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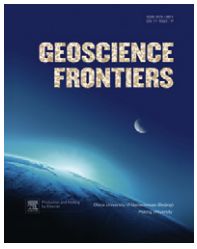

RESEARCH PAPER

Variation of culturable bacteria along depth in the East Rongbuk ice core, Mt. Everest

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Abstract Ice melt water from a 22.27 m ice core which was drilled from the East Rongbuk Glacier, Mt. Everest was incubation in two incubation ways: plate melt water directly and enrichment melt water prior plate, respectively. The abundance of cultivable bacteria ranged from 0–295 CFU mL^{-1} to 0–1720 CFU mL^{-1} in two incubations with a total of 1385 isolates obtained. Comparing to direct cultivation, enrichment cultivation recovered more bacteria. Pigment-producing bacteria accounted for an average of 84.9% of total isolates. Such high percentage suggested that pigment production may be an adaptive physiological feature for the bacteria in ice core to cope with strong ultraviolet radiation on the glacier. The abundances of cultivable bacteria and pigment-producing isolates varied synchronously along depth: higher abundance in the middle and lower at the top and bottom. It indicated that the middle part of the ice core was hospitable for the microbial survival. Based on the physiological properties of the colonies, eighty-nine isolates were selected for phylogenetic analysis. Obtained 16S rRNA gene sequences fell into four groups: *Firmicutes*, *Alpha-Proteobacteria*, *Gamma-Proteobacteria*, and *Actinobacteria*, with the *Firmicutes* being dominant. Microbial compositions derived from direct and enrichment cultivations were not overlapped. We suggest that it is a better way to explore

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the culturable microbial diversity in ice core by combining the approaches of both direct and enrichment cultivation.

1. Introduction

Mountain glaciers are widespread in the Tibetan Plateau. Comparing to the glaciers in Arctic and Antarctic, they have the traits of locating in lower latitude and higher altitude, being adjacent to densely populated human habitat area and having vital significance in studying the impacts on environmental and climatic change caused by human activities (Yao et al., 2003). There have been several crucial progresses on the relationship between glaciers and climatic change in the Tibetan Plateau (Qin et al., 2000, 2002; Kang et al., 2001, 2002; Ren et al., 2003). Ice core not only records important chemical information and substantial biological signature, but also reflects the past climatic and environmental change in the Tibetan Plateau (Thompson et al., 1989; Yao et al., 1996; Abyzov et al., 1998; Battarbee et al., 2002; Bulat et al., 2011). Microorganisms in the ice core were mostly precipitated from the atmosphere and were largely buried in the glacier via eolian sedimentation in different historical periods which could span up to thousands of years (Jouzel et al., 1999). Therefore, the microorganisms in deep ice core recorded the changes of the climate and the environment on the glaciers (Zhang et al., 2001, 2008; Xiang et al., 2004, 2005a). As a possible biological indicator for climatic change, the studies about microorganisms on glaciers have been a hot-spot of the scientists. Abyzov and colleagues have taken ocean ice thawed water from the depth of 2750 m in Vostok Lake and observed various forms of microorganisms, in which the density of cell distribution was about 10^3–10^4 cells mL^{-1}. And the fluctuations in the number of microorganisms correlated with changes in the number of mineral particles, which depended on alternations of warm and cold periods on Earth (Abyzov et al., 1998). In the Tibetan Plateau, microbe and their relationship with climatic change were studied on the Malan ice core (Zhang et al., 2001), the Puruogangri ice core (Zhang et al., 2006), the Guliya ice core (Christner et al., 2003), the Rongbuk ice core (Xiang et al., 2004; Zhang et al., 2010), and the Muztagata ice core (Xiang et al., 2005a,b). The concentration of cultivable microorganisms in ice core ranged from 0 to 760 CFU (colony forming unit) mL^{-1} (Zhang et al., 2001, 2006, 2010; Christner et al., 2003; Xiang et al., 2004, 2005a,b). In general, it was asserted that the cultivable microorganisms were abundant in the north while rare in the south over plateau. But the highest abundance of cultivable microorganisms was found in the Puruogangri ice core in the middle part of the plateau (Zhang et al., 2006).

