopri.re.kr
[1]Present address : Institute of Marine and Coastal Science, Rutgers University, New Brunswick, NJ 08901, USA

al. 1999). These studies further demonstrated that portions of the cells are viable by showing signs of metabolism or by recovering them in cultures (Christner *et al.* 2003, 2001; Karl *et al.* 1999; Miteva *et al.* 2004). Finite descriptions on the physiology or metabolic features of cells would only be available from the cultures, i.e., living cells, but the vast majority of natural bacterial cells are not culturable (Lee and Fuhrman 1991). Often employed in searching for the bacterial diversity is the culture-independent molecular tool that uses 16S ribosomal DNA (rDNA) sequences directly obtained from environmental DNA (Pace *et al.* 1986; Ward *et al.* 1990), bypassing the need of cultures. One can characterize the diversity of prokaryotic phylotypes, based upon the 16S rDNA base sequences that are obtained from cloned 16S genes of mixed natural populations.

The scope of the clone library was vastly expanded, from 16S gene to the whole genomic DNA, with the technique of bacterial artificial chromosome (BAC) cloning (Shizuya *et al.* 1992), which was first incepted by Schmidt *et al.* (1991). The artificial chromosome technique was quickly deployed to microbial ecology for natural environmental samples, having the advantage of maintaining metagenomics without the 'culture' problem (Béjà *et al.* 2000). This approach interprets individual functional genes as a proxy to the metabolic features in real life, and thus making the living cultures not necessarily a must to study their physiology.

One of the ultimate goals of the metagenomics of environmental DNA is the reconstruction of individual single genome from a mixture of multiple genomes. The technique would require substantial amount of time and cost to sort out the myriad fragments of mixed multiple genomes. However, the sequence information obtained from the BAC cloning is yet useful in unveiling the genes and genomic structures of natural bacteria. For example, protein-coding gene sequences obtained from a BAC clone library measure the evolutionary distances between similar genes spread across different taxa in a quantitative term, i.e., nucleotide substitutions. With this technique, one can investigate the phylogenetic standings and metabolic features of bacteria simultaneously by identifying their phylotypes from the 16S gene as well as the functional genes from the total genome (Béjà *et al.* 2002). Genomic organization, genes, and their sequences from the ancient prokaryotes preserved in glacier ice should thus be a valuable asset of unparalleled significance in understanding the evolution of genes and species.

A recent study reported a discovery of a small lobe of an ancient glacier that avoided any reworking processes such as freeze-thaw cycles or sublimation, from the Dry Valley region in Antarctica (Sugden *et al.* 1995). In the study, age dating by $^{40}Ar/^{39}Ar$ analysis on the volcanic ashes deposited *in situ* indicated the ice is at least 8 million years old. They also presented multiple lines of evidence that the remnant glacier was derived from the expansions of the nearby Taylor glacier, and survived for the millions of years by being buried under the moisture-saturated frozen till layer (Marchant *et al.* 2002). This ice is the oldest known glacier ice on Earth to date.

We extracted environmental DNA from this ancient glacier ice. The DNA was suitable for molecular research, such as, diversity studies by polymerase chain reaction (PCR) amplification of 16S gene, cloning, and sequencing. BAC clones were successfully generated, and throughput sequencing is currently underway. In this study, we describe the earlier stages of our full-bore research, for example, methods for DNA sample preparations, the purity and integrity of the DNA, PCR work, and controls and blanks we ran parallel. Also presented are the preliminary results.

2. Materials and methods

Ice samples were from Beacon Valley in East Antarctica, where the Taylor glacier flows nearby and merges midway in the valley (Fig. 1). Ice sample EME98-03 (EME) is from the central Beacon, and DLE98-12 (DLE) is from the Mullins that is a short tributary valley in the upper Beacon (Fig. 1C). Both ice samples contained soil particles and rock debris of a broad size range, from fine silt to coarse sand granules (Fig. 2). This solid phase was poorly sorted and non-homogeneously distributed in the ice samples, and occasionally displayed faint patterns of irregular strata (not shown). Further descriptions on the sampling site and the glacier ice can be found in Sugden *et al.* (1995) and Marchant *et al.* (2002).

The ages of the ice bodies, determined with the volcanic ash on top of the sediment cap by cosmogenic-nuclide exposure-age dating method, are minimum 8.1 million years for the EME and 100,000 to 300,000 years for the DLE, respectively (Sugden *et al.* 1995). Data from ice crystallography, oxygen stable-isotope analysis, and ash dating, suggested that the EME ice is a remnant of the ancient basal glacier derived from an expansion of the adjacent Taylor glacier. The data further indicate that the ice bodies have been well preserved frozen during the entire age with no reworking processes (Marchant *et al.* 2002). All these results from the geological assays indicate

