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Bacterial Diversity in Malan Ice Core from the Tibetan Plateau

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ABSTRACT. Three ice core samples were collected from the Malan ice core drilled from the Tibetan Plateau, and three 16S rDNA clone libraries by direct amplification from the ice-melted water were established. Ninety-four clones containing bacterial 16S rDNA inserts were selected. According to restriction fragment-length polymorphism analysis, 11 clones were unique in the library from which they were obtained and used for partial sequence and phylogenetic analysis, and compared with 8 reported sequences from the same ice core at depth 70 m. Differences among the samples were apparent in clone libraries. The phylotypes were dominated by the *Proteobacteria* group, *Acinetobacter* sp. and *Cytophaga–Flavobacterium–Bacteroides* (CFB) group. They accounted for 92.5 % (*Proteobacteria*), 100 % (*Acinetobacter* sp.), 34.4 % (CFB) and 100 % (β-*Proteobacteria*) in the clone libraries from the samples at ice depths 35, 64, 70, and 82 m, respectively. The *Acinetobacter* sp. was only found in the deposition at ice depth 82 m and closely clustered with γ-*Proteobateria*. Two members (Malan A-21 and 101) of α-*Proteobacteria* from the sample of 35 m and two (Malan B-26 and 48) of β-*Proteobacteria* of 64 m were loosely clustered (<95 %similarity) with known bacteria, represented new genera in ice bacteria.

With the increasing interest in polar microbes and the application of molecular biological techniques to ecological science, microbial diversity studies of deep glacial sheets have become feasible. For example, the 16S rDNA sequences of bacterial isolates from the Antarctic Vostok ice core at depth 3593 m and from Guliya Ice core (The Tibetan Plateau, China) and Sajama (Bolivia), Canada glacier (CanClear), and from Taylor Dome, and Siple Dome (SIA) in Antarctica were compared by using the polymerase chain reaction (PCR; Christner *et al*. 2000). These studies led to the conclusion that deep-glacial ice-sheet bacteria are diverse and largely represent novel groups of organisms, at least compared to the tropical and temperate continental habitat from which most of the sequences in the database have been retrieved.

The Tibetan Plateau, one of the most imposing topographic features on the Earth, has a mean elevation of about 4.5 km and a glacial area of about 5700 km^2 (Shi and Wang 1981), and displays relatively new features of the global glacial environment. The extreme cold and the resulting formation of deep ice sheets offer a unique habitat where microorganisms entrapped by aeolian processes into glacial ice have to develop a mechanism to adapt to the extreme condition under a long term of selected stress, and numerous bacteria have survived for a long time (Abyzov 1993; Christner *et al*. 2000; Zhang *et al*. 2002; Wahlström and Danilov 2003). The current collection of the plateau glacial microbial small-subunit ribosomal RNA-encodning DNA sequences (16S rDNA) also provides a good independent evolution frame with the ice core depth.

The top question concerning ice core prokaryotes is how the composition of bacterial communities that evolved in perennial ice cover has diverged substantially from those in terrestrial sediments. The general view is that the bacteria recovered from deep glaciers are very similar to those in continental sediments. For example, the study of viable bacteria immured in different glacier ice from polar or nonpolar such as Sajama (Bolivia) ice core and Guliya ice core from the Tibetan Plateau (China) showed that most of the bacteria so far isolated are closely related to species found ubiquitously in environmental samples from around the world (Christner *et al*. 2000). On the other hand, in terms of 16S rDNA sequences, some bacteria recovered from these different glaciers appeared to be highly similar to those from Antarctic sea ice and from deep marine sediment, or to species of *Bacillus*, mycobacteria, *Micrococcus*, *Brevibacterium*, *Planococcus*, *Arthrobacter*,

Clavibacter, and *Friedmanniella* that were isolated previously from Siberian permafrost and tundra soil, the Canadian high Arctic, Dry Valley rock and soil, or sea ice (Siebert *et al*. 1988; Gosink *et al*. 1995; Bowman *et al.* 1997; Schumann *et al*. 1997; Shi *et al.* 1997; Zhou *et al.* 1997; Junge *et al.* 1998). Moreover, some psychrophilic novel strains from one glacier are related to isolates from other ice cores. For instance, a 200-year-old Guliya ice isolate closely related to *C. michiganensis* was obtained from 12 000-year-old Sajama ice samples (Christner *et al*. 2000). The consistent isolation of related microbes from such geographically diverse frozen environments suggests that these species may indeed have features that confer resistance to freezing and extended survival under frozen conditions.

