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Differences in community composition of bacteria in four glaciers in western China

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Abstract. Microbial community patterns vary in glaciers worldwide, presenting unique responses to global climatic and environmental changes. Four bacterial clone libraries were established by 16S rRNA gene amplification from four ice layers along the 42-m-long ice core MuztB drilled from the Muztag Ata Glacier. A total of 151 bacterial sequences obtained from the ice core MuztB were phylogenetically compared with the 71 previously reported sequences from three ice cores extracted from ice caps Malan, Dunde, and Puruogangri. Six phylogenetic clusters *Flavisolibacter*, *Flexibacter* (*Bacteroidetes*), *Acinetobacter*, *Enterobacter* (*Gammaproteobacteria*), *Planococcus/Anoxybacillus* (*Firmicutes*), and *Propionibacter/Luteococcus* (*Actinobacteria*) frequently occurred along the Muztag Ata Glacier profile, and their proportion varied by seasons. Sequence analysis showed that most of the sequences from the ice core clustered with those from cold environments, and the sequence clusters from the same glacier more closely grouped together than those from the geographically isolated glaciers. Moreover, bacterial communities from the same location or similarly aged ice formed a cluster, and were clearly separate from those from other geographically isolated glaciers. In summary, the findings provide preliminary evidence of zonal distribution of microbial community, and suggest biogeography of microorganisms in glacier ice.

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1 Introduction

Variation in the communities of microorganisms in the deep glaciers (ice cores) worldwide reflects a response of microorganisms to global climatic and environmental change (Christner et al., 2000; Muller et al., 2004; Yergeau et al., 2007; Miteva et al., 2009). Analysis of a 102-m-long ice core drilled from the Malan ice cap (Xiang et al., 2004), a shallow ice core extracted at an elevation of 6350 m from the Muztag Ata Glacier (Xiang et al., 2005) as well as ice cores from the Arctic, Antarctica and other mountain glaciers (Christner et al., 2000) showed differences in the phylogenetic relationship of bacteria between the glacier ice and mild environments. These results suggest an important selective effect of the extreme cold glacier environments on microorganisms in glacier ice. However, it is not well known how changes in the

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patterns of microbial communities in glacier ice are related to climatic and environmental changes.

Recent analysis of glacial snow pits from glaciers Kuytun 51 (Xiang et al., 2009b), Guoqu (Liu et al., 2009) and Rongbuk (Liu et al., 2006) revealed apparent differences in community composition in the different glacial snow layers, suggesting the effects of seasonal conditions on the microbial communities. Phylogenetic comparison of bacterial communities in the surface snow and snow pits among the isolated glaciers showed less of a shift between seasonal communities than between those extending over a large spatial scale (Xiang et al., unpublished). A recent investigation of the Greenland ice core GISP2 demonstrated a large difference in the proportion of the main phylogenetic phyla during the distinct geographical periods which occurred from 30–80 Ka, suggesting a strong temporal effect of aeolian activities on community composition of microorganisms in Greenland (Miteva et al., 2009). These results suggest strong spatial and temporal effects on the microbial communities in glacier ice-snow.

To initially investigate microbial biogeography in glacial ice over extended scales of time and space, the present study extended earlier preliminary investigations on the glacier surface (Xiang et al., 2009c) and deep snow (Xiang et al., 2009b), and further investigated the composition of bacterial communities along the four ice core profiles extracted from geographically isolated glaciers. First, to investigate the seasonal community changes in glacier ice, four bacterial clone libraries were established by 16S rRNA gene amplification from the ice core MuztB drilled at an elevation 7000-m Muztag Ata Glacier. Second, the geographical effects on the evolution of microorganisms in glacier ice were preliminarily evaluated by a comparison of pooled sequences from the surrounding mild environments and various geographically isolated glaciers. Finally, to investigate the community shift at a large spatial scale, four clone libraries from the ice core MuztB were phylogenetically compared with eight previously recovered bacterial clone libraries from ice cores drilled from three ice caps Dunde (Zhang et al., 2009), Malan (Xiang et al., 2004; Zhang et al., 2009) and Puruogangri (Zhang et al., 2009). This study attempted to provide interpretations of biogeography of microorganisms in the glacier ice. The potential limitation of conclusions on biogeography of microorganisms in ice cores was also discussed in this study.

2 Materials and methods

2.1 Study sites and sample collection

The data used in this study were collected from four ice cores from the Muztag Ata Glacier (38°17′ N, 75°04′ E; Tian et al., 2006), Dunde ice cap (38°06′ N, 96°24′ E; Zhang et al., 2009), Puruogangri ice cap (33°44′–34°04′ N, 89°20′–89°50′ E; Zhang et al., 2009), and Malan ice cap (35°48.40′ N, 90°35.34′ E; Xiang et al., 2004), respectively. The Muztag Ata Glacier is located in the most western periphery of the Tibetan Plateau, where precipitation is derived from the air masses from arid and semi-arid regions, including the deserts Sary-Ishykotrau, Muyun Kum, Kyzyl Kum, Kara Kum, Taklimakan and Gurbantunnut (Fig. 1). The Dunde ice cap is located in the north margin of the Qaidam Basin and in the Qilian mountain region on the northeastern Tibetan Plateau (Fig. 1). During the winter, precipitation results from the incursion of westerly depressions along the southern slopes of the Hymalayas (Murakami, 1987; Davis et al., 2005). In addition, during the summer, Indian monsoon circulation transports moisture from the Bay of Bengal to the central Hymalayas, and extends to the Qaidam Basin (Davis et al., 2005). Moreover, the numerous large depressions in the Takalamakan Desert and Daidam Basin cause strong winds and snowstorms as well (Dregne, 1968; Chen and Bowler, 1986). The Malan and Puruogangri ice caps are located in the centre of the Tibetan Plateau, where precipitation is derived from a westerly direction during winter, and Indian monsoons in the summer (Wang, 1989; Wake et al., 1993; Shi and Liu, 2000).

All of the four ice cores with 10-cm diameter were drilled from the glaciers at the high altitudes, >5300 m a.s.l. (above sea level). The first ice core MuztB (42-m-long) was extracted at 7010 m a.s.l. from the Muztag Ata Glacier in the summer of 2003 (Tian et al., 2006), the second ice core Dunde (140 m long) was extracted at an elevation of 5325 m on the Dunde ice cap in 1987 (Thompson et al., 1990). The third ice core Puruogangri (86-m-long) was extracted at an elevation of 5970 m from the Puruogangri ice cap in October 2000 (Thompson et al., 2006), and the fourth ice core, Malan (102-m-long), was extracted at an elevation of 5620 m on the Malan ice cap in 1999 (Wang et al., 2003). Theses four glaciers have an extremely cold ice temperature at a range of ice-core borehole temperature from $<-3.7\,^{\circ}C$ to $-26.17\,^{\circ}C$ (Thompson et al., 1990; Pu et al., 2002; Wu et al., 2003; Li et al., 2004). The visible stratigraphic features were recorded immediately after ice core drilling. All ice cores were returned frozen to the freezer room (air temperature between $-18\,^{\circ}C$ to $-24\,^{\circ}C$) at the Key Laboratory of the Ice Core and Cold Regions Environment of the Chinese Academy of Sciences. Each ice column of the obtained ice cores was split lengthwise into four sections within a walk-in freezer, and stored in a refrigerated room at $-18\,^{\circ}C$ to $-24\,^{\circ}C$. All ice core sub-samples (around 30 to 50 cm-long ice columns) for further microbial analysis were handled at temperatures below $20\,^{\circ}C$ within a sterile and positive-pressure laminar flow hood by following the previously described procedure (Xiang et al., 2005). An annulus (10 mm) was successively cut three times from the surface of each core sample using three clean, sterilized saw-tooth knifes. The remaining inner core was washed, and the samples were allowed to melt at $4\,^{\circ}C$ in covered, autoclaved containers and then later used