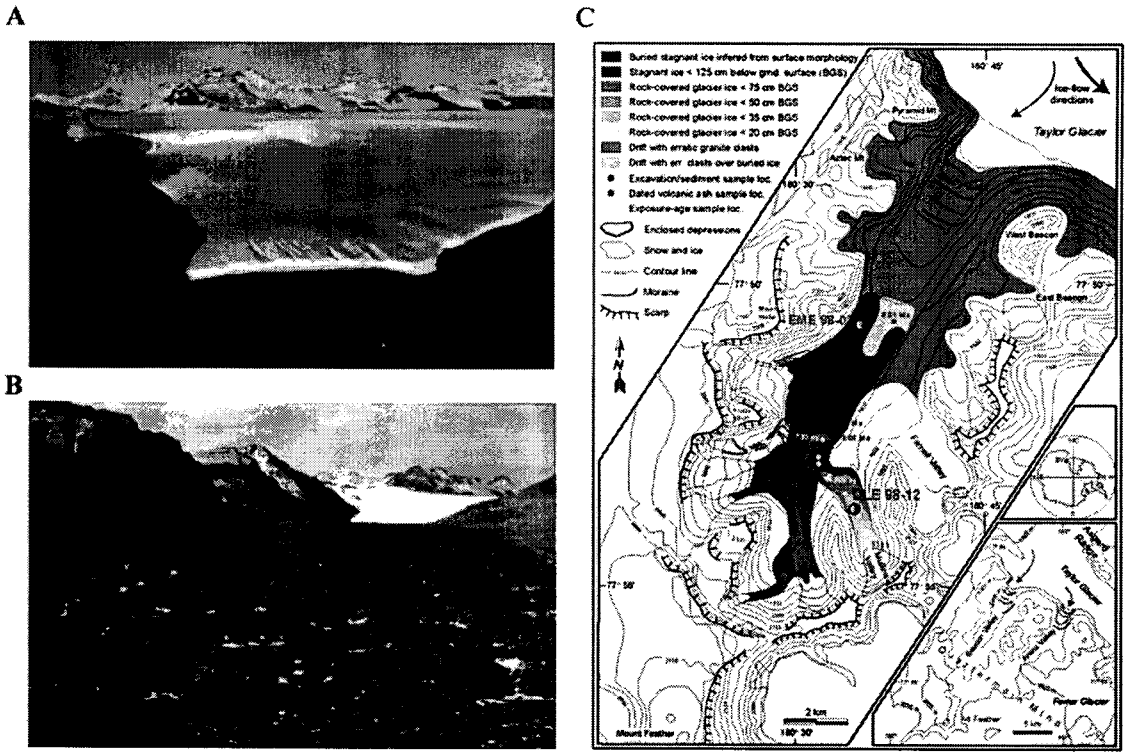

Fig. 1. Sampling sites in the Dry Valley region, Antarctica. Panel A, Taylor Glacier viewed at the entrance of the Beacon Valley. Panel B, Beacon Valley viewed down from the upper valley, note the surface soil till layer that covers the buried ice underneath. Panel C, aerial map of the sampling sites (denoted as EME 98-3 and DLE 98-12), with insets for further geographic reference.

Fig. 2. EME ice, a chunk piece separated from the sample body. Note the brownish color and opaqueness of the ice due to soil particles, which were also present in DLE ice but to a less extent. The two round drill-bit holes were made when aseptically subsampling for cultures.

that the bacteria secluded in the ice have been separated and preserved since late Miocene for EME, and late Pleistocene for DLE.

The ice was analyzed for pH, conductivity, and other selected biochemical properties. Chemistry of the both ice samples varied within a sample (data not shown), which is probably due to the non-homogeneous distribution of the solid phase (soil particles) within the ice. As shown in Fig. 2, the ice contained numerous soil particles of a wide size range. It is well documented that particle-associated microzones are relatively enriched in both biomass and organic substrate as compared to the surrounding frozen liquid, and thus serve as a source of rich biota in this barren environment (Priscu *et al.* 1998).

Bacterial cells in the melt water of the ice were first viewed by 4',6-diamidino-2-phenylindole (DAPI) epifluorescence microscopy (Porter and Feig 1980). However, viewed under the microscope, the melt water contained numerous abiotic particles of the size and the fluorescence intensity overlapping to those of bacteria (Fig. 3; panels A1 & B1). Though we could identify fluorescent bacterial cells, the interference from the abiotic particles complicated exact