A related question concerns the similarities or differences of bacterial populations in the ice core. Although air masses leading to precipitation associated with ice microbes in the region are the summer monsoon from the Indian Ocean and from the Bay of Bengal, and a subtropical westerly jet stream, the intensity of the summer monsoon and the distribution of precipitation in this region with a time scale are quite different. The isolation of ice core with the global air circulation suggests that ice core prokaryotes at different depth may have evolved independently, as have many other polar species (Gosink *et al.* 1998). However, there is little information on the phylogenetic composition of bacterial assemblages and the similarities or differences of the microbial communities found in these different ice core depths. Although data are emerging for the Tibetan Plateau glaciers (Christner *et al*. 2000; Zhang *et al.* 2002), there have been no comparable studies of the plateau ice sheets.

This study presents the sequence analyses of the 16S rDNA gene amplified directly from the Malan ice core with two objectives: (*1*) to provide phylogenetic characterization of ice core bacteria that could be used to test evolution hypotheses about bacterial biogeography, and (*2*) to evaluate the spatial and temporal variation in the distribution of the Malan glacier main phylotypes by a cloning and sequencing approach, recognizing the effect of global climate on the community composition.

MATERIALS AND METHODS

Sample collection. The ice core used in this study was drilled from the Malan ice cap (35°48.40'N, 90°35.34'E, 6000 m asl); we obtained three ice samples at 35 m (Malan A), 64 m (Malan B), and 82 m (Malan D) depths. The samples were selected after examining the curve of δ^{18}O of 30 points smoothing of the melt water. These samples were selected to represent a cool, a warm and a cool period (Yao *et al.* 2003), respectively. The 10 mm outer layer of the ice core sections was sliced from the surface with a saw-toothed knife sterilized with ethanol. The inner core was rinsed with 95 % cold ethanol. All core handling was conducted under a sterile, positive-pressure laminar flow hood; sterile gloves, clean laboratory clothing, and hair covering were worn during manipulation. The decontaminated samples were then completely melted at room temperature in a clean and sterile glass beaker. The control core was processed and melted by methods identical with those of the sample.

Direct amplification of 16S rDNA molecules from melt water. All reagent transfers were undertaken within a sterilized laminar flow hood. Before use, all reaction tubes and micropipette tips were autoclaved, and all solutions, except for the *Taq* DNA polymerase (2.5 U; *Promega*), were passed through sterile 0.2-μm filters. Melt water (1, 30 or 50 μL aliquots) was added directly to PCR mixtures (final volume of 100 μL) that contained 5 pmol of the primers 8f (forward, 5′-AGA GTT TGA TCA TGG CTC AG-3′) and 1492r (reverse, 5′-CGG TTA CCT TGT TAC GAC TT-3′). Reaction mixtures were placed for 9 min at 95 °C, and then subjected to 43 cycles of PCR amplification by incubation for 1 min at 94 °C, 1 min at 52 °C and 2 min at 60 °C with a final extension for 5 min at 60 °C. Samples of the PCR products were subjected to agarose gel eletrophoresis and visualized by ethidium bromide staining. DNA molecules of the expected length (≈1500 bp) were amplified.

Clone library construction. Products of three parallel PCR were combined and precipitated to concentrate the DNA for cloning. DNA was ligated in the pMD 18-T vector according to the protocol of the manufacturer (*TakaRa*, Japan). Ligation reaction mixtures were purified and transformed into competent *E. coli* JM109 cells. The transformed cells were plated on Luria–Bertani (LB) plates containing 100 μg/mL ampicillin. Recombinant transformants were selected by blue and white screening.

ARDRA analysis. Amplified rDNA restriction analysis (ARDRA) was performed to analyze the diversity of clones within each ice core layer. Isolated plasmid DNA of 16S rDNA clones were used as templates for insert amplification. The PCR was performed as follows: 1 cycle for 7 min at 95 °C; 30 cycles for 1 min at 94 °C, for 1 min at 56 °C, for 2 min at 72 °C, and 1 final extension for 2 min at 60 °C. PCR products were purified and aliquots of 200 to 400 ng of the amplified insert were digested with 7.5 U of the restriction

endonuclease *Hae*III (*TakaRa*) for 3 h at 37 °C. The resulting fragments were analyzed on a 1 % agarose gel, and restriction patterns within each ice layer were compared.