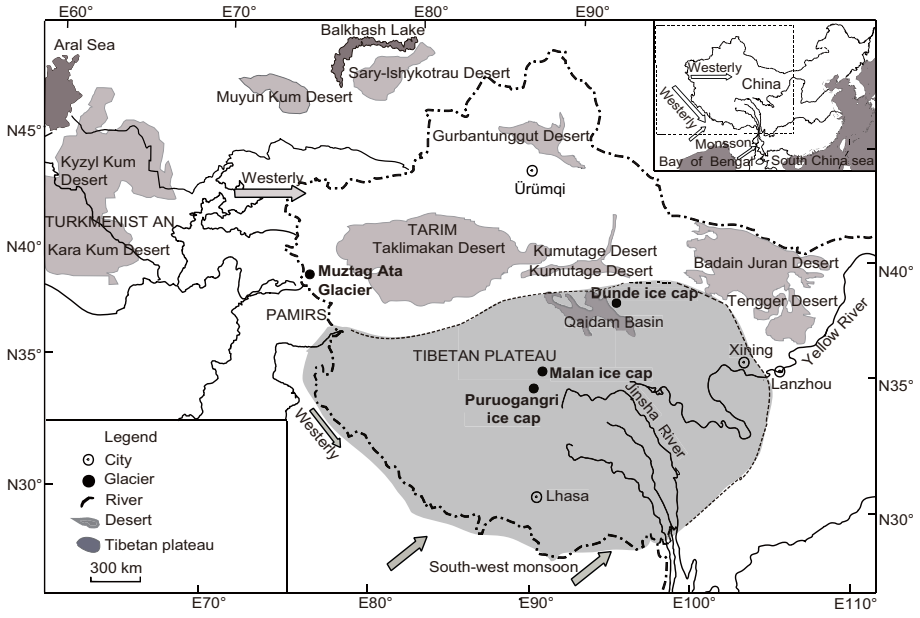

Fig. 1. Map showing the locations of four geographically isolated glaciers Muztag Ata, Puruogangri, Malan and Dunde (Map was adapted from Xiang et al., unpublished).

for further analysis. Microbial data of the three ice cores Dunde, Puruogangri, and Malan were collected from published reports (Zhang et al., 2009; Xiang et al., 2004). Four ice columns MuztB30, MuztB16, MuztB28, and MuztB30 used in this study were subsections of ice core MuztB. They and other ice colums from glaciers Malan, Puruogangri and Dunde, were cautiously dated to AD 1970 to 1988 (Tian et al., 2006), 1600 to 1800 (Wang et al., 2003), 1750 to 1920 (Thompson et al., 2006) and 1780 to 1830 (Thompson et al., 1990), respectively.

2.2 Biomass analysis and clone library establishment of the bacterial 16S rRNA gene amplified from the Muztag Ata Glacier

The fresh melt-water (10 ml) obtained from the ice core MuztB was used for the determination of total and live biomass by flow cytometric (FCM) analysis by following the previously reported protocols (Xiang et al., 2009c). The 100 µl of diluted sample was added to the known concentration of fluorescent-dyed bead solution Trucount with 100 µl autoclaved and filtered phosphate-buffered saline (PBS, 50 mM; pH 7; containing 1 mM dithiothreitol, DTT) containing either filtered (0.22-µm Nuclepore) PI dye (final concentration 5 µM, Sigma, P4170), cFDA (final concentration: 10 µM, Sigma, F7378) or cFDA/PI mixture. The stained sample mixture was incubated in the dark for 15 min at 22 °C to allow the cells to stain. Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) equipped with an air-cooled argon ion laser emitting 15 mW of blue light at 488 nm and with the standard filter setup (see the detailed information in Xiang et al., 2009c).

Approximately 400 ml of melt-water was used for DNA extraction. DNA extraction and further clone library establishment procedure were conducted by following the same protocols as previously used in a microbial analysis of the Kuytun 51 Glacier samples (Xiang et al., 2009c). The 16S rRNA gene amplicons used for the establishment of clone libraries from the Muztag Ata Glacier were generated by PCR amplification with the bacterial universal primer pair 8f (5′-AGAGTTTGATCATGGCTCAG) and 1492R (5′-CGGTTACCTTGTTACGACTT) (Lane, 1991; Weisenburg et al., 1991). To avoid possible bias, the pooled PCR products were used to establish clone library from each ice column. A total of 151 clones were selected for sequencing by *Hae*III-based ARDRA (amplified rRNA restriction analysis) screening out of the 352 clones from the ice core MuztB obtained from the Muztag Ata Glacier. Each sequence was named using the initial of Muztag Ata Glacier (MuztB, B was noted for the ice core drilled at an elevation of 7010 m to distinguish it from the Muztag Ata ice core MuztA at 6350 m a.s.l. in the summer of 2002, Xiang et al., 2005), along with the ice column tube number (13, 16, 28 and 30) followed by the clone number (1 to 148). For example, clones MuztB13–132, MuztB16–36, MuztB28–125, and MuztB30–118 were representative bacterial clones from the ice core columns 13 (1988 spring), 16 (1984 autumn–1985 winter), 28 (1972 spring–summer), and 30 (1970 winter) which were obtained from the ice core MuztB drilled at 7010 m a.s.l. of the Muztag Ata

Glacier. The time series of the ice core MuztB was determined based on the visible annual layers, seasonal fluctuation in the oxygen isotope ratios, and the beta activity in the melt water as a reference (Tian et al., 2006). The accession numbers of the cloned sequences obtained from the ice core MuztB in GenBank are: GU246831–GU246982.

2.3 Statistical analysis of the bacterial communities in the four glaciers in western China

To investigate the biogeography of microorganisms in glacier ice over extensive spatial and temporal scales, all 151 sequences from the Muztag Ata Glacier were compared with the 71 previously reported sequences obtained from the three glaciers Malan (accession number AY322483-AY322493 in Xiang et al., 2004 and AY121823-AY121830 in Zhang et al., 2009), Dunde (accession number AY313918, AY313919 and DQ076445-DQ076456, Zhang et al., 2009), and Puruogangri (accession number AY313907-AY313917, AY313920-AY313922, and DQ076420-DQ076444, Zhang et al., 2009), as well as the closest relatives obtained by Blast search (Altschul et al., 1990) and aligned with reference sequences obtained using ClustalX (Thompson et al., 1997). A Neighbor-Joining (NJ, Tamura et al., 2004) phylogeny for the aligned sequences was constructed using MEGA 4.0 (Tamura et al., 2007: http://www.megasoftware.net/) pairwise deletion mode for gaps (with bootstrap analysis, 100 replicates) and subroutines Maximum Composite Likelihood (MCL) for substitutions. The archaeal 16S rDNA sequences from *Methanosaeta harundinacea* strain 8Ac (accession no. AY817738) and *Methanosaeta concilii* strain NW-1 (accession no. DQ150255) were used as outgroup references on all trees instead of a closer related outgroup, since the bacterial *Thermotogae* referent sequences AB039768 and U89768 collapsed the outgroup into either one or two branches of the tree, which was possibly due to broad taxonomic phylotypes identified in our study. The relations between bacterial subphyla in the tree were not affected by the tree construction without and with the presence of long distant archael outgroup sequences (Daubin et al., 2001). All the obtained sequences from the glaciers were identified by the recognized species, and related to the ecological clusters (e.g., *Rhodoferax* sp. and *Variovorax* sp. in the *Betaproteobacteria* subphyla). Sequences obtained displaying similarities of >97% with known species were identified as the reported species. Most of the obtained clones were related to known cultivated genera or genus clones (e.g., *Acinetobacter* sp., *Cryobacterium* sp., and *Sphingomonas* sp.). A few clones had <97% similarity with reported species, and thus were designated separately.

The diversity (Shannon-Wiener index H') and evenness (E) indices were based upon the distribution of unique sequence OTUs (operational taxonomic units) obtained from the clone libraries using equations: $H'=-\mathrm{SUM}\{p_i \cdot \ln(p_i)\}$ and $E=H/\ln(S)$, respectively (Hill et al., 2003), where p_i = the proportion of the i-th clone in the total clones in each individual library, and S is the single unique sequence richness. Bacterial phylotype abundance and coverage estimators were calculated with the software program EstimateS (Kemp and Aller, 2004, the EstimateS program is available online at http://www.aslo.org/lomethods/free/2004/0114a.html).

To further investigate the biogeographic effect on the community patterns of microorganisms in the ice cores drilled from the geographically different glaciers in western China, the representative sequences were pooled together, and compared among four glaciers Muztag Ata Glacier, Malan (Xiang et al., 2004; Zhang et al., 2009), Dunde (Zhang et al., 2009), and Puruogangri (Zhang et al., 2009). The bacterial community composition was statistically analyzed using the UniFrac software package (Lozupone and Knight, 2005). Differences between the clone libraries were estimated with the weighted Unifrac algorithm (Lozupone and Knight, 2005). The clone numbers of the tested sequences were also used for the UniFrac analysis. A sequence jackknifing technique was applied to each cluster to determine the sensitivity of the relationships to sample size.

