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Phylogenetic analysis of bacteria in sea ice brine sampled from the Canada Basin, Arctic Ocean

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Abstract Bacterial diversity in sea ice brine samples which collected from four stations located at the Canada Basin, Arctic Ocean was analyzed by PCR-DGGE. Twenty-three 16S rDNA sequences of bacteria obtained from DGGE bands were cloned and sequenced. Phylogenetic analysis clustered these sequences within γ-proteobacteria, *Cytophaga-Flexibacter-Bacteroides* (CFB) group, Firmicutes and Actinobacteria. The phylotype of *Pseudoalteromonas* in the γ-proteobacteria was predominant and members of the CFB group and γ-proteobacteria were highly abundant in studied sea ice brine samples.

Key words sea ice brine; Arctic; bacteria; 16S rDNA; phylogenetic

1 Introduction

Majority of the Arctic Ocean is covered by sea ice for most of the year, with the area varying between 7 and 14×10^6 km^2 seasonally (Gradinger and Zhang 1997). Sea ice with temperature ranging from 0 to − 35℃ (Haas *et al.* 1997) is an important and extreme habitats on Earth for marine life. Once sea ice formed from seawater, it is a semi-solid matrix being permeated with a labyrinth of brine-filled channels and pockets. Such a semi-enclosed or even completely enclosed ecosystem is a microhabitat characterized by highly changeable salinity, acidity, dissolved gas and light signatures (Eicken *et al.* 2000; Thomas and Dieckmann 2002), and in which, very heavy, highly active and diverse sea-ice microbial communities (SMCO) develops annually (Horner *et al.* 1992; Grossmann and Dieckmann 1994). Sea-ice microbial communities live mainly in brine channels and pockets in the ice matrix, which play a significant role in the marine food webs of the Arctic Ocean. It was believed that heterotrophic bacteria represent a major group within sea ice microbial communities (Sullivan and Palmisano 1984; Staley and Gosink 1999; Mock and Thomas 2005).

The phylogenetic diversity of sea-ice microbial communities has been studied mainly in the Antarctic (Bowman *et al.* 1997; Brown and Bowman 2001; Brinkmeyer *et al.* 2003). Just recently has the diversity of Artic sea-ice bacteria been investigated by Brown and Bow-

man (2001) and Brinkmeyer *et al*. (2003, 2004), combining both cultivation and cultivation-independent methods. It has been found that the Arctic and Antarctic sea ice bacteria fall into 4 major phylogenetic groups including α-proteobacteria, γ-proteobacteria, *Cytophaga-Flexibacter-Bacteroides* (CFB) group and Gram-positive ones (Brown and Bowman 2001; Junge *et al*. 2002; Brinkmeyer *et al*. 2003; Groudieva *et al*. 2004). In addition, the phylotypes of both regions were found to be highly similar each other. In this study, the phylogenetic diversity of bacteria in sea ice brine samples of four stations located at the Canada Basin, Arctic Ocean was studied using PCR-DGGE.

2 Materials and methods

2.1 *Sampling*

Four Sea ice brine samples used in this study were collected from the Canada Basin of the Arctic Ocean during the Louis S. St-Laurent JWACS/NOAA Canada Basin expedition from 19th to 28th August 2002 (Table 1). After the sea ice cores were taken by MARK (七) at 4 stations, the brine samples were collected into sterile plastic bottles and kept at 4℃. When returning to the lab, the brine samples (1500~1900 ml) were filtered through a Whatman filter (0.22 μm pore size). The filter was put in a sterilized 2 ml centrifuge tube and kept frozen at −80℃ until DNA extraction.

Table 1 Sites and time of brine samples

Station	Designated sample	Latitude and longitude	Date	Volume (ml)
1	8	72°52.588′N, 136°32.218′W	19th Aug. 2002	1500
2	12	73°30.059′N, 136°59.012′W	21st Aug. 2002	1900
3	22	73°30.596′N, 136°59.980′W	22nd Aug. 2002	1600
4	45	76°49.154′N, 148°18.438′W	28th Aug. 2002	1900

2.2 *DNA extraction*

For total DNA extraction, microorganisms were washed off the filter by rinsing the filter with lytic buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 50 mM sucrose). After centrifugation, the cells were re-suspended in 0.5 ml lytic buffer. 15 mg of lysozyme was added in and mixed. The mixture was incubated at room temperature for 10 min. After adding SDS and proteinase K to final concentrations of 1% (v/v) and 100 μg/ml respectively, the mixture was incubated at 37℃ for 30 min and then at 55℃ for 10 min. DNA was obtained from the lysates using standard phenol-chloroform extraction and ethanol precipitation procedure. The pellet was suspended in 20 μl sterilized Milli-Q water. RNA was removed by incubating the DNA solution with 5U DNase-free RNase at 37℃ for 15 min.