Cultivable bacteria recovered from Tibetan Plateau ice core could be majorly divided into four groups: *Firmicutes*, *Proteobacteria*, *Cytophaga/Flavobacterium/Bacteroides* (CFB), and *Actinobacteria*. The microorganisms in the cold periods own higher abundance and diversity than those in the warm periods (Yao et al., 2003). Cultivable microorganisms in the Malan ice core were mainly composed of bacteria, fungi, and algae, but the last one occupying a small part (Zhang et al., 2001). The number of bacteria was in negative correlation with the temperature indicated by oxygen isotope but it was closely related to the dirty layer of the glacier (Zhang et al., 2001). The vertical distribution of dominant groups of bacteria showed a clear layered distribution, reflecting the response of the microorganisms to the climatic change represented by the depth of the ice core in different periods (Xiang et al., 2004, 2009a,b). Bacteria in Puruogangri ice core had different phenotypes and hereditary features, but did not show observable correlation with the depth (Zhang et al., 2006). In ice core from Mt. Everest, the combination of culture and culture-independent methods indicated layer distribution of bacterial community in different sections of the ice core, which might reflect the ecological environments on glacier at the time of their deposition (Zhang et al., 2010).

The purpose of this study is to explore the diversity of cultivable microorganisms along depth in the East Rongbuk ice core. An integrated approach including cultivation and 16S rRNA gene phylogenetic analysis was employed.

2. Materials and methods

2.1. Sample collection and pretreatment

A 22.27 m ice core was drilled from the East Rongbuk Glacier in Mt. Everest (Fig. 1). It was longitudinally cut into two parts and one of them was used for microbial analysis. The ice core was cut into 173 sub-sections in interval of 10–20 cm. The 1 cm thick ice on the surface of the samples was chipped off by a sterilized blade to avoid contamination during transportation and the sectioning process. All the processes were performed in the aseptic environment. The divided ice cores were put in sterilized beakers and were melted slowly in 4 °C.

2.2. Cultivation of culturable microorganisms in the ice core

For direct cultivation, a volume of 200 μL ice core thawed water was separated onto two sterilized dishes with R2Asolid medium (http://www.dsmz.de/media/med830.htm), which were incubated at 24 °C for 3 months. For enrichment cultivation, 200 μL of ice core thawed water was added in the cube containing 5 mL liquid R2A medium. After it was incubated at 4 °C for 1 week, 200 μL of enrichment broth was spread onto solid R2A medium (complemented with 1.5% agar) and then were incubated at 24 °C for 3 months. Bacteria colonies were visible on the culture dishes after 1 week and they were under continuous observation for 3 months.

2.3. DNA extraction and 16S rRNA genes PCR of the culturable microorganisms in the East Rongbuk ice core

A total of 89 isolates were chosen for DNA extraction and sequencing according to the phenotype and color of the obtained colonies. Bacterial DNA was extracted as follows (Johnson et al., 1981): 1.5 mL fresh broth was taken and centrifuged at 12,000 rpm for 5 min. Then discard the supernatant, added 567 μL TE, 30 μL 10% SDS and Protease K, 37 °C bain-marie for 1 h. After the bain-marie, 100 μL 5 mol L^{-1} NaCl, 80 μL CTAB/NaCl