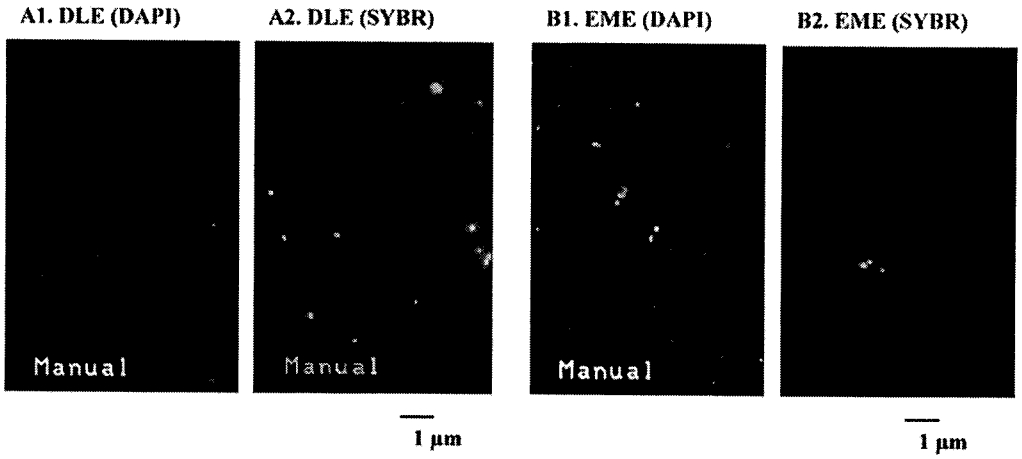

Fig. 3. Bacterial cells in the ice samples viewed by epifluorescence microscopy. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI; A1, B1), or SYBR Green I (A2, B2). Note the numerous, amorphous, dimly fluorescent particles in A1 and B1 (DAPI). SYBR Green I stain subdued the non-specific fluorescence from the abiotic particles in the background, and allowed a better estimation of the cell concentrations.

enumeration of the cells. In order to reduce the non-specific fluorescence and to obtain the cell concentrations with better precision, we later switched to SYBR Green I stain (Noble and Fuhrman 1998). From the SYBR-stained cell images (Fig. 3; panels A2 & B2), we estimated bacterial cell concentrations by including only those images we were positive for the bacterial cells. Estimates of the cell concentrations were, 5×10^5 cells ml^{-1} for DLE, and 3×10^4 cells ml^{-1} for EME, respectively. The cell concentrations of our ice samples are comparable to those from similar polar environments, such as, Antarctic lake water (Pearce 2003), lake ice cover (Priscu *et al.* 1998), cryoconite hole in ice (Säwström *et al.* 2002), and polar sea ice (Brinkmeyer *et al.* 2003), within an order of magnitude. However, the cell concentrations reported from Antarctic snow (Carpenter *et al.* 2000) or subglacial lake ice accretion (Karl *et al.* 1999) are ca. 2-3 orders lower than ours.

We attempted to recover viable cells from the ice melt water by smearing the melt water on moderately enriched plate media, but no colonies developed over a 5-month period of incubation. However, we successfully recovered and isolated viable cells in later attempts, where we pre-conditioned the cells for 2 months in the ice melt water *in situ* spiked with nutrients, and then spread the liquid culture on plate media. These results will be presented elsewhere.

Large chunk (ca. 1 kg) of ice, separated from the sample body, was thoroughly rinsed with copiously flowing Milli-Q water that ran through a 0.22-μm filter cartridge. The rinse ablated the exterior of the ice a few millimeters away. The cleaned ice was thawed in a sterile plastic bag at 18°C over night, and the melt water was handled aseptically afterwards from this point. Bacterial cells in the liquid phase were collected on 0.2-μm polycarbonate filters, and the cells associated with the solid phase were collected by centrifugation at 15,000 g for 20 min. For the DNA extraction, filters that collected particulate (>0.2 μm) material from the liquid phase were shredded and treated as the solid phase. We extracted genomic DNA from both the filters and the solid phase with SoilMaster kit (Epicentre), paying particular attention not to shear the high molecular weight genomic DNA.

From the genomic DNA, we amplified the eubacterial (*Bacteria*) 16S rDNA by PCR with 27F (forward) and 1492R (reverse) primers (Lane 1991; numbers refer to equivalent base positions of *Escherichia coli*). Because of the co-extraction of the impurities from soil particles, which often inhibit the enzymatic reaction, we had to optimize the PCR parameters. The PCR product was size-selected and cleaned by gel electrophoresis (QIAgen), and clone libraries (118 EME clones and 140 DLE clones) were generated from the 16S amplicon with Invitrogen TOPO TA Cloning kit and TOP10 chemically competent *E. coli* cells. We attempted to amplify the 16S rDNA of *Archaea* from EME and DLE ice with *Archaea*-specific primers (21F and 958R; DeLong 1992) under the same optimized PCR conditions.

The cloned 16S genes were sequenced with ABI BigDye kit for PCR and ABI 3100-Avant. The clones were first

de-replicated based on the similarity of the partial sequences (ca. 700 bp) generated with 341F primer (Lane 1991), and thus identical or similar (<2% sequence similarity) clones were grouped together. We selected unique clones, either standing alone or a representative of a group, for complete (ca. 1450 bp) sequencing with 27F and 1492R primers for flanking sides. Sequences that were suspected as heteroduplex or chimera were discarded by checking them on-line at the Ribosomal Database Project II web site (CHIMERA_CHECK). For an additional precaution, we also look for the robustness of the branching order in phylogenetic trees built with different portions of the 16S rDNA (Ludwig *et al.* 1997).

Despite the extreme usefulness, it is well known that PCR technique are vulnerable to the bias associated with the PCR amplification procedure, and to the contaminants potentially present in lab reagents or accidentally mixed in during sample handling (Suzuki and Giovannoni 1996; Tanner *et al.* 1998). We performed wipe test on the sample-handling tool occasionally; we smeared the wipe onto plate media, and incubated at room temperature. No contaminants were found from the wipe test.