3 Results

3.1 Differences in biomass and diversity of dominant bacteria along the Muztag Ata Glacier depth profile

There was great variation in the diversity of dominant bacteria along the Muztag Ata Glacier profile, although there was a slight change in the total and live biomass at a range from 1.03×10^5 to 1.99×10^5 cells ml^{-1} and 4.28×10^3 to 4.98×10^3 cells ml^{-1}, respectively, in the four tested ice layers (Table 1). The ice layer in the warm spring-summer season of 1972 contained the most diverse bacteria, as estimated by the Schao1 value, the winter 1970 ice layer contained the highest Shannon index value, and the ice layer from autumn 1984 to winter 1985 contained the lowest OTUs of bacteria in both the Schao1 and Shannon indices (Table 1). This indicated a great heterogeneity of bacteria along the glacier depth profile.

The dominant bacteria in the four ice layers were investigated by 16S rRNA gene amplification, as well as clone library and sequencing techniques. BLAST results of the 16S rDNA sequences obtained from the Muztag Ata ice core showed that the bacterial phyla *Gammaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* frequently occurred throughout the glacier depth profile (Fig. 2). However, there was a great difference in the proportion of the main phylogenetic groups along the glacier depth profile (Fig. 2). Phyla *Alphaproteobacteria* and *Firmicutes* were dominant in the spring ice layer of 1988, *Gammaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*,

Table 1. Biomass and diversity of dominant bacteria in the Muztag Ata Glacier based on 16S rRNA gene sequence analysis.

Biomass and diversity	Clone library (ice age, year)			
	MuztB13 (1988 spring)	MuztB16 (1984 summer–1985 winter)	MuztB28 (1972 spring–summer)	MuztB30 (1970 winter)
Total cells (10^5 cells ml^{-1})	1.23	1.95	1.99	1.03
Live cells (10^3 cells ml^{-1})	4.47	4.90	4.98	4.28
No. of OTUs predicted (S_{chao1})	55	36	76	58
Coverage C_{ACE} (%)	84	84	74	84
No. of OTUs observed	45	23	49	31
Shannon index	3.60	2.90	3.05	4.01
Eveness	0.94	0.94	0.78	1.10

and *Bacteroidetes* phyla were dominant in the ice layer from 1984 autumn to 1985 winter, *Gammaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* were common in the 1972 spring-summer ice layer, and *Gammaproteobacteria*, *Betaproteobacteria*, and *Actinobacteria* dominated the community in the 1970 winter ice layer. Interestingly, the *Firmicutes* phylum was dominant in the spring seasons of both 1988 and 1972, but rarely occured in the winter seasons of 1970 and 1985.

3.2 Similarities and differences in the main phylogenetic bacterial groups among the four isolated glaciers

To investigate the biogeography of microorganisms in glacier ice, the sequences obtained from the Muztag Ata Glacier were compared with those from the geographically isolated glaciers Puruogangri, Dunde and Malan. The sequence data showed that all of the ice core clones were related (with 97% to 100% similarity) to those from the different environments of urban aerosols, soil, from the river to the Antarctic (Fig. 3a–d). For example, two clones MuztB30–40 and TD-21 were 97% similar to *Comamonadaceae* sp., clone XJ-L144 (with accession number EU817496), a betaproteobacterial family from the Xiangjiang River, clone MuztB30–61, which was 100% identical to a *Pelomonas saccharophila* strain (AF368755) from the ultrapure water from an industrial system, and the two clones MuztB28–21 and MuztB28–32, which were similar with 99% similarity to a *Methylibium fulvum* isolate (AB245356) from ginseng field soil (Fig. 3a). However, most of the glacier bacterial clones were closely clustered with those from the cold environments, such as the glaciers and Antarctica (Fig. 3a–e). The two clones MuztB28–21 and MuztB28–32 were easily clustered with clone Kuy-SL-42 (EU263707) from the Kuytun 51 Glacier in the *Methylibium* sp. group

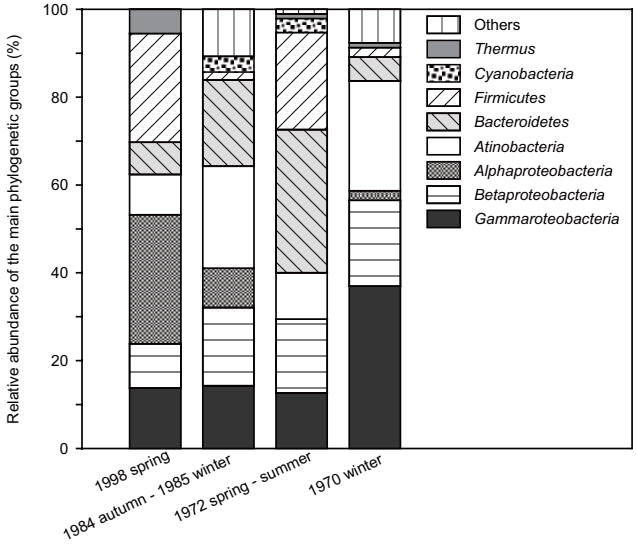

Fig. 2. Relative abundance of the main bacterial phyla based on the Blast result of 16S rRNA gene sequences in each of the clone libraries in the four different ice layers along the Muztag Ata Glacier profile.

within the family of *Betaproteobacteria* (Fig. 3a). Clones MuztB28–125, MuztB16–36, P80–5, P80–18, P200–23 and P60–49 were closely clustered with the betaproteobacterial clone KuyT-IWPB-41 (EU263727) from the Kuytun 51 Glacier and a clone (DQ675477) from the Hymilayan glacier, respectively (Fig. 3a). Other clones from the Muztag Ata, Puruogangri, and Dunde glacier clustered with the Antarctic clones H12_ELL02 (EF220189) and H07_ELL02 (EF220180) within the *Flexibacter* sp. group in the family of *Bacteroidetes* (Fig. 3c).

There were some common species in the different glaciers. For example, in the *Rhodoferax* sp. group in the