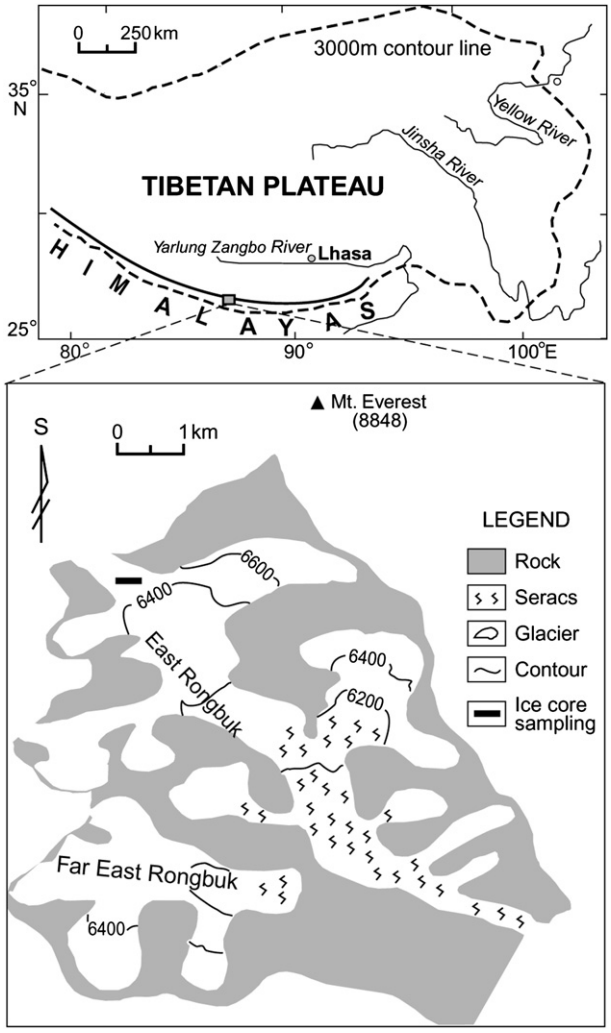

Figure 1 Sampling site of the ice core analyzed in this study (86°57′ E, 28°01′ N).

was added and then continued the process of bain-marie at 65 °C. After that, added 800 μL of the mixture of phenol, chloroform and isoamyl alcohol (25:24:1). Then were centrifuged at 12,000 rpm for 5 min. Supernatant was discarded and the DNA pellet was washed by using 1 mL of ethanol (conc. is 70%). The resulting DNA was air dried for 5 min, and then the DNA was dissolved into 40 μL ddH_2O.

16S rRNA genes were PCR amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and1492R (5′-CGGTTACCTTGTTACGACTT-3′) (Embley et al., 1991). PCR was carried out in a final volume of 50 μL using 2 μL template DNA, 3 μL $MgCl_2$, 4 μL each dNTP, 0.5 μL each primer, 0.2 μL Taq DNA polymerase, and 35 μL ddH_2O. Reactions were performed in the thermo cycler (ABI PCR System) with the following cycling parameters: 94 °C for 5 min for an initial denaturation, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

2.4. 16S rRNA gene phylogenetic analysis

The bacterial 16S rRNA gene sequences obtained in this study were blasted in the GenBank (http://www.ncbi.nih.gov). The blasted sequences and selected closest references were pooled and aligned using ClustlW. Phylogenetic analysis was performed using distance-based Neighbor-Joining method with MEGA version 5.0 (Tamura et al., 2011). The sequences determined in this study were deposited in the GenBank database under accession numbers: JN698965-JN698983.

3. Results and discussion

3.1. Phylogenetic characterization of culturable microorganisms in the East Rongbuk ice core

A total of 89 isolates were sequenced (Table 1, Fig. 2) and 16S rRNA gene phylogenetic analysis showed that these isolates belonged to four groups, *Actinobacteria*, *Firmicutes*, *Alpha-Proteobacteria*, and *Gamma-Proteobacteria*. *Firmicutes* were the most abundant one with a percentage of 74%. The other three groups occupied 17%, 7% and 2%, respectively. This result was consistent with previous study on another ice core in the same glacier (Zhang et al., 2010). However, we did not obtain the *Deinococcus-Thermus* isolates. Such difference could be attributed to the difference of the cultivation media employed: we used the R2A medium in this study, whereas they used the PYGV medium (http://www.dsmz.de/media/med830.htm).

All sequences were grouped into twelve OTUs at the 97% cut off (Fig. 3a, b) (Schloss and Handelsman, 2005). The phylogenetic trees indicated that culturable microorganisms in the East Rongbuk ice core were similar to those from air, earth, sea water, frozen soil, snow, and ice environments (Fig. 3a, b). This suggested that ice core microorganisms were from the locations that relevant to atmospheric circulation of Tibetan Plateau, which was in accordance with the views of former researchers (Zhang et al., 2001; Yao et al., 2003; Xiang et al., 2005a).