Sequencing and phylogenetic analysis. Representatives of all major ARDRA patterns were chosen for sequencing. Plasmid DNA from selected 16S rDNA clones was sequenced (partially) by Sequencer ABI PRISM 377-96 with universal rRNA-specific primers. A total of 11 clones were sequenced partially. All sequences were checked for chimera formation with CHECK-CHIMERA software of the Ribosomal Database Project (Maidak *et al.* 1996). Sequence data were analyzed by pairwise sequence alignment and by multi-alignment with the Lasergene program Megalign (*DNAStar*), and compared to known sequences by using the basic local alignment search tool (BLAST) (Altschul *et al.* 1990). Phylogenetic analysis was performed by MEGA (Molecular Evolutionary Genetics Analysis 1.01; Kumar *et al*. 1993) by using evolutionary distances and the neighbor-joining method.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper appeared in the GenBank nucleotide sequence databases under the accession no. AY322483–AY322493.

RESULTS AND DISCUSSION

Populations of 16S rDNA molecules were amplified directly from the melt water of three samples (Malan A, Malan B, and Malan D) at different ice core depths by using universal primers. A total of 210 white clones were chosen from the 3 clone libraries at random and used for plasmid analysis. Of the 210 clones screened, 94 contained inserts. Eleven phylotypes (Unique ARDRA patterns or operational taxonomic unit, OTUs) were detected by restriction fragment-length polymorphism analysis (RFLP) and used in the phylogenetic analyses, and the phylogenetic tree was drawn from the total of 19 sequences including 8 cloned sequences from Malan C of the Malan ice core at depth 70 m (Zhang *et al.* 2003) (Table I). Fig. 1 represents the phylogenetic affiliations and similarity values of the most closely related GenBank sequences for all of the sequences obtained in four samples from the Malan ice core at depth 35 m (Malan A), 64 m (Malan B), 70 m (Malan C), and 82 m (Malan D). The sequences were assigned to 5 phylogenetic clades.

Table I. Summary of the 16S rDNA sequences identified in the Malan ice core

Cluster[a]	Malan phylotype	%[b]	Clones[c]	Closest sequence[d] (Accession no.)
α	A-21	14.8	8	*Ochrobactrum anthropi* (AJ242578)
	A-101	18.5	10	uncultured bacterium (AY274164)
	A-104	27.8	15	uncultured bacterium (AY274164)
β	A-7	7.40	4	*Aminomonas minovorus* (AY027801)
	A-33	14.8	8	β-*Proteobacterium* (AJ318109)
	B-26	40.0	8	*Aquabacterium* sp. (AF089858)
	B-48	60.0	12	*Pseudomonas lanceolata* (AB021390)
γ	A-86	9.20	5	*Halomonas* sp. BYS-1 (AY062217)
	C-33	6.56	4	uncultured bacterium (AJ290044)
	D-10	60.0	12	*Acinetobacter johnsonii* (AF188300)
	D-11	40.0	8	*Acinetobacter* sp. (AY055373)
CFB[e]	A-38	7.40	4	uncultured bacterium (AY187882)
	C-2	26.2	16	*Flectobacillus* sp. (AF182020)
	C-P39	8.20	5	*Flavobacterium* sp. (U85888)
	C-P45	6.56	4	uncultured bacterium (AJ290028)
Other eubacterial groups	C-13	9.84	6	agricultural soil bacterium (AJ252627)
	C-37	16.4	10	*Marinobacter* sp. (U85863)
	C-9	4.92	3	uncultured eubacterium (AJ232784)
	C-P22	4.92	3	uncultured bacterium (AJ277700)

[a]Phylogenetic cluster (*see* Fig.1).
[b]Cluster percentage in the clones with inserts from the clone library where they were obtained.
[c]Number of clones corresponding to the type sequence based on direct sequence comparison or inferred from ARDRA patterns.
[d]Determined by BLAST.
[e]Two cloned sequences from Malan C sample, which sequenced from prime 1492r, were not used for phylogenetic analysis.