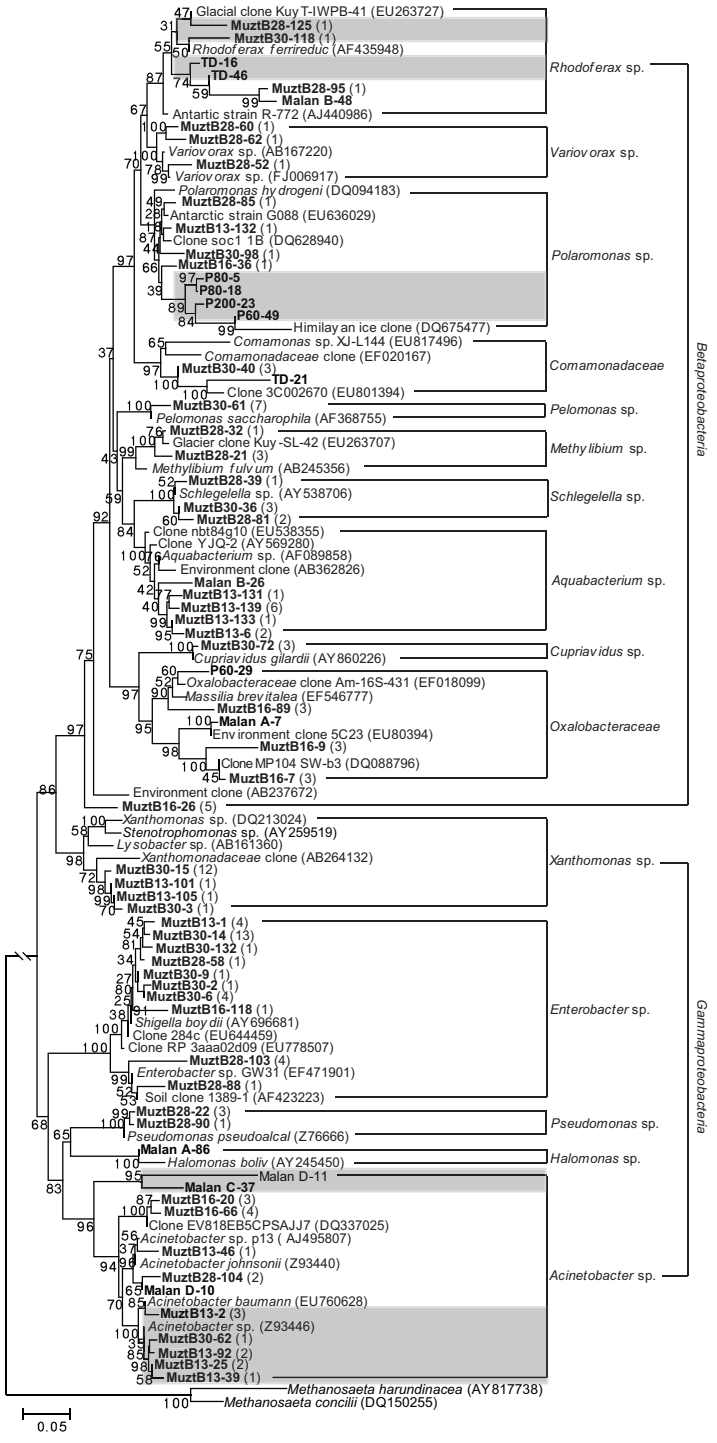

Fig. 3a. Phylogenetic analysis of the 16S rRNA genes for *Betaproteobacteria* and *Gammaproteobacteria* clones from the four ice cores and the closest relatives. The tree was generated by the Neighbour-Joining method after sequence alignment, and rooted with two *Methanosaeta harundinacea* strains (accession no. AY817738 and DQ150255). Bootstrap values (100 replications) were specified for each Node. Numbers of the obtained snow-ice clones (had the same ARDRA pattern to the sequenced representatives listed on the tree) and relative sequence affiliations corresponding to GenBank accession number were provided in parentheses. Scale bar indicated 0.05 substitutions per nucleotide. The sequences discussed in this study were noted bold. The Muztag Ata Glacier clones were represented by MuztB13 (1988 spring), MuztB16 (1984 autumn–1985 winter), MuztB28 (1972 spring–summer) and MuztB30 (1970 winter), the Puruogangri Glacier clones were noted by P60 (at 52 m depth, AD 1850–1920), P80 (62 m depth, AD 1750–1800), and P200 (89 m depth, AD 1600–1700), while the Malan Glacier clones were noted by Malan A (35 m depth, AD 1800), Malan B (64 m depth, AD 1600), Malan C (70 m depth, AD 1560), and Malan D (82 m depth, AD 1400). The typical endemic cluster was indicated in the gray shaded areas (same for the following Figs). See a detailed description for the assigned sequence references and numbers in materials and methods.

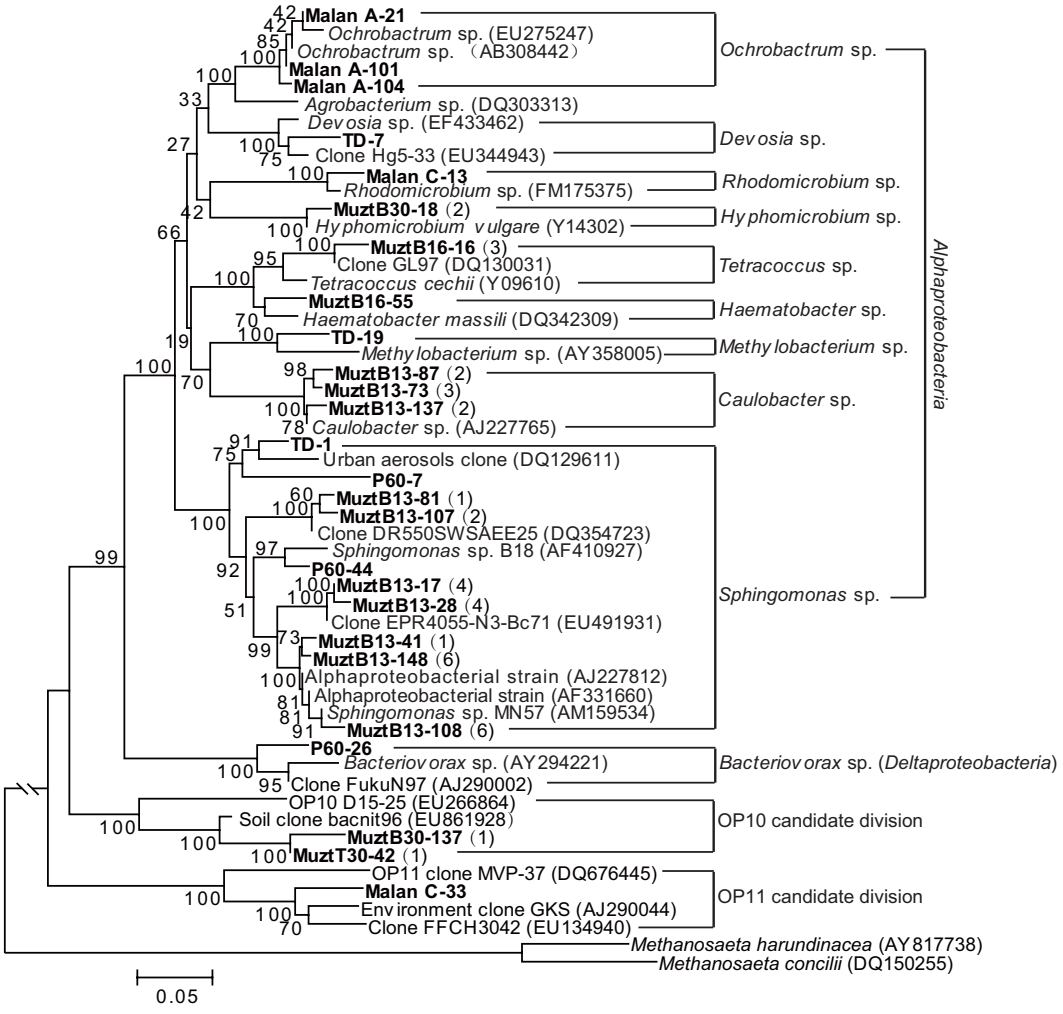

Fig. 3b. Phylogenetic analysis of the 16S rRNA genes for the *Alphaproteobacteria*, *Deltaproteobacteria*, OP10 and OP11 clones from the four geographically isolated glaciers and the closest relatives. The tree was constructed by following the protocol as described in Fig. 3a. Scale bar indicated 0.05 substitutions per nucleotide.

Betaproteobacteria phylum, clone MuztB28–95 from the Muztag Ata Glacier was 99% similar to clone Malan B-48 from the Malan Glacier (Fig. 3a). Clone Malan D-10 clustered with the Muztag Ata glacier clone group (e.g., clones MuztB28–104 and MuztB13–2) and fell into the *Acinetobacter* sp. group among the members of the *Gammaproteobacteria* phylum (Fig. 3a).

Common bacteria not only occurred in the geographically isolated glaciers, but also in the different ice layers throughout the ice core profile, which was very evident along the MuztB ice core profile (Fig. a–e). In the *Schlegella* sp. cluster within the *Betaproteobacteria* phylum, two clones MuztB28–39 and MuztB28–81 from the ice column 28 (1972 spring - summer) closely clustered with clone MuztB30–36 from the 1970 winter column 30 (Fig. 3a). Two clones (MuztB30–15 and MuztB30–3) from ice column 30 (1970 winter), MuztB30–15 and MuztB30–3, were 98% similar to two clones from the spring 1988 ice column 13 in the *Xanthomonas* sp. subgroup within the members of *Gammaproteobacteria* subphylum (Fig. 3a). In *Chamaeshiphon* sp. cluster within the *Cyanobacteria* phylum, clone MuztB16–114 from ice column 16 (1984 autumn–1985 winter) was 100% identical to the clone MuztB28–129 from ice column 28 (1972 spring–summer) (Fig. 3c). In the *Thermus* sp. cluster (*Thermus*), clone MuztB30–117 from ice column 30 (1970 winter) was 100% identical to clones MuztB13–91, MuztB13–59 and MuztB13–58 from ice column 13 (1988 spring) and clone MuztB28–56 from the 1972 ice column 28 (Fig. 3c). Similarly, *Anoxybacillus* sp. (*Firmicutes*) occurred throughout the ice columns of MuztB13 (e.g., clones MuztB13–102, MuztB13–14, and MuztB13–78 etc.), MuztB16 (e.g., clone MuztB16–76), MuztB28 (e.g., clones MuztB28–123, MuztB 28-16, MuztB 28-5, MuztB28–57) and MuztB30