Microbial compositions derived from direct and enrichment cultivations were not overlapped. At the genus level, seven genera of *Bacillus*, *Staphylococcus*, *Novosphingobium*, *Paracoccus*, *Brevundimonas*, *Nocardioides*, and *Brachybacterium* were obtained by direct cultivation and five genera including *Paenibacillus*, *Bacillus*, *Pseudomonas*, *Micrococcus*, *and Kocuria* were obtained by enrichment cultivation. The common genera appeared in both situations is *Bacillus* while the *Paenibacillus*, *Staphylococcus*, *Paracoccus*, *Brevundimonas*, *Novosphingobium*, *Nocardioides*, *Brachybacterium*, *Pseudomonas*, *Micrococcus* and *Kocuria* were unique to different situations. The result suggested that combining two approaches could recover more diverse of bacteria from ice core.

3.2. Diversity of culturable microorganisms along depth

The relative abundance of the eleven obtained genera changed along depths (Fig. 4). At the depths of 0–5 m and 6–10 m, the predominant genus were both *Bacillus*, accounting for more than 90% and 70% of total isolates. Genus *Staphylococcus* was predominant at the depths of 10–15 m and 15–20 m accounting for 50%. And at the depths of 20–23 m, *Bacillus* and *Staphylococcus* took the same ratio of 40%. The diversity of the culturable microorganisms was lower in the surface layer, and then became higher in the middle and then lower again at the bottom part of the ice core. Two and three genera were obtained at the top and bottom part of the ice core, but five genera were recovered at the middle (10–15 m). The possible reason for such changing regularity may be that the climate in the surface layer was very severe;

Table 1 Summary of the bacteria isolated in the East Rongbuk ice core.

Depth (cm)	Isolate ID	Phylum	Depth (cm)	Isolate ID	Phylum	Depth (cm)	Isolate ID	Phylum
38	R02_4_2	*Firmicutes*	575	R07_5	*Firmicutes*	893	RC11_8_1	*Proteobacteria*
131	RC01_2	*Firmicutes*	575	RC07_5	*Actinobacteria*	944	RC12_2	*Proteobacteria*
131	RC02_3_1	*Firmicutes*	625	RC08_2_1_1	*Actinobacteria*	972	R12_2	*Firmicutes*
147	RC02_3_2	*Firmicutes*	625	RC08_2_1_2	*Actinobacteria*	972	RC12_2_1	*Firmicutes*
180	RC03_1	*Firmicutes*	625	RC08_2_2	*Actinobacteria*	972	RC12_2_2	*Firmicutes*
199	RC03_2	*Firmicutes*	635	RC08_3_1	*Actinobacteria*	978	R12_3	*Firmicutes*
226	RC03_4_1	*Firmicutes*	635	RC08_3_2	*Actinobacteria*	978	RC12_3_1	*Firmicutes*
226	RC03_4_2	*Firmicutes*	635	RC08_3_2_1	*Actinobacteria*	1020	RC12_7	*Firmicutes*
286	R04_3_1	*Firmicutes*	648	R08_4	*Firmicutes*	1079	RC13_4_1_2	*Proteobacteria*
286	R04_3_1_2	*Proteobacteria*	676	R08_6	*Firmicutes*	1198	R14_6	*Proteobacteria*
286	R04_3_2	*Firmicutes*	691	R09_1	*Firmicutes*	1243	R15_3	*Proteobacteria*
314	RC04_5	*Firmicutes*	726	R09_4	*Firmicutes*	1258	R15_4	*Actinobacteria*
339	RC05_1	*Firmicutes*	737	R09_5	*Firmicutes*	1290	RC15_6_2	*Firmicutes*
351	RC05_2_2	*Firmicutes*	737	RC09_5_2	*Actinobacteria*	1290	RC15_6_3	*Firmicutes*
425	R05_7	*Firmicutes*	746	R09_6_1	*Firmicutes*	1290	RC15_6_1_1	*Actinobacteria*
445	R06_1_2_1	*Firmicutes*	746	RC09_6_1	*Actinobacteria*	1367	RC16_6	*Firmicutes*
445	R06_1_2_2	*Firmicutes*	746	RC09_6_1_2	*Actinobacteria*	1367	RC16_6_2_1	*Firmicutes*
445	RC06_1_2_1	*Firmicutes*	746	RC09_6_2	*Actinobacteria*	1367	RC16_6_2_2	*Firmicutes*
528	R07_1	*Firmicutes*	746	RC09_6_4_1	*Actinobacteria*	1416	RC17_4_A	*Firmicutes*
539	R07_2_1	*Firmicutes*	746	RC09_7_1	*Actinobacteria*	1498	R18_3	*Firmicutes*
539	R07_2_2	*Firmicutes*	746	RC09_6_3_1	*Firmicutes*	1559	R19_1_1_2	*Firmicutes*
539	R07_2_3	*Firmicutes*	746	RC09_6_4_2	*Firmicutes*	1613	R19_5_1	*Firmicutes*
539	R07_2_4	*Firmicutes*	756	R09_7	*Firmicutes*	1748	R21_5_2	*Firmicutes*
539	R07_2_5	*Firmicutes*	756	RC09_7_2	*Actinobacteria*	1784	RC21_8	*Firmicutes*
550	RC07_3	*Firmicutes*	756	RC11_4	*Firmicutes*	2065	R25_4	*Firmicutes*
550	RC07_3_1	*Actinobacteria*	771	R09_8	*Proteobacteria*	2103	R26_1	*Firmicutes*
550	RC07_3_2	*Actinobacteria*	837	R10_6	*Firmicutes*	2118	R26_2	*Firmicutes*
563	RC07_4	*Actinobacteria*	862	R11_1	*Firmicutes*	2146	R26_4	*Firmicutes*
563	RC07_4_2	*Actinobacteria*	882	R11_3	*Firmicutes*	2160	R26_5	*Firmicutes*
			893	R11_4	*Firmicutes*	2160	RC26_5	*Firmicutes*