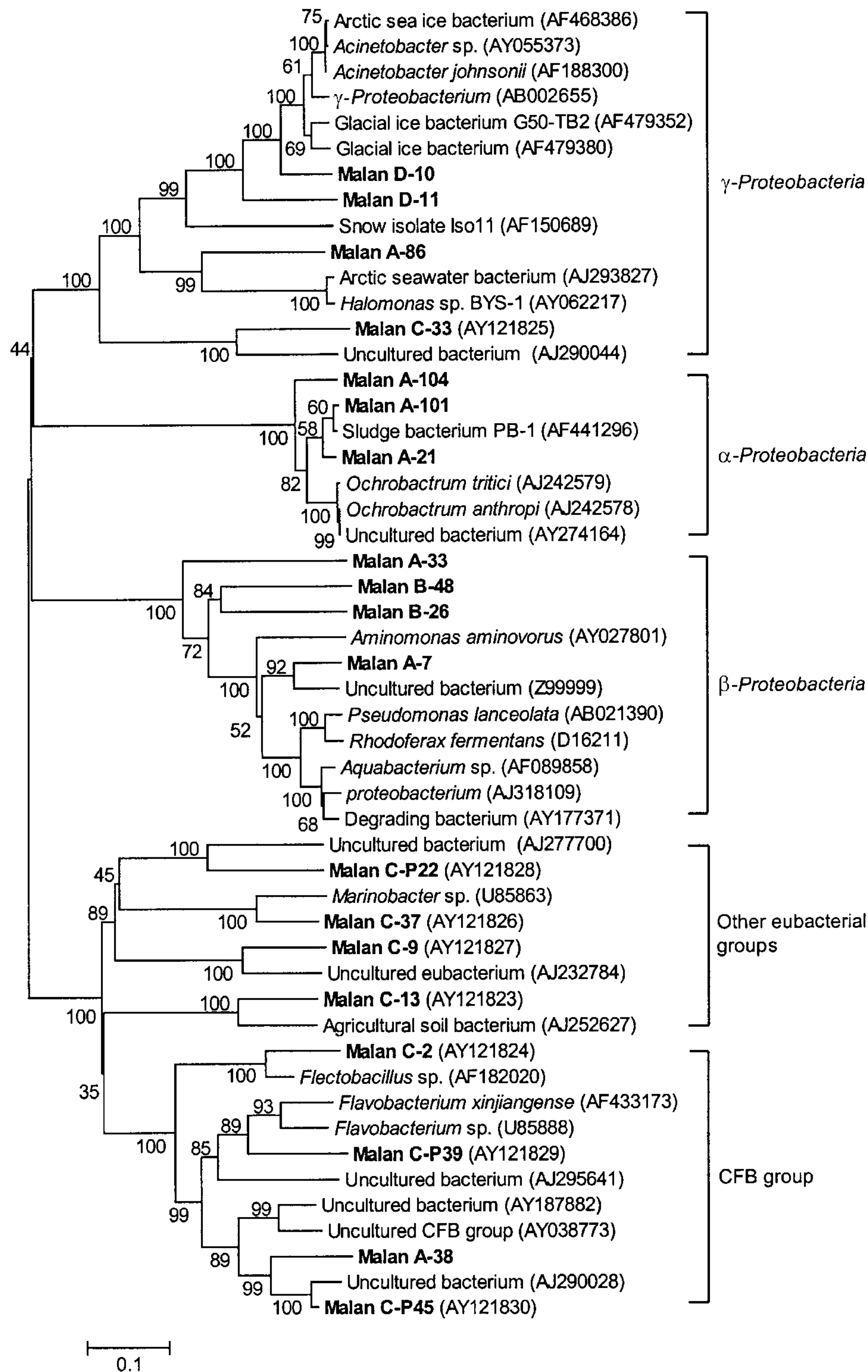

Fig. 1. Neighbor-joining tree showing phylogenetic relationships of 16S rDNA sequences cloned from four ice samples (Malan **A**, **B**, **C**, and **D**) to closely related sequences from GenBank; bootstrap values of >50 % (of 100 iterations) were obtained by maximum parsimony analysis for bootstrap sampling of 100; *scale bars* indicates *p*-distance.

The majority of the sequences were associated with *Proteobacteria* (groups α, β, and γ) (Table I and Fig. 1), of which 61.1 % were α-*Proteobacteria* in Malan A sample, 100 % were γ-*Proteobacteria* in Malan D sample, 22.2 % and 100 % grouped with β-*Proteobacteria* in Malan A and B sample, respectively. Our results agree with the previous analysis of a subset of ice sample from Lake Vostok accretion ice in the Antarctic. The

Proteobacteria group dominated in the clone library from Vostok accretion ice, and the cloned sequences fell into *Proteobacteria* (groups α and β) and accounted for 82.5 % (Christner *et al*. 2000).