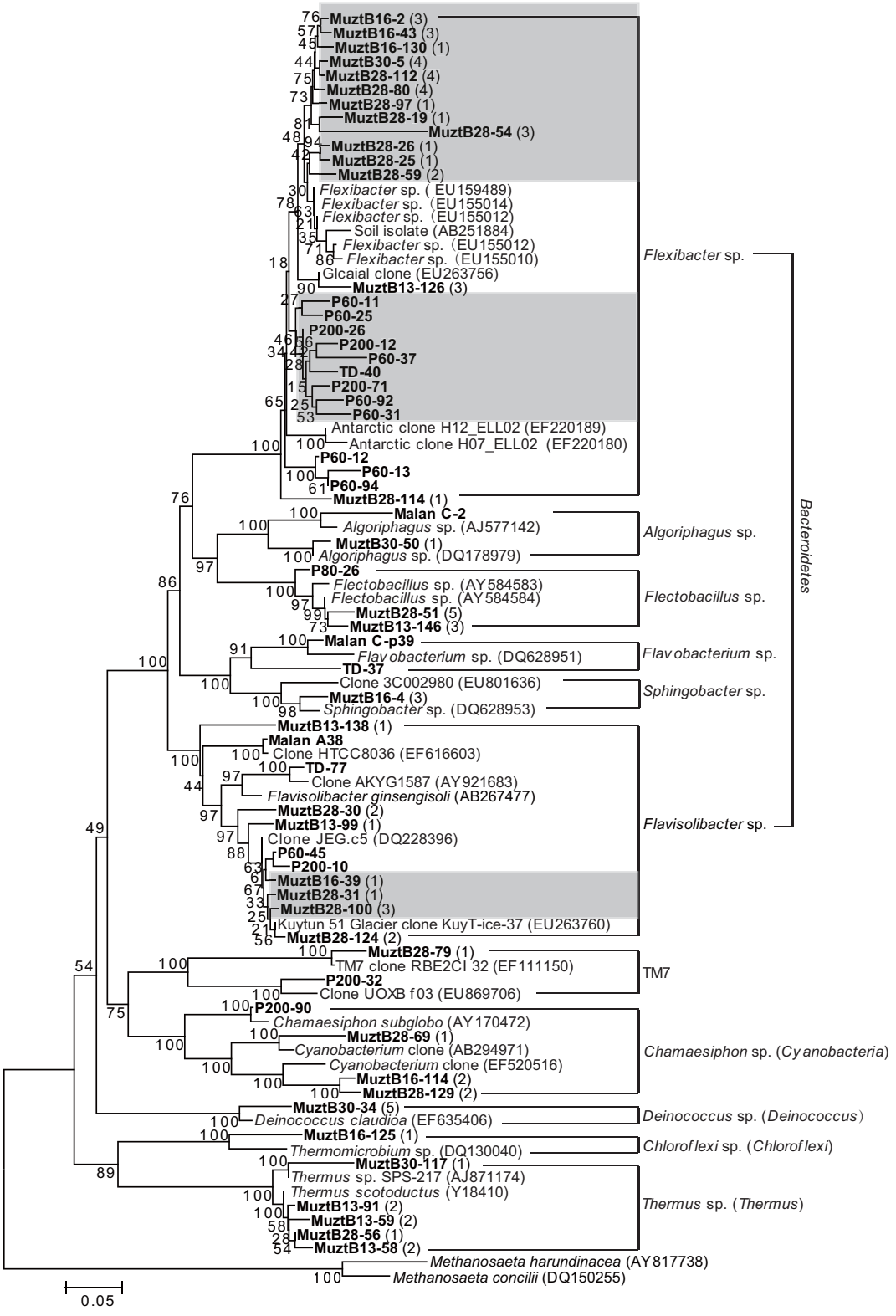

Fig. 3c. Phylogenetic analysis of the 16S rRNA genes for the *Bacteroidetes*, TM7, *Cyanobacteria, Deinococcus*, *Chloroflexi*, and *Thermus* clones from the four geographically isolated glaciers and the closest relatives. The tree was constructed by following the protocol as described in Fig. 3a. Scale bar indicated 0.05 substitutions per nucleotide.

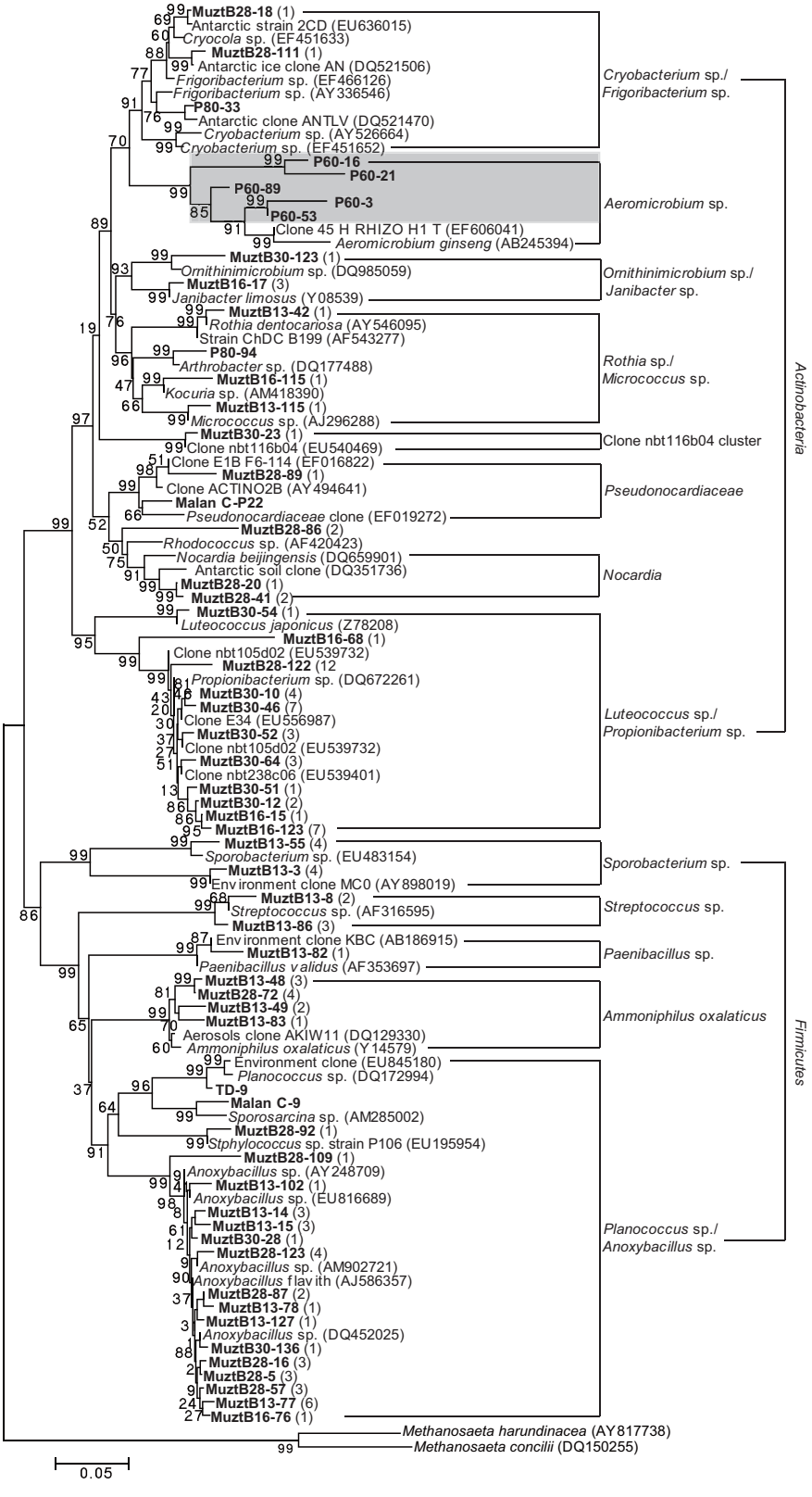

Fig. 3d. Phylogenetic analysis of the 16S rRNA genes for the *Actinobacteria* and *Firmicutes* clones from four geographically isolated glaciers and the closest relatives. The tree was constructed by following the protocol as described in Fig. 3a. Scale bar indicated 0.05 substitutions per nucleotide.