the microorganisms were in the state of deep physiological dormancy and could not be easily recovered. The middle part of the ice core was relatively hospitable, and the sub-sections in this depth were not so old; Furthermore, microorganisms in this part have enough available organic matter. So, the microorganisms in this layer were more active and more species could be recovered. The amount of the culturable microorganisms in the depths of 10–15 m was the highest (Fig. 5). However, the bottom part was so old that the available organic matter at such depth may be depleted. Thus, the variety of the microorganisms decreased. For example, the genus *Staphylococ*cus appeared from the depth of 10 m and increased between 15 and 20 m but decreased after the depth of 20 m. The possible reason might be that the surroundings were suitable for them survival at the depth of 10 m. Then the amount of the *Staphylococcus* reached peak value. Our finding about the higher diversity of culturable bacteria appeared in the middle part of the ice core was consistent with Xiang et al. (2005a).

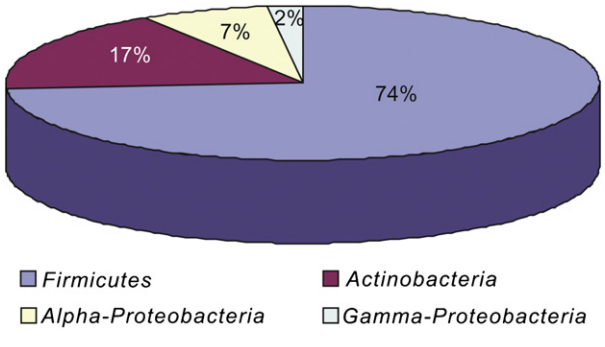

Figure 2 Pie chart showing the composition of the cultivated bacteria identified via bacterial 16S rRNA gene sequences affiliated with the major phylogenetic groups.

3.3. Abundance of culturable microorganisms varied along depth in different culture conditions

The number of culturable bacteria in East Rongbuk ice core changed remarkably along depth (Fig. 4). The concentration was 0–1720 CFU mL^{-1} in general, which was higher than the concentration (0–5.6 CFU mL^{-1}) obtained by Zhang et al. (2010). One of the reasons that caused the consequences might be the individual differences of the sample drilled and the local factors that drove bacterial community composition over a wide range of spatial scales (Gucht et al., 2007). But the main reason might be the differences in the cultivation process employed.