In order to check for the contaminants that may have originated from the Milli-Q rinsing, routine lab handling, and lab reagents, we ran a PCR blank control in parallel, that was made of Milli-Q water. This blank control was 1 liter of Milli-Q water, which was frozen, thawed, filtered on a 0.2-μm polycarbonate filter, and subjected to the SoilMaster kit. The blank control was processed exactly the same as we did for the glacier ice up to the point of the PCR amplification of the 16S gene. We serially diluted the material that was extracted with the SoilMaster kit from the blank control filter, used it as PCR template, and examined by gel electrophoresis to check for any false-positive PCR product generated at different dilution levels.

We generated BAC clone libraries from the ice genomic DNA. A BAC cloning kit (Epicentre) was used; in brief, the genomic DNA was digested with BamH1 restriction enzyme (RE), ligated to a vector, introduced into a host cell, and the transformed cells were screened for on selective plates. We did not size-selected for the insert DNA, because we were handling extremely small amount (<100 ng) of very rare DNA. The vector and the host cell were specially designed to accommodate larger fragment insert (a few tens of kb) and able to maintain a single copy of ligate, i.e., plasmid vector harboring the insert, which is stable for an extended time period until multiple copies are chemically induced as needed. A small subset of the DLE BAC clone library was selected, and their plasmid DNA was prepared from the clone cultures. We examined the size of the inserts by running the plasmid on an agarose gel before and after the BamH1 digestion. From the plasmid prep, we sequenced the both end parts (ca. 650 bp-long) of the inserts, as a preliminary test prior to a throughput processing at The Institute for Genomic Research (TIGR; Rockville, MD, USA).

Even larger fragments (>35 kb) can be inserted and maintained as an artificial chromosome library by a fosmid cloning kit commercially available (Epicentre). The fosmid insert is the end-repaired genomic DNA that is sheared to an optimum size range (ca. >40 kb), which is greater than that of the BAC cloning. The costly genome reconstruction could benefit from the larger insert, which reduces the number of clones that have to be screened, because the whole genome is to be pieced together from the fragments. We are currently at the testing stage for fosmid cloning with a test DNA, using a fosmid library kit (Epicentre). The fosmid cloning was done as follows; the genomic DNA was first end-repaired, insert fragments were size-selected (larger than ca. 35 kb) by pulse-field gel electrophoresis, and ligated to a vector. The vector was then packaged into phage particles, and host cells were infected with the phage.

3. Results and discussion

In the earlier stages, we experienced difficulties in amplifying the target fragments by PCR from the ice DNA prepared with the SoilMaster kit, due presumably to the high levels of the impurities that co-eluted with the genomic DNA. We modified the protocols through a series of tests to reduce the excessive levels of the co-eluted impurities. In short, aliquots of the sample volume we loaded per purifying chromatographic column of the SoilMaster kit were reduced to less than half of the manufacturer's recommendation, in order to maximize the column performance and enhance the removal of the impurities. PCR optimization was also carried out; we reduced the PCR template amount, and thus the concentration of the enzyme-inhibiting impurities that co-existed with DNA could be lowered. In return to the reduced template, we increased the number of thermal cycles, which would compensate for the low initial amount of the template to begin with. In the end, we managed to obtain cleaner genomic DNA and PCR product subsequently. Fig. 4 shows the ice genomic DNA and PCR products amplified from 16S gene of *Bacteria*. We did not obtain any positive results with the *Archaea*-specific primers

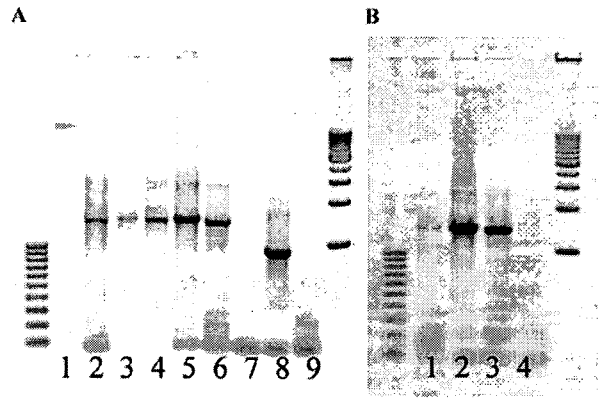

Fig. 4. Ice DNA and their PCR product with 16S gene primers. Panel A; genomic DNA from DLE ice (lane 1); *Bacteria* primers (27F, 1492R) to the DLE ice genomic DNA (lane 2); PCR product size-selected and purified from lane 2 (lane 3); *Bacteria* primers to a positive control template, e.g., *E. coli* DNA (lanes 4-6) and to a negative control (lane 7); *Archaea* primers (21F, 958R) to a positive control (*Haloferax volcanii*; lane 8) and to the DLE DNA (lane 9). Panel B; *Bacteria* primers to EME DNA (lane 1), to a positive control template (lanes 2 and 3), and to a negative control (lane 4). No PCR product was obtained from EME with *Archaea* primers. Flanking two lanes in each panel are molecular weight marker, 0.1-1.0 kb range with 0.1 kb spacing (left) and 1-15 kb range with 1 kb spacing (right).