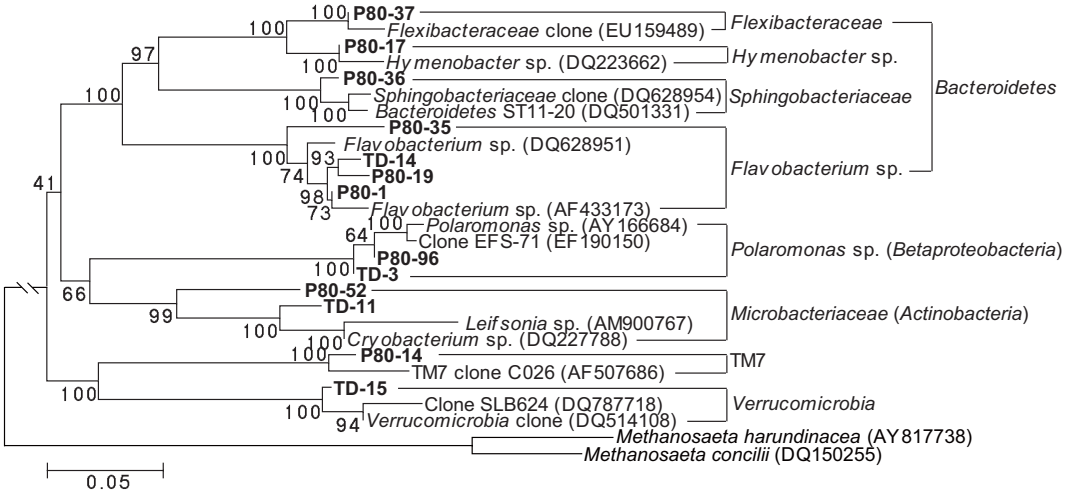

Fig. 3e. Phylogenetic analysis of the 16S rRNA genes for the *Bacteroidetes*, *Betaproteobacteria*, *Actinobacteria*, TM7, and *Verrucomicrobia* clones (with later portion of the 16S rRNA gene sequences, corresponding to regions 800–1452 of the *Escherichia coli* 16S rRNA molecule) from the Puruogangri and Dunde Glaciers and the closest relatives. The tree was constructed by following the protocol as described in Fig. 3a. Scale bar indicated 0.05 substitutions per nucleotide.

(e.g., clones MuztB30–28 and MuztB30–136), in the ice layers spring 1988, autumn 1984–winter 1985 , spring–summer 1972 and winter 1970 , respectively (Fig. 3d).

However, a comparison of sequences among the four geographically isolated glaciers showed that the bacterial clones from the same location readily formed a group, and were clearly distinct from those recovered from the geographically isolated glaciers (Fig. 3a–e), which presented a phenomenon of zonal distribution of microorganisms in glacier ice on a large spatial scale. This was apparent in the dominant clusters, as indicated by the gray shaded areas within the members of *Betaproteobacteia*, *Gammaproteobacteria*, *Bacteroidetes* and *Actinobacteria* phylum (Fig. 3a–e). In the *Rhodoferax* sp. cluster within the *Betaproteobacteria* phylum, the Muztag Ata Glacier clones MuztB28–118 and MuztB28–125 were clearly distinct from two clones TD-16 and TD-46 from the Dunder Glacier (Fig. 3a). Four clones, MuztB28–85, MuztB13–132, MuztB30–98, and MuztB16–36 from the Muztag Ata Glacier, formed a cluster distinct from four clones from the Puruogangri Glacier in the *Polaromonas* sp. cluster within the *Betaproteobacteria* phylum (Fig. 3a). The two clones Malan D-11 and Malan C-37 from the Malan Glacier were clearly distinct from eight Muztag Ata Glacier clones MuztB16–20, MuztB16–66, MuztB13–46, MuztB28–104, MuztB13–2, MuztB30–62, MuztB13–92 and MuztB13–25 in the *Acinetobacter* sp. cluster within the *Gammaproteobacteria* pylum (Fig. 3a). Similarly, the clones from the Muztag Ata Glacier (e.g., clones MuztB16–2 and MuztB16–14), were clearly separate from those from the Pruogangri Glacier (e.g., clones P60-11 and P60-25) in the *Flexibacter* sp. cluster within the *Bacteroidetes* phylum (Fig. 3c). The Malan Glacier clone Malan A38 was distinct from the Dunde Glacier clone TD-77 and the Muztag Ata Glacier clones (e.g., clones MuztB28–30 and MuztB13–99) in the *Flavisolibacter* sp. cluster within the *Bacteroidetes* phylum (Fig. 3c); while two clones MuztB28–18 and MuztB28–111 from the Muztag Ata Glacier were distinct from clone P80-33 from the Puruogangri Glacier in the *Cryobacteria/Frigoribacter* cluster within the *Actinobacteria* phylum (Fig. 3d).

The proportion of main phylogenetic groups varied throughout the depth profile of the Muztag Ata Glacier (Fig. 4). Six clusters, *Planococcus/Anoxybacillus*, *Propionibacter/Luteococcus*, *Flavisolibacter* sp., *Flexibacter* sp., *Acinetobacter* sp., and *Enterobacter* sp. were common throughout the ice core profile, but they varied at different frequencies along the profile. *Propionibacter/Luteococcus* dominated in the cold winter ice layers in 1970 (Fig. 4d) and 1985 (Fig. 4b), but rarely occurred in the spring ice layers in 1972 (Fig. 4c) and 1988 (Fig. 4a), indicating their prevalence in dry cold seasons. *Flexibacter* sp. dominated in the ice layers during summer 1972 and winter 1985, while *Enterobacter* sp. dominated in the ice layer in winter 1970. Other bacteria only occurred in certain specific seasons, as indicated by the solid triangles (Fig. 4), likely indicating an opportunist bacterial event in the glacier. A combination of prevailing, opportunistic, and other minor species constituted the community of bacteria in the ice-snow layers of specific seasons along the Muztag Ata Glacier profile.

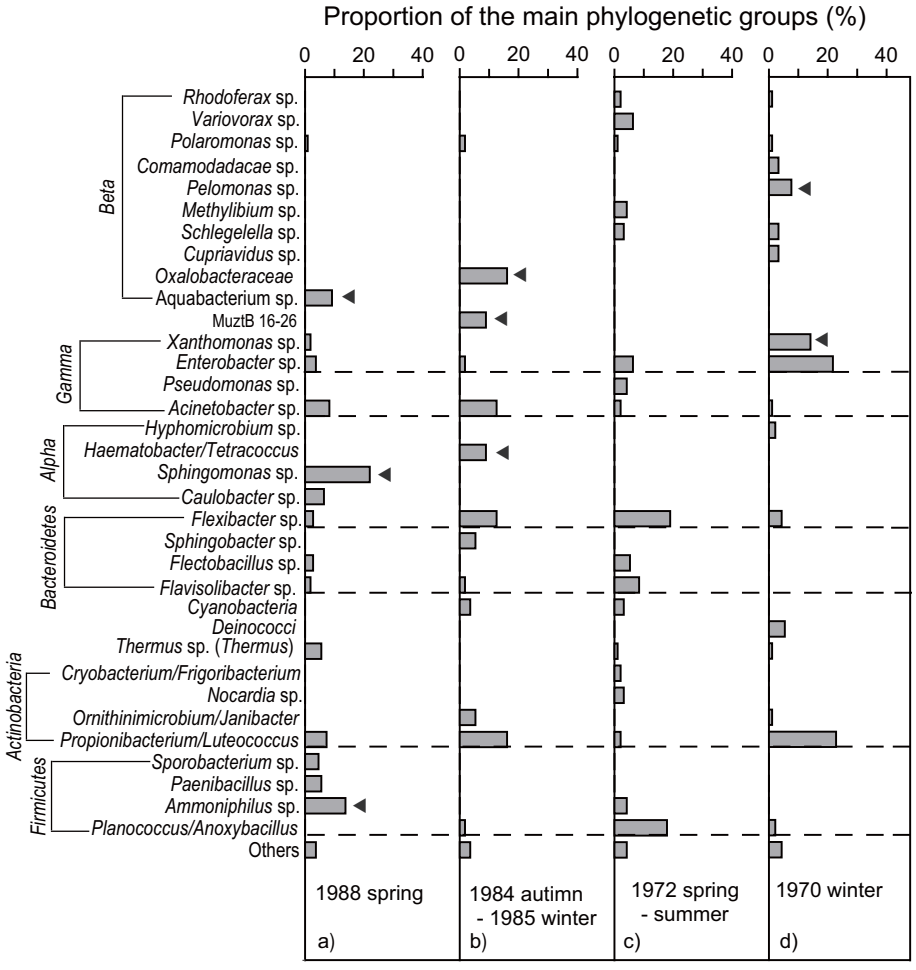

Fig. 4. Proportion of the main phylogenetic clusters (genera) based on the Blast result of 16S rRNA gene sequences in each of the clone libraries in the different ice layers along the MuztB ice core profile: **(a)** MuztB13, **(b)** MuztB16, **(c)** MuztB28, and **(d)** MuztB30. *Alph*, *Beta*, and *Gamma* = *Alpha-*, *Beta-*, and *Gamma-proteobacteria*.