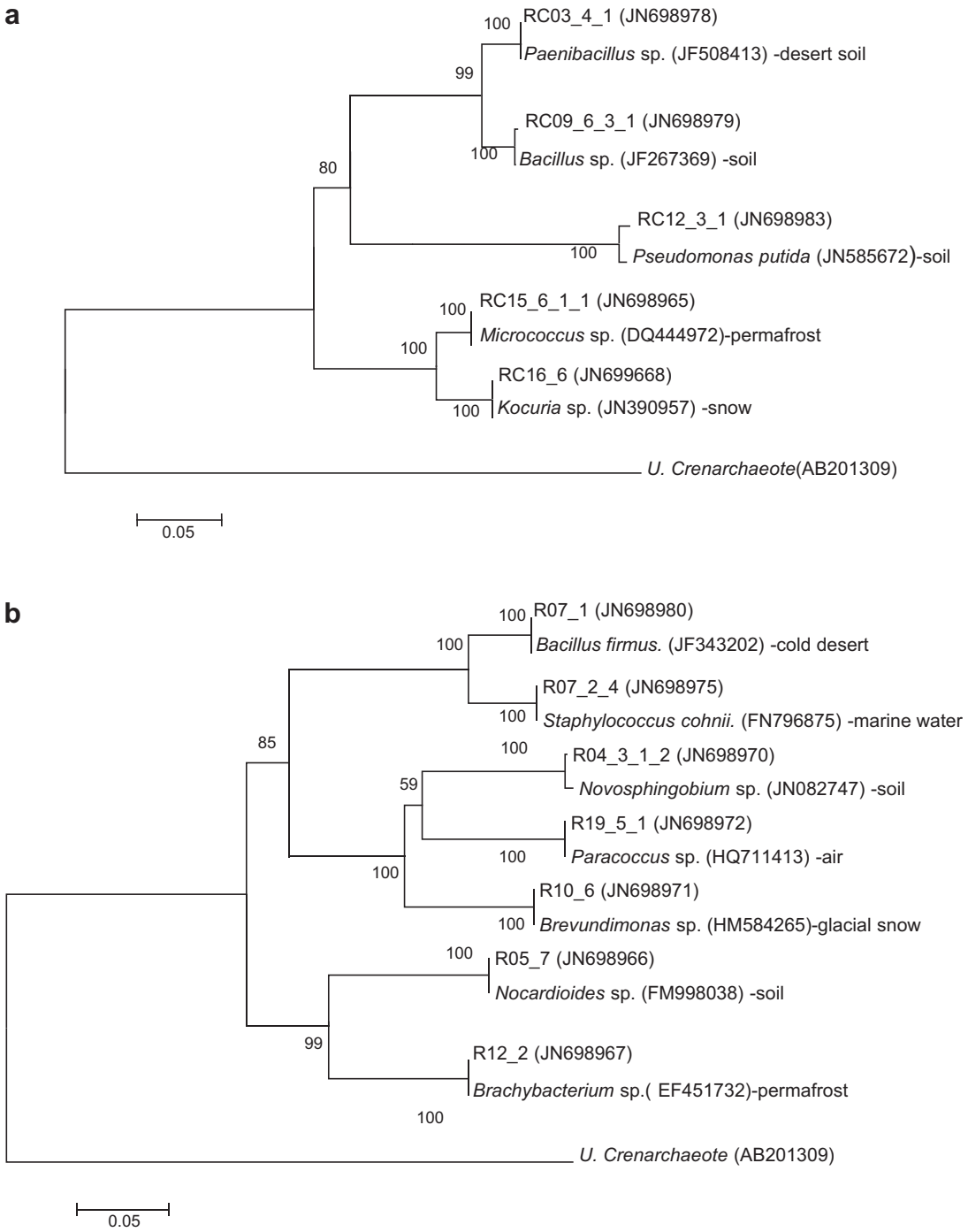

Figure 3 Neighbor-joining trees showing the phylogenetic relationships of bacteria 16S rRNA gene sequences from the East Rongbuk ice core to closely related sequences from the GenBank database. Panel a shows the phylogeny of isolates obtained through enrichment cultivation at 4 °C; Panel b shows the phylogeny of isolates obtained through direct cultivation.