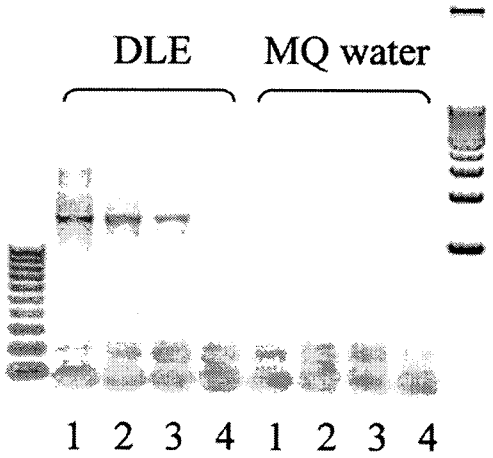

Fig. 5. PCR amplifications with serially diluted template to check for contaminations from potential sources, or during the sample handling. *Bacteria* primers were used. Label DLE denotes the template is DLE DNA. The 1-liter Milli-Q blank control (see text) was the template for the MQ water lanes. Flanking two lanes are molecular weight marker, 0.1-1.0 kb range with 0.1 kb spacing (left) and 1-15 kb range with 1 kb spacing (right). Lanes 1-4 present the template serially diluted, 1x, 5x, 25x, and 125x, respectively. Note that none of the blank control (MQ water) showed PCR product, whereas the PCR product disappears at 125x dilution of the DLE template. Dilution factors we estimate from our routine sample handling indicate that the presence of the Milli-Q water in our PCR reactions would be at the level << 125x. Our blank control verified that contaminations from whatsoever sources were not likely.

(21F and 958R; DeLong 1992), from neither EME nor DLE

No PCR product was detected from the blank control made of Milli-Q water (Fig. 5). The amount of Milli-Q water that might get into our sample preparation would be a few tens of ml at maximum, which is ca. 1/50 volume of the Milli-Q water that was filtered (1L). For the PCR amplification, we routinely diluted the template (ice genomic DNA) 10-50 times, as described above in the PCR optimization. Consequently, the final dilution factor of the Milli-Q water that might have been present in a sample PCR tube is ca. 1/500-1/2500 of the 1-liter control, i.e., 1× Milli-Q control (this is lane 1 of the MQ water in Fig. 5). This much of dilution is equivalent to 4 to 20-fold dilutions from the lane 4 (1/125) of the MQ water (Fig. 5), where no signals could be found. These results confirmed that there was little or no chance of false-true signals from any source of contamination during the sample handling, neither from the well-known potential contaminants in the reagents routinely used in such experiment, nor by accidental breach of the sterility. None of the 16S gene sequences we obtained was found closely related to the sequences from common contaminants reported by Tanner *et al.* (1998).

From the 16S gene clone library and sequencing, 11 phylotypes (EME) and 19 phylotypes (DLE) were finally identified. Fig. 6 shows the diversity of the ice microbial communities, with individual phylotypes and their frequencies, featuring a few dominant phylotypes and the rest in low frequencies. This is a typical pattern of the frequency distribution one would normally observe in nature, although the clone library may not be the exact replica of the true community composition, due to the potential bias in PCR (Suzuki and Giovannoni 1996). Instead, the cloned phylotypes and their frequencies would rather be on a probability basis for the re-presentation of the true 16S gene sequences and their frequencies. The kind of the phylotypes and their relationships to extant

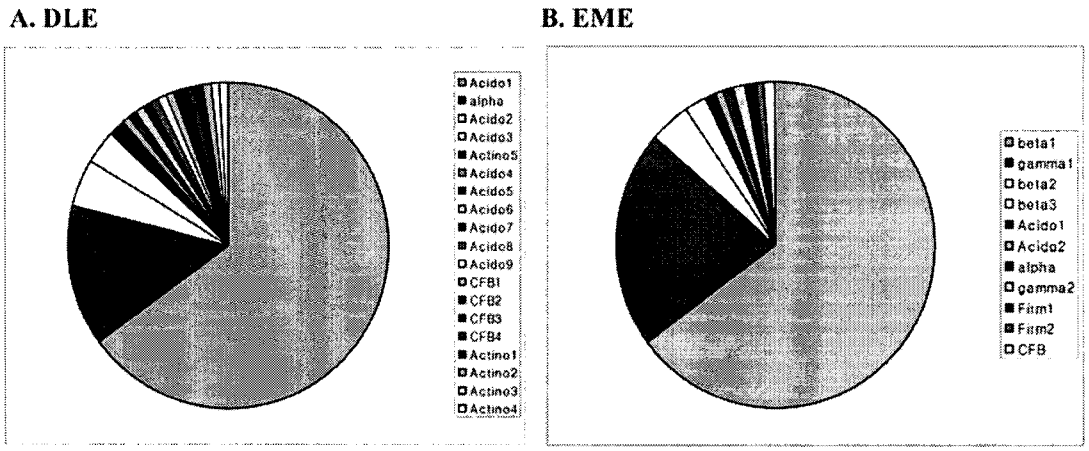

Fig. 6. Frequency of phylotypes. Abbreviations; Acido, *Acidobacteria*; Actino, *Actinobacteria*; alpha, beta, and gamma are the subdivisions of the *Proteobacteria*; CFB, *Cytophaga-Flavobacterium-Bacteroid* division; Firm, *Firmicutes*. Labelling and the pattern fills are given arbitrarily; identical ones between the two pie diagrams do not necessarily indicate the same phylotype.