3.3 Community comparison of bacteria among the four geographically isolated glaciers

UniFrac analysis of the pooled bacterial 16S rRNA gene sequences from four glaciers Muztag Ata, Dunde, Malan, and Puruogangri revealed a biogeography of bacterial communities (Fig. 5). Generally, the obtained 9 clone libraries of bacteria generated four clusters corresponding to the geographical distance, with the bacterial communities from the same glacier clustering with one another, clearly separated from those of other geographically isolated glaciers. This was particularly evident for the bacterial communities from the ice layers along the Puruogangri and Muztag Ata ice core profiles. All four bacterial clone libraries from the Muztag Ata Glacier fell into the same cluster, and the same trend was found for the two clone libraries from the Puruogangri Glacier.

Interestingly, the bacterial communities also appeared to cluster with one another at a temporal scale (Fig. 5). Five bacterial clone libraries from the ancient ice layers of $>$200 years were clearly separated from four clone libraries from the modern ice layers of $<$40 years. Two clone libraries from around AD 1600 and AD 1800 ice layers in the Malan Glacier formed two distant branches, and were distinct from other younger ice layers in the Muztag Ata Glacier. The clone library from the ice layer TD obtained from the Dunde Glacier of AD 1780–1830 clustered with the two clone libraries from the Puruogangri Glacier of similar ice age, i.e. AD 1750–1800 and 1850–1920, respectively.

4 Discussion

Previous reports of the deep glaciers (ice cores) demonstrated a great variability in the proportion of the main phyla of

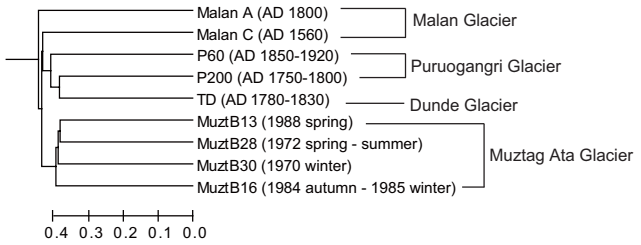

Fig. 5. Hierarchical cluster showing the overall phylogenetic differences amongst bacterial communities from four geographically isolated glaciers Puruogangri (two clone libraries containing the forward portion of 16S rRNA gene sequence corresponding to 8 to ∼800 of *Esherichia coli* 16S rRNA molecule, with sequence accession number AY313907-AY313917, DQ076421-DQ076430, and DQ076441-DQ076444, Zhang et al., 2009), Dunde (with sequence accession number AY313918, AY313919 and DQ076445-DQ076456, Zhang et al., 2009), Malan (two clone libraries containing >7 unique clones with sequence accession number AY322483-AY322489 in Xiang et al., 2004 and AY121823-AY121830 in Zhang et al., 2009) and Muztag Ata (this study). UniFrac scale indicated differences amongst the bacterial communities.

bacteria at the different depths of glaciers worldwide (Christner et al., 2000; Xiang et al., 2005; Liu et al., 2009), which is also evident in our present analysis of an ice core extracted from the Muztag Ata glacier. This suggests temporal biogeographic effects on microbial communities in glacier ice. However, in this current study, the analyses of pooled 16S rDNA sequences from the four geographically isolated glaciers, Muztag Ata, Dunde, Puruogangri and Malan showed a clear separation of microorganisms at species-genera and whole community levels, corresponding to the geographic pattern of glaciers. This suggests that spatial isolation of glaciers might have stronger influences than temporal stresses on the distribution of microbial community in glacier ice.

4.1 Methodological considerations

Ice core records have been used to reconstruct a history of climatic and glacial changes, presenting consistent results with the available climatic data (Naftz et al., 2002; Thompson et al., 1989, 2000; White et al., 1997; Yao, et al., 1999). The quality of the ice core records at the extremely high altitude (>5300 m a.s.l.) is much less influenced by the interruption of snow-melting, snow-layer-overturning and sublimation than at low altitude. Moreover, all four ice cores used in this study were cautiously dated to 48 to 400 years before today (Wang et al., 2003; Thompson et al., 1990, 2006; Tian et al., 2006), and the MuztB ice dating was confirmed by the peak in β activity (Tian et al., 2006). The evident correlation (coefficient of 0.67) of annual variation in values of oxygen isotope ratios with the annual air temperature changes at the nearby meteorological station Taxkorgen (Tian et al., 2006) indicated a reliability of temperature records in the ice core MuztB. The organic matter concentrations are extremely low, at a range from 20 to 120 ng g^{-1} along the Muztag Alta Glacier profile (Xu et al., 2009), and 0.3 to 2.8 ppm along the Puruogangri Glacier profile (Zhang, 2006). Despite the lack of data in the Malan and Dunde glaciers, the organic matter concentrations were reported at 10–350 ng g^{-1} and around 30 g m^{-2} in the high-altitude glaciers on the Tibetan Plateau (Xu et al., 2006, 2009; Takeuchi and Li, 2008). The high quality of ice core records and overall extremely cold and oligotrophic glacier environments at the high altitudes ensures the preservation of microbial information in the ice, and makes it possible for a community comparison of microorganisms in the geographically isolated glaciers.

The DNA sequence analysis of 16S rRNA gene clone libraries is a currently popular molecular method for surveying microbial communities (Pace, 1997). However, there are resolution limitations of the highly conserved 16S rRNA genes (De Rijk et al., 1992), polymerase chain reaction (PCR)-derived biases caused by intrinsic differences in the efficiency of amplification from environmental samples (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998; Crosby and Criddle, 2003), and sequence artifacts involved in the formation of chimerical molecules, heteroduplex molecules, and mismatch of *Taq* DNA polymerase with DNA templates (Acinas et al., 2005; De Rijk, 1992; Farrelly et al., 1995; Ishii and Fukui, 2001; Sipos et al., 2007; Thompson et al., 2002). Despite the disadvantages of PCR-derived methods, all chimeras, artifact and poor quality sequences (<300 bp) can be identified and removed in the further sequence analysis. PCR bias can also be reduced by the optimum amplification protocols (Ishii and Fukui, 2001). Moreover, this approach provides good phylogenetic resolution and makes it possible to compare the obtained bacterial sequence data with the previously reported sequences of bacteria in the geographically isolated glaciers.

4.2 Ubiquity and biogeography of bacteria in glacier ice

Previous investigations showed that most of the bacterial sequences from glaciers worldwide are closely related to those from certain environments, such as agricultural soil, river water and urban aerosol (Christner et al., 2000; Xiang et al., 2005; Miteva et al., 2004), indicating the ubiquity of microorganisms on earth. The ubiquity of microorganisms was also apparent in the bacteria found in the four geographically isolated glaciers, with 96% to 100% sequence similarity to those from mild environments (Fig. 3a–e). Previous studies, however, also reported phylogenetic differences between bacteria from glacier ice and the surrounding mild environments. These glacier clone sequences closely cluster with those from cold environments such as sea ice, Antarctic soil and other snow-ice (Xiang et al., 2005; Zhang et al., 2009). Microorganisms are transported from outside environments

onto the glacier surface and trapped in the ice (Gloster et al., 1982; Shuval et al., 1989; Prospero et al., 2005). Microorganisms present in ice may be in dormant, representing a viable but metabolically inactive state. They may even metabolize within ice for maintenance but not growth, which possibly make them more resistant to freezing than other populations in mild environments (Price and Sowers, 2004; Price, 2007). This was obvious for the biogeography of microorganisms in glacier ice when all of the sequences from the four geographically isolated glaciers Muztag Ata, Malan, Dunde and Puruogangri, along with the nearest relatives from other environments, were subjected to phylogenetic analysis (Fig. 3a–e). Moreover, 60% of the total bacterial clones from the same glacier (e.g., the Muztag Ata Glacier) were easily clustered together, clearly separating them from those from other, geographically isolated glaciers, or the surrounding environments (Fig. 3a, c, and d). The biogeography of microorganisms in glacier ice may be attributed to the geographic distance and the consequently isolated effect on the evolution of microorganisms in glacier snow-ice, which will be discussed below.