The specific depths with a large number of microorganisms were concentrated, in the depths of 5–7.5 m and near the depth of 15 m. The number of cultivable microorganisms was in a sharp increase at small interval (5.5 m, 5.63 m, 5.75 m, 6.76 m, 7.37 m, 7.46 m, 8.62 m, 10.2 m and 14.16 m). This suggested that the microorganisms were brought in by snowfall events or it might be the result of sand storm.

If 30 CFU mL^{-1} was regarded as a relatively high concentration range of microorganisms (Xiang et al., 2005a), nine periods, represented by the depths of 5.5 m, 5.63 m, 5.75 m, 6.76 m, 7.37 m, 7.46 m, 8.62 m, 10.2 m and 14.16 m, during which, higher amounts of dust were brought into the East Rongbuk Glacier. The abundance of cultivable bacteria from direct cultivation was 0–295 CFU mL^{-1}. In contrast, the concentration of culturable bacteria from enrichment cultivation was much higher, with a value of 0–1720 CFU mL^{-1}. The reasons for such result may be that some dormant bacteria could be recovered by enrichment cultivation. While direct cultivation may lead to the

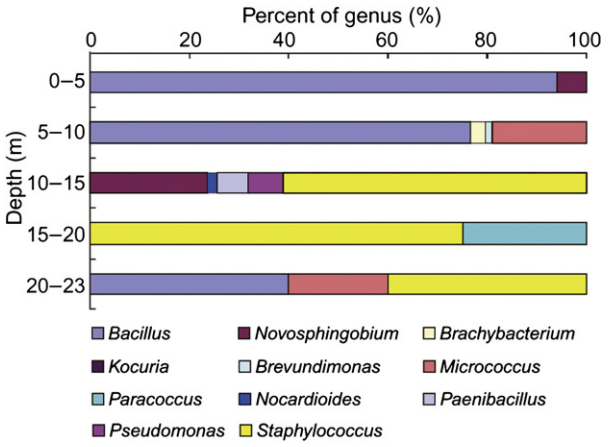

Figure 4 Bar graphs showing the frequencies of the obtained bacterial 16S rRNA gene sequences affiliated with the major phylogenetic groups along depth of the ice core.

drastic change of the external environment and some bacteria could not be recovered. Previous studies showed that some culturable bacteria could not be obtained without the employ of enrichment cultivation (Christner et al., 2003; Miteva et al., 2004).

3.4. Abundance of the pigment-producing isolates in different culture conditions along depth

With direct cultivation, a total of 348 isolates were obtained, among which 69.3% isolates (241 out of 348) produced pigments. With enrichment cultivation, a total of 1037 isolates were obtained, among which 90.2% isolates (935 out of 1037) produced pigments. In total of 1385 isolates were obtained, 84.9% of which produced pigments (Fig. 6). The features of cold, dry, oligotrophic and strong ultraviolet (UV) of pole region and the Tibetan Plateau endowed some unique patterns and physiological features of the microorganism there: smaller cell volume, capability of producing spores, trait of thinner cell walls, ability of producing pigment and cold-active enzymes etc (Fogg, 1998; Miteva et al., 2004; Cavicchioli, 2006; Zhang et al., 2008; Reddy et al., 2009). Producing pigment is an effective way for the microorganisms to survive against the UV. Pigments absorb ultraviolet light to prevent its lethal damage to cells. Under the circumstances mentioned above, the percentages of pigment-producing isolates obtained by the two cultivations were different significantly. 90.2% of the isolates obtained from enrichment cultivation produced pigment. But 69.3% in direct cultivation. Consider the number and diversity of the microbial community, enrichment cultivation may be a better way to study the adaption of bacterial to the environment in the ice core. The abundance variation of the pigment-producing bacteria was in accordance with the distribution of the total amount of culturable bacteria in different depths (Figs. 5 and 6).