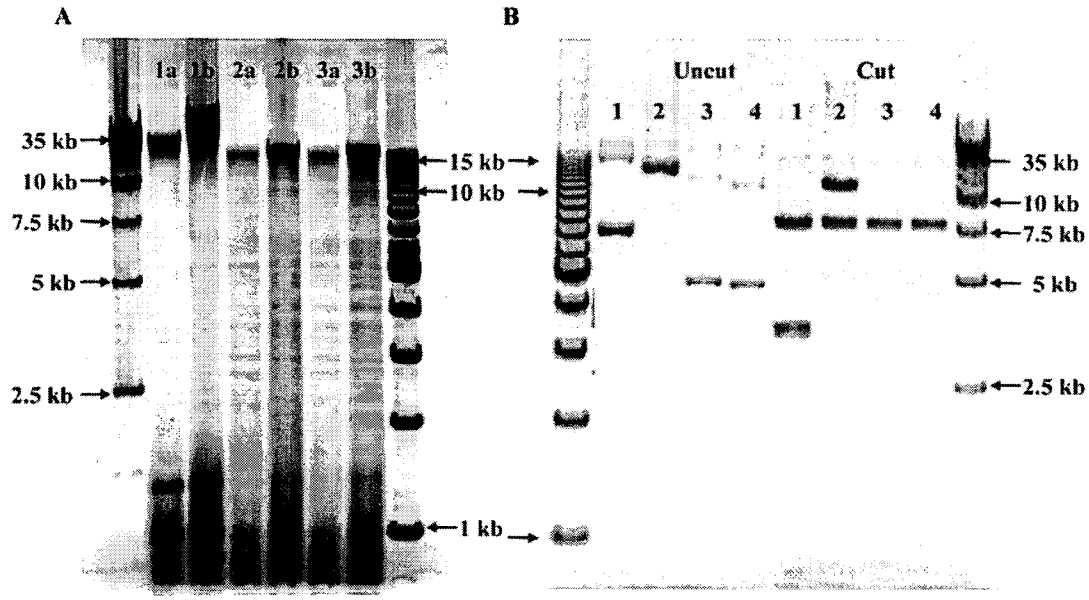

Fig. 7. BamH1 digested DNA (panel A) and the insert size of the BAC clone plasmid (panel B). Molecular markers are in the two flanking lanes in each panel (1-15 kb range with 1 kb spacing at the center, and 2.5 kb spacing from 2.5 to 35 kb at both sides). We used *E. coli* DNA for the presentation in panel A; lanes 1a and 1b, undigested DNA (a and b denote different concentrations); lane 2, 8 hr incubation; lane 3, 20 hr incubation. Panel B; intact plasmid DNA from 4 BAC clones (1 to 4 Uncut lanes) compared to the same plasmid DNA but digested with BamH1 (1 to 4 Cut lanes) to release the insert. Linearized vector appears at ca. 7.5 kb size in panel B Cut lanes.

species by BLAST match will be presented elsewhere.

Since the ice DNA that was available for the lab experiments was in an extremely small quantity (<100 ng), we could not afford to spend it for routine tests and gel picture presentations. Presented in Fig. 7A is not from the ice DNA, but from *E. coli* DNA that has been treated equally as the sample ice DNA. There is no guarantee that the test DNA would behave exactly the same as the ice DNA, but the test DNA could help render guidance in search of the optimal settings for the real samples without

wasting them. Under the BamH1 restriction enzyme (RE) digestion conditions we set up, incubation times of >4 hrs yielded visible RE-digested fragments (data not shown). Twenty hours of RE digestion did not show any recognizable difference from the 8-hr RE digestion (Fig. 7A). We chose 12-hr incubation for the BamH1 digestion of the ice DNA, a complete digestion rather than a partial one that may produce DNA fragments unable to ligate to the vector due to the absence of the RE site on one end. Consequently, partial digestion may result in a low ligation efficiency, which was not deemed desirable under the circumstances that we had very little DNA. The gel picture of Fig. 7A shows that the DNA fragments (bands) produced from 8 & 20-hr RE digestions are wide spread over ca. 3-30 kb ranges.

We prepared plasmid DNA from the BAC clones of DLE DNA in order to verify the presence of the insert and its size. We also obtained preliminary sequence data from the inserts. Fig. 7B shows the gel picture of the plasmid prep before and after BamH1 digestion, where the inserts were separated from the vector by the RE digestion. The vector, of which size is 8 kb according to the manufacturer, shows up at corresponding positions, slightly above 7.5 kb, on the right side of Fig. 7B (the 'cut' plasmid). In Fig. 7B, plasmid in the lane #1 and #2 turned out to contain inserts of 3.5 and 11 kb, respectively, however those in lane #3 and #4 are self-ligated (closed without insert). Judging from the migration distance of the self-ligated plasmid compared to the RE-digested (cut) one, it seems like the self-ligated plasmid has a secondary folding or coiled structure that alters the migration rate from the linear (cut) form.