4.3 Biogeography of bacterial community in glacier ice

Several bacterial genera frequently occurred throughout the depth profile from the Muztag Ata Glacier, indicating their prevalence in this specific glacier (Fig. 4). The six phylogenetic clusters *Planococcus/Anoxybacillus*, *Propionibacter/Luteococcus*, *Flavisolibacter* sp., *Flexibacter* sp., *Acinetobacter* sp., and *Enterobacter* sp. constituted the main community constituents throughout the depth profile, suggesting they might be the prevalent bacteria in the Muztag Ata mountain regions. In particular, *Acinetobacteria* sp. were not only found in all four of the established clone libraries from the ice core MuztB profile extracted at an elevation of 7010 m in the Muztag Ata Glacier (Fig. 4–d),, but also frequently isolated from the four ice layers from the ice core drilled at an elevation of 6350 m in the same location (Xiang et al., 2005). This confirmed that the culture-independent based results on the dominant bacteria in the ice core was consistent with our earlier culture-based data from the Muztag Ata Glacier, and thus it was sufficient for a comparison of dominant bacteria in the different seasons (Fig. 4). The prevalent bacteria in the ice core may represent the biological indicators of climatic and environmental conditions in the air mass over the Muztag Ata Glacier, and also on the subsequent glacier surface during the current deposition period. The phenomenon of prevalent bacteria in the local regions can be found in other reports as well. For example, *Bacillus* sp., and *Microbacter*/*Arthrobacter* sp. were frequently isolated from the ice layers along the Guliya Glacier profile (Christner et al., 2000), potentially serving as a microbial indicator of the air mass over the glacier and in the surface snow-ice. *Propionibacterium* sp. and *Bacillus* sp. frequently appeared in three clear ice layers along the Greenland GISP2 ice core profile (Miteva et al., 2009), constituting the prevalent bacteria at the specific deposition time, and thus may be considered as the biological indicator of the prevailing air mass over Greenland during the current deposition period. Likely, *Flexibacter* sp. and *Polaromonas* sp. frequently occurred in three ice layers along the Puruogangri ice core profile (Fig. 3a and c), constituting the main components of bacterial community in the ice core (Fig. 1 in reference of Zhang et al., 2009), and thus may be the biological indicator in the prevailing air mass over the Puruogangri Glacier. Three clusters *Arthrobacter* sp. (*Actinobacteria*), *Pseudomonas* sp. (*Gammaproteobacteria*) and *Sphingomonas* sp. (*Alphproteobacteria*) were the dominant components of the microbial community in the Guoqu (Geladaindong) Glacier (Yao et al., 2008).

4.4 Climatic and environmental implications of microbial communities in glacier ice

Microorganisms vary in geographically isolated glaciers, not only at the species-genera level (Fig. 3–e), but also at the whole community level (Figs. 4 and 5). When subjected to the UniFrac analysis, the pooled sequences of bacterial 16S rRNA genes amplified from the four glaciers Malan, Puruogangri, Dunde, and Muztag Ata showed a clear separation of microbial communities, corresponding to the glacier's spatial pattern (Fig. 5). This suggests that spatial isolation of glaciers might have stronger influences than temporal stresses on the distribution of microbial community in glacier ice. The Muztag Ata Glacier community is dominated by the six phylogenetic clusters *Planococcus/Anoxybacillus*, *Propionibacter/Luteococcus*, *Flavisolibacter* sp., *Flexibacter* sp., *Acinetobacter* sp., and *Enterobacter* sp. (Fig. 4). In contrast, the Puruogangri Glacier community is dominated by three main phylogenetic clusters, *Cryobacterium/Aeromicrobium* sp., *Polaromonas* sp., and *Flexibacter* sp. (Fig. 3a, c, d, and e; Zhang et al., 2009). This distinct phylogenetic community composition may be attributed to a combination of microbial deposition through aeolian activities over a glacier as well as post-deposition selection on the community structure of microorganisms in the surface snow-ice (Xiang et al., 2009a). On the one hand, the Muztag Ata Glacier is located in the most western periphery of the Tibetan Plateau, surrounded by the vast arid and semi-arid regions of central Asia (Fig. 1). It receives precipitation mainly derived from the western dry air mass (Wang, 1989; Wake et al., 1993), in which the frequent dust storms (Li et al., 2003) may carry abundant microorganisms to the glacier surface. The Dunde, Malan, and Puruogangri ice caps are located in the northern to middle part of the Tibetan Plateau, where the air masses are derived from the cold westerly mass in winter and mild south Asia monsoon in the warm summer seasons. They are also affected by the powerful winds and snowstorms caused by the numerous large depressions in the Tibetan Plateau, and in the local mountain regions (Fig. 1; Wang, 1989; Wake et al., 1993; Dregne, 1968; Chen and Bowler, 1986). The dramatic

changes of the air masses may lead to differences in the microbial species pool and thus result in the distinct community composition of microorganisms across the glaciers. On the other hand, differences in the local climatic and environmental conditions, such as temperature, light intensity, meltwater availability and nutrient concentrations in the glacier ice (Takeuchi et al., 1998, 2001; Takeuchi and Li, 2008; Takeuchi and Kohshima, 2004) may cause significant variation in the growth rate of tolerant microorganisms, which in turn may lead to the subsequent changes in the community composition of microorganisms in glacier ice. Variations in the phylogenetic population pool as a result of both aeolian and post-deposition processes lead to the apparent zonal distribution of microbial communities, which clearly corresponds to the distances across the four geographically isolated glaciers (Fig. 5). The preliminary data on the zonal pattern of microbial communities across the four deep glaciers suggests an ecological selection effect on the phylogenetic community composition of microorganisms in the air masses and under the glacier system.

The results suggest that both spatial and temporal stresses might exert influences on the community patterns of dominant bacteria across the four geographically isolated glaciers. First, most bacterial species from the same glacier more closely grouped together than those from the separated glaciers (e.g., the gray shaded areas in Fig. 3a, c, and d). Second, several phylogenetic clusters contributed to the community shift across the glaciers on the Tibetan plateau (Fig. 4). Finally, variations in the proportion of the main phylogenetic clusters at the species/genera level resulted in a seasonal community shift along the glacier depth profile. Although the high quality of ice core records at the extremely high altitude showed seasonal changes of major species, it cannot absolutely exclude the uncertainty of minor species trend in different seasons since the data was based on a series of single-ice-core results. In this current study, the community shift of microorganisms was related to the changes in the air masses over the glaciers on the Tibetan plateau. This has important implications for research on global climatic and environmental changes using microbial indices in glacier ice. There are still open questions as to how the processes of microbial deposition influence the community shift of microorganisms in the glacial regions considered, and how the climatic and environmental changes and microbial processes co-regulate the temporal and spatial patterns of microbial communities across the glaciers. This preliminary study provides only a glimpse of microbial biogeography over extensive spatial and temporal scales. However, it is uncertain to some extent for the spatial and temporal biogeography of microorganisms in glacier ice since limited data is available for a community comparison of microorganisms from the geographically isolated glaciers without an absolute similarity in physical-chemical characteristics. More data on the meteorologic, physico-chemical, and biological characteristics of the surface snow and consistent ice core data of microbial analysis will be crucial for truly understanding the community dynamics of microorganisms in glacier ice. More sequence data based on multiple-loci genes such as *rec*A (encoding the multi-functional DNA-binding protein involved in homologous recombination; Story et al., 1992; Kumar et al., 1993), *gyr*B (encoding the DNA gyrase B protein; Hallett et al., 1990), *wzm* (encoding the inner membrane protein; Reeves et al., 1996), and *rpo*B (encoding the β-subunit of RNA polymerase B; Landick et al., 1990) of the chromosome of microorganisms in glacier ice may provide the necessary detailed information on microbial biogeography, since the sequence data in this study was based on only a single 16S rRNA gene analysis.

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