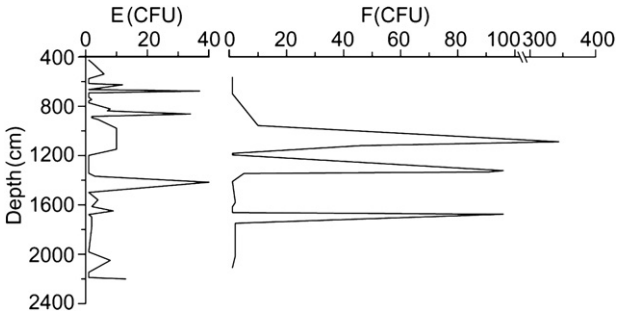

Figure 6 Abundance of pigment-producing isolates along depth (E: direct cultivation for 3 months; F: enrichment cultivation at 4 °C for 3 months).

The pigment-producing microorganisms showed the pattern of high concentration appeared in the middle layer of the ice core (Fig. 6). The possible reason might be that the microorganisms in the surface layer did not have enough time to acclimatization after they were brought in glacier. In contrast, those microorganisms in the middle layer had enough time to cope with UV. At the bottom part of the ice core, the microorganisms were in the stage of decaying, and could not reveal their adaptive features to the special environment in the Tibetan Plateau.

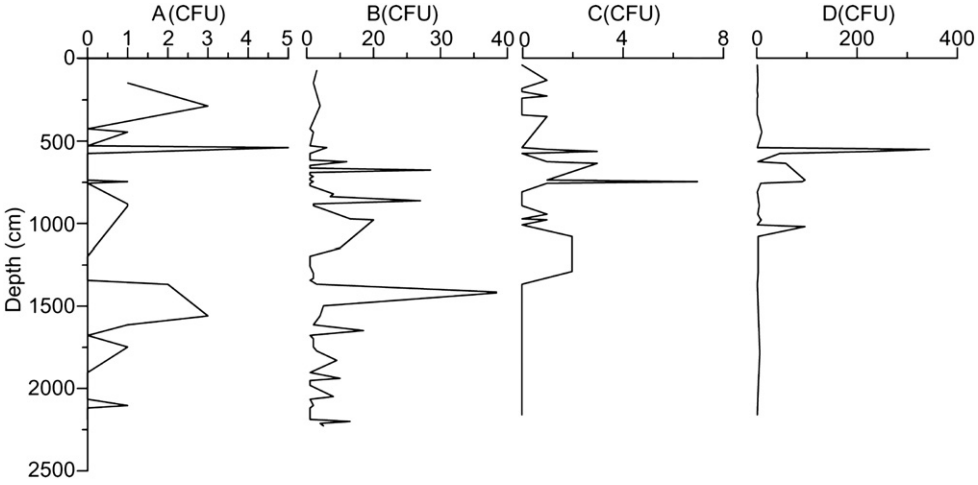

Figure 5 CFU counts at different cultivation conditions (A: direct cultivation for 1 month; B: direct cultivation for 3 months; C: enrichment cultivation at 4 °C for 1 month; D: enrichment cultivation at 4 °C for 3 months) along depth of the ice core.

4. Conclusion

With the approaches of cultivation and 16S rRNA gene phylogenic analysis, diversity and abundance of culturable bacterial were investigated in a 22.27 m ice core drilled from the East Rongbuk Glacier. The abundance of culturable bacteria in the ice core was 0–295 CFU mL^{-1} and 0–1720 CFU mL^{-1} for direct and enrichment cultivations, respectively. The culturable bacteria could be divided into 4 groups: *Firmicutes*, *Alpha-Proteobacteria*, *Gamma-Proteobacteria*, and *Actinobacteria*. Both microbial abundance and diversity were highest in the middle part of the ice core, indicative of special historical periods when large amounts of microorganisms were brought together with nutrition in the ice core by historical events such as sand storms or snow falls. A high percentage of culturable bacteria produced pigments, suggested that producing pigments might be one of the physiological features of the microorganisms adapting to the strong UV in the Tibetan Plateau. In addition, enrichment cultivation could provide a more hospitable recovering environment than direct cultivation, and thus the former could obtain more microorganisms. So the combination of direct and enrichment cultivations could retrieve a more representative microbial community.

Acknowledgments

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