Sequence data were obtained from the end portions (ca. 650-bp long) of the inserts, as we utilized the forward and reverse priming sites on the vector located at two flanking positions nearby the open gap for the insert. The nucleotide sequence was BLAST-matched to known protein sequences at the NCBI site. We were not able to identify many of the sequences, because the protein BLAST matching did not fetch known amino acid sequences, due primarily to the lack of the data base, which is yet to grow at this moment. However, many sequences matched with >60% identity and scores of >200 bits to well-known sequences, for example, phenylacetic acid degradation protein paaA, chromosome segregation ATPases, or cold shock protein B. A complete sequencing of the BAC clone libraries is underway through a collaboration with TIGR. Once revealed, gene sequences, genetic structure and organization, and other genetic information from the ancient bacteria will

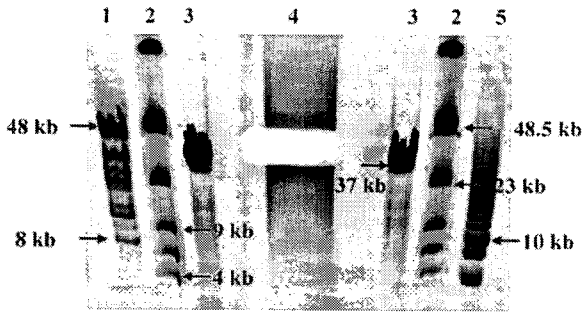

Fig. 8. Pulse-field gel elctrophoresis of end-repaired DNA for fosmid library generation. Lanes 1, 2, 5 are molecular markers; lane 3 is the molecular standard (37 kb) for fosmid inserts; lane 4 is the end-repaired test DNA. Insert to the fosmid vector was purified from the white block of lane 4.

contribute a tremendous asset to the foundation for understanding the ancient life forms and their evolution.

The size of the inserted DNA fragments in the BAC clones turned out relatively short, although short inserts do have their own advantage in practical applications. Seeking a method for larger inserts, we looked into the possibility of fosmid clone library made from the ice DNA. Via fosmid cloning, even larger fragments (>35 kb) can be inserted and maintained as an artificial chromosome library. Costly genome reconstruction could benefit from the larger insert size, which could reduce the number of clones to be screened. Fig. 8 shows the selected size range of the fragments to be inserted into fosmid clones from pilot tests with *E. coli* DNA. We have not run the ice samples yet, but from pilot tests performed with *E. coli* DNA, we obtained positive colonies on selective media. We also verified the inserts and their size from the test fosmid clones (data not shown).

We have successfully extracted and purified the environmental genomic DNA that was preserved in a glacier ice for millions of years. We have proven the potential of the ancient DNA by demonstrating that the DNA was suitable for a variety of leading-edge molecular applications that would bring significant findings. Many of prokaryotes retain the physiological features of the earlier life forms that were best adapted to then prevailing environmental conditions. It is their metabolic versatility that made them so successful and ubiquitous along the evolutionary pathway. Further investigations on the ancient environmental DNA will shed light on the genetic features of the earlier life forms, with greater impacts ultimately on the evolution and adaptations of life on Earth.

We are currently investigating on the viability of the

bacterial cells from the ancient glacier ice. We were able to recover viable and culturable cells from the DLE ice (300,000 yr old), but not yet from the 8 Myr-old EME ice. Recent studies proposed the significance of the microbial biota associated with particles, as they could serve as a reserve and seed of life in this barren region (Priscu *et al.* 1998). If bacterial cells could survive millions of years in this protected reserve, there is a possibility of life surviving the time needed for inter-planetary travel. Dazzling research ideas may also rise from questions like whether the revived bacteria would tread on the same evolutionary track as their siblings did in the past on this planet, or anywhere else, if any.

Acknowledgements

This study is supported by Rutgers University Fellowship for visiting scientist (S. Lee), US National Science Foundation Polar Program, Korea Antarctic Research Program (PP04102, PP04104), and Moore Foundation.

References

Brinkmeyer, R., K. Knittel, J. Jürgens, H. Weyland, R. Amann, and E. Helmke. 2003. Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. *Appl. Environ. Microbiol.*, 69, 6610-6619.

Béjà, O., L. Aravind, E.V. Koonin, M.T. Suzuki, A. Hadd, L.P. Nguyen, S.B. Jovanovich, C.M. Gates, R.A. Feldman, J.L. Spudich, E.L. Spudich, and E.F. DeLong. 2000. Bacterial rhodopsin: Evidence for a new type of phototrophy in the sea. *Science*, 289, 1902-1906.