α-*Proteobacteria*. The α-*Proteobacteria* group was only detected in sample Malan A. One cloned sequence (Malan A-104) was closely related to the uncultured bacterium *Integron* (100 % similarity) from 'heavy'-metal-contaminated mine tailings, and 100 % similar to *Ochrobactrum anthropi* and denitrifying strain *Ochrobactrum trici* TG23 capable of degrading phenol and reducing nitrate in soil. Two clones contained a sequence (Malan A-21 and Malan A-101) that clustered (82 %) with *O. anthropi*, and *O. trici* TG23, and were less similar (58 % and 60 %, respectively) to PAH-contaminated sludge bacterium PB-1 from an acidic oil refinery sludge. The two sequences with lower similarity to known sequences in GenBank perhaps belong to new genera. Although there were only three α-*Proteobacteria* clones in all detected Malan ice core samples, they dominated in ice sample Malan A, and played an important role in the microbial biomass at 35 m deposition where the total microbial concentration was very high (320 cells per mL). The clones from a deposition during the most extreme cold period with very low average value of δ^{18} (–162 ppm) (Yao *et al.* 2003) indicate their capacity to adaptation to an extremely cold environment.

β-*Proteobacteria*. A total of 32 sequences (22 and 100 % of all sequences obtained in sample Malan A and Malan B, respectively) belonged to the β-*Proteobacteria*. One sequence (Malan A-33) was a deep lineage within *Aquabacterium* sp. (100 % similarity) isolated from a drinking water biofilm, and was closely similar (100 %) to a phenanthrene-degrading bacterium from soil during long-term exposure to phenanthrene. One cloned sequence (Malan A-7) was highly clustered (100 % similarity) with *Aminomonas aminovorus*, and was closely related (92 % similarity) to an uncultured freshwater bacterium. The sequences contained in clones Malan B-48 and Malan B-26 were related (72 % similarity) to the subcluster of *A. aminovorus*, *Pseudomonas lanceolata*, and *Aquabacterium* sp. The occurrence of the member of β-*Proteobacteria* in both sample A and B indicated some similarity of the community composition at different ice depth, although these two ice depositions represented different climatic conditions (*i.e.*, A as a representative of warm period, and B as one of cool stage). The difference in the β-*Proteobacteria* group from two ice depositions also reflects the influence of the climatic and environmental change on bacterial evolution. As shown in Fig. 1, the sequence Malan A-7 closely clustered with Malan A-33 from the melt-water at the same ice depth and so did the sequence from Malan B-26 with Malan B-48.

γ-*Proteobacteria*. Twenty nine clones from samples Malan A, C, and D grouped into the γ-*Proteobacteria*, and were closely related (99–100 %) to the isolates from Arctic seawater, sea ice, glacial ice in the Antarctic and on the Tibetan Plateau, and snow ice in the Elbe River of Germany. One cloned sequence Malan A-86 was 99 % similar to phenylacetic-acid-degrading strain *Halomonas* sp. Bys-1, and Arctic seawater bacterium. One sequence containing inserts was 100 % identical to uncultured bacterium GKS2-30 from lake water. Only two cloned sequences were retrieved in Malan D sample, and belonged to the γ-*Proteobacteria*. Both sequences could be assigned to *Acinetobacter* sp. and highly clustered with sea ice and glacial bacterium. One clone Malan D-10 was highly similar to *Acinetobacter johnsonii* from waste water, and closely associated with Arctic sea ice bacterium and glacial ice bacterium G50-TB2 isolated from Guliya ice core on the Tibetan Plateau (Christner *et al*. 2000). Another clone from Malan D sample was assigned to *Acinetobacter* sp. The similar sequences from the Malan ice core and see ice, Guliya ice core, and other Antarctic ice cores argue that these species probably have physiologically adapted for life in an extremely cold environment.