Béjà, O., M.T. Suzuki, J.F. Heidelberg, W.C. Nelson, C.M. Preston, T. Hamada, J.A. Eisen, C.M. Fraser, and E.F. DeLong. 2002. Unsuspected diversity among marine aerobic anoxygenic phototrophs. *Nature*, 415, 630-633.

Carpenter, E.J., S. Lin, and D.G. Capone. 2000. Bacterial activity in South Pole snow. *Appl. Environ. Microbiol.*, 66, 4514-4517.

Christner, B.C., E. Mosley-Thompson, L.G. Thompson, and J.N. Reeve. 2003. Bacterial recovery from ancient glacier ice. *Environ. Microbiol.*, 5, 433-436.

Christner, B.C., E. Mosley-Thompson, L.G. Thompson, and J.N. Reeve. 2001. Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. *Environ. Microbiol.*, 3, 570-577.

DeLong, E.F. 1992. Archaea in coastal marine environments. *Proc. Nat. Acad. Sci. USA.*, 89, 5685-5689.

Karl, D.M., D.F. Bird, K. Björkman, T. Houlihan, R. Shackelford, and L. Tupas. 1999. Microorganisms in the accreted ice of Lake Vostok, Antarctica. *Science*, 286, 2144-2147.

Lane, D.J. 1991. 16S/23S rRNA sequencing. p. 115-175. In: *Nucleic acid techniques in bacterial systematics*. eds by E. Stackebrandt and M. Goodfellow. Wiley, New York.

Lee, S. and J. Fuhrman. 1991. Species composition shift of confined bacterioplankton studied at the level of community DNA. *Mar. Ecol. Prog. Ser.*, 79, 195-201.

Ludwig, W., S.H. Bauer, M. Bauer, I. Held, G. Kirchhof, R. Schulze, I. Huber, S. Spring, A. Hartmann, and K.H. Schleifer. 1997. Detection and in situ identification of representatives of a widely distributed new bacterial phylum. *FEMS Microbiol. Lett.*, 153, 181-190.

Marchant, D.R., A.R. Lewis, W.M. Phillips, E.J. Moore, R.A. Souchez, G.H. Denton, D.E. Sugden, N. Potter, Jr., and G.P. Landis. 2002. Formation of patterned ground and sublimation till over Miocene glacier ice in Beacon Valley, southern Victoria Land, Antarctica. *Geol. Soc. Am. Bull.*, 114, 718-730.

Miteva, V.I., P.P. Sheridan, and J.E. Brenchley. 2004. Phylogenetic and physiological diversity of microorganisms isolated from a deep Greenland glacier ice core. *Appl. Environ. Microbiol.*, 70, 202-213.

Noble, R.T. and J.A. Fuhrman. 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquat. Microb. Ecol.*, 14, 113-118.

Pace, N.R., D.A. Stahl, D.J. Lane, and G.J. Olsen. 1986. The analysis natural microbial populations by ribosomal RNA sequences. *Adv. Microb. Ecol.*, 9, 1-55.

Pearce, D.A. 2003. Bacterioplankton community structure in a maritime Antarctic oligotrophic lake during a period of holomixis, as determined by denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridization (FISH). *Microb. Ecol.*, 46, 92-105.

Priscu, J.C., E.E. Adams, W.B. Lyons, M.A. Voytek, D.W. Mogk, R.L. Brown, C.P. McKay, C.D. Takacs, K.A. Welch, C.F. Wolf, J.D. Kirshtein, and R. Avci. 1999. Geomicrobiology of subglacial ice above Lake Vostok, Antarctica. *Science*, 286, 2141-2144.

Priscu, J.C., C.H. Fritsen, E.E. Adams, S.J. Giovannoni, H.W. Pearl, C.P. McKay, P.T. Doran, D.A. Gordon, B.D. Lanoil, and J.L. Pinckney. 1998. Perennial Antarctic lake ice: an oasis for life in a polar desert. *Science*, 280, 2095-2098.

Porter, K.G. and Y.S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.*, 25, 943-948.

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°C). *Polar Biol.*, 25, 591-596.

Schmidt, T.M., E.F. DeLong, and N.R. Pace. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.*, 173, 4371-4378.

Shizuya, H., B. Birren, U.J. Kim, V. Mancino, T. Slepak, Y. Tachiiri, and M. Simon. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA

in *Escherichia coli* using an F-factor-based vector. *Proc. Nat. Acad. Sci. USA.*, 89, 8794-8797.

Sugden, D.E., D.R. Marchant, N. Potter, Jr., R.A. Souchez, G.H. Denton, C.C. Swisher III, and J.-L. Tison. 1995. Preservation of Miocene glacier ice in east Antarctica. *Nature*, 376, 412-414.

Suzuki, M.T. and S.J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixture of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.*, 62, 625-630.

Tanner, M.A., B.M. Goebel, M.A. Dojka, and N.R. Pace. 1998. Specific ribosomal DNA sequences from diverse environmental settings correlated with experimental contaminants. *Appl. Environ. Microbiol.*, 64, 3110-3113.

Ward, D.M., R. Weller, and M.M. Bateson. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature*, 345, 63-65.

Received Dec. 30, 2004
Accepted Apr. 26, 2005