Cytophaga–Flavobacterium–Bacteroides *(CFB) and other eubacteria group.* A total of 29 cloned sequences from two samples (Malan A and C) formed four ARDRA patterns and fell into the CFB group. Of these, 25 contained inserts which formed three ARDRA patterns and accounted for 41 % in the library from the Malan C sample, indicating that the CFB group plays an important part in the structure of bacterial community at the ice deposition of 70 m. The fourth cloned sequence constituted 7.4 % of the clones in the library from sample Malan A. The cloned sequence (Malan C-2) was 100 % similar to *Flectobacillus* sp. from marine water, and 100 % identical with *Flavobacterium xijianggens* from the Chinese no. 1 glacier, while the sequence from Malan C-P39 was only 89 % similar to the glacial bacterium and *Flavobacterium* sp., but was highly related (100 % similarity) to *Flectobacillus* sp. and 99 % similar to the uncultured CFB group. The sample Malan C-P45 had a high similarity (100 %) to uncultured bacterium from a lake sample, and was 99 % identical with another sequence from sample Malan A-38 and 99 % with *Flavobacterium* sp. The occurrence (*i.e.*, Malan A-33) of the member of phenanthrene-degrading bacterium at the ice layer 35 m and the CFB group at ice depth 35 m and 70 m suggests that the ice habitats may have relationship with the complex organic compounds. These compounds were virtually detected from the Himalayan Dasuopu glacial ice (Xie *et al.* 2000). It is likely that these air organic compounds provide suitable growth habitats and a medium for aerosolized microbes to travel large distances on atmospheric currents, and were embedded in ice associated with micro-particles and accreted microorganisms.

Fig. 1 also shows the diversity of eubacteria from sample Malan C in the Malan ice core. The sequence of Malan C-13 clone was 100 % similar with an agricultural soil bacterium, the Malan C-P22 cloned sequence closely grouped with an uncultured bacterium from rice paddy soil, and the Malan C-9 also with an eubacterium from plant root, and Malan C-37 closely clustered (100 %) with *Marinobacter* sp. from Antarctic sea ice. This group was only found at ice deposition of 70 m, but played a great role in the contribution to the bacterial diversity of community composition in the Malan ice core.

The scanning electron observations of the Antarctic ice core revealed that many large particles trapped in atmospheric dust would have also transported attached microorganisms into the ice cores (Priscu *et al.* 1999; Christner *et al.* 2000). These microbes attached to particles were deposited annually on the surface of the glacier, and embedded into cracks and bubbles of the ice core with winter freezing and summer melting processes of ice (Squyres *et al.* 1991; Anderson *et al*. 1993; Adams *et al*. 1998; Fritsen *et al*. 1998; Priscu *et al*. 1998). The distribution of microbes at different positions in the ice core may reflect the prevalent climate, wind direction, and individual events that occurred at the time of deposition. Fig. 1 revealed the variation of similar sequences with depth. Four similar sequences from ice core depth 35 m (Malan A) and 64 m (Malan B) fell into the β-*Proteobacteria* group but the sequences from the same deposition had a higher similarity. It revealed the effect of the prevalent climatic condition on the evolution of microorganisms.

Phylogenetic analysis of 16S rDNA clones from the Malan ice core on the Tibetan Plateau showed a limited diversity among the cloned sequences, with similarity to known sequences ranging from 99 to 100 % (only a few sequences with <95 % similarity). In general, the database sequences most closely related to our plateau glacial sequences are from some extreme environments such as sludge contaminated by some complex organic compounds, and sea ice water, and geographically diverse glacial ice cores, as predicted by their closer proximity to major cold biological ecosystems. The predominance of *Proteobacteria* in different ice positions indicated that the structurally simple, prokaryotic consortia along microscale biogeochemical gradients is perhaps a unique and effective strategy for meeting the requirements of life in what appears to be an otherwise inhospitable environment.

Our report further expands the known diversity of the proteobacterial plankton assemblages from the initial reports of the prevalence of this group associated with microbial mats (Priscu *et al.* 1999; Christner *et al.* 2000; Zhang *et al.* 2002). If the phylogenetic diversity of this group and the diversity of habitats from which they have been isolated such as tundra, sea ice and glacial ice cores, and from more vertical depth, it is difficult to speculate what their role in the extreme cold, desiccated and presumably obligotrophic (low-nutrient, low-biomass and low-energy flux) ice environment might be and in the evolution of bacteria with time. The predominant proteobacteria, CFB group, and *Acinetobacter* sp. similar to many reported ice bacteria detected in this study may reflect the capacity of these bacteria to colonize extreme habitats.

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