2.3 *Amplification of 16S rRNA genes*

Nearly full-length 16S rDNA was amplified from genomic DNA following the method described by Bosshard *et al.* (2000) with the primers 8f 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1492r 5′-GGT TAC CTT GTT ACG ACT T-3′. Amplification was carried out in 50 μl reaction volume containing 1 × buffer, 1.5 mM $MgCl_2$, 200 μmol dNTP each kinds, 1U *Taq* DNA polymerase (Promega, Germany), 10 pmol primers (each direction), and 1 μl template DNA. PCR was carried out by denaturing at 95℃ for 3 min, followed by 25 cycles of denaturing at 95℃ for 1 min, annealing at 50℃ for 1 min and extending at 72℃ for 2 min, and a final extension at 72℃ for 10 min. For DGGE analysis, a nest-PCR was carried out. The primers for amplifying eubacterial 16S rRNA genes were 341f with GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CC TAG GGG AGG CAG CAG-3′) and 534r (5′-ATT ACC GCG GCT GCT GG-3′) as described by Muyzer *et al.* (1993). 1 μl of the first PCR product was used as the template of the second amplification in a 50 μl reaction volume containing 200 μmol each of dNTP, 10 pmol each of primers, 1U of *Taq* DNA polymerase (Promega, Germany), and 5 μl of 10 × PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3). The thermocycling program included an initial denaturation at 94℃ for 4 min, followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 58℃ for 30 s and extension at 72℃ for 1 min, and a final extension at 72℃ for 7 min. An aliquot of PCR products was electrophorezed in 0.8% agarose gel. Gel was stained with ethidium bromide and recoded using an ultraviolet analysis system.

2.4 *DGGE analysis*

DGGE analysis was carried out with the D-CODE universal mutation detection system (Bio-Rad, USA). Approximately, 700 ng of PCR product was loaded each lane. PCR products were separated in 0.75mm 8% polyacrylamide denaturant gradient gel (acrylamide: bisacrylamide, 37.5: 1) in 1 × TAE buffer at 200V and 60℃ for 4hrs. Denaturant gradient ranged from 30% to 60% linearly (100% denaturant was defined as 7M urea and 40% formamide). The gels were stained for 30 min with ethidium bromide and visualized under UV irradiance. The number of operational taxonomic unit (OTU) for each sample was defined as number of DGGE band found in that sample.

2.5 *16S rDNA sequencing*

A total of 23 bands (Fig. 1) were excised from the DGGE patterns. Excised band was suspended in 20 μl of sterilized MilliQ water, and kept at – 20℃ overnight. The supernatant was collected after centrifugation at 12, 000 g for 1 min at room temperature. 1 μl of such supernatant was used for re-amplification with primers 341f with GC clamp and 534r. The PCR product was ligated into pMD-18 T Vector (TAKARA, JAPAN) following manufacturer's instruction, and transformed into *E. coli* DH5α. The plasmid containing inserts were sequenced with M13 forward primer using an ABI PRISM automatic sequencer (model

377).

2.6 Phylogenetic analysis

The sequences most similar to the obtained ones were found through BLAST search (http://www.ncbi.nlm.nih.gov) against GenBank Database and retrieved. All 16S rDNA sequences were aligned using clustal multiple-alignment program (ClustalW). In such alignment, primer corresponding region were included. Phylogenetic trees were infered, and bootstrap analysis (1000 replicates) was performed with MEGA version 3.1 (Kumar *et al.* 2004).

2.7 Nucleotide sequence accession numbers

Nucleotide sequences have been deposited in the GenBank database under the accession numbers DQ838467 to DQ838489.

3 Results

3.1 DGGE

The V3 region of 16S rRNA gene of bacteria retrieved from the sea ice brine samples collected from the Canada Basin were amplified and profiled using DGGE. As showed in Fig. 1, DGGE profiles of different samples were complex and distinguishable each other, and that each sample was composed of a set of bands (at least 10 distinguishable bands). Many of the DGGE bands were weak, implying they correspond to relatively low abundance of template (DeLong and Pace 2001). DGGE revealed also that some bands were shared by samples.

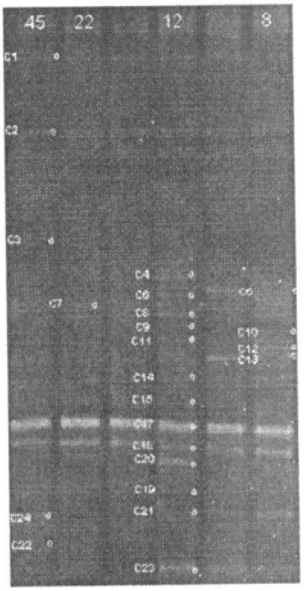

Fig. 1 DGGE profiles of 16S rDNA V3 region of sea ice brine samples. The bands marked with "o" were excised, cloned and sequenced.

Table 2 Phylogenetic affiliations of 16S rDNA sequences in this study and their detection in sea ice brine samples (+ positive, − negative)

Band	Phylogenetic affiliation	Most closely related sequence and the accession number	Similarity(%)	Source	Detection in brine samples			
					45	22	12	8
C1	γ-proteobacteria	AY167313 *Pseudomonas* sp. ANT92	99	Antarctic pack ice	+	+	+	+
C2	γ-proteobacteria	AM111024 *Stenotrophomonas* sp. 7022	98	Deep sea sediment	+	+	+	+
C3	γ-proteobacteria	AY771728 *Pseudoalteromonas nigrifaciens* S3-28	99	Arctic bacteria	+	−	−	−
C4	CFB	DQ186953 *Polaribacter* sp. clone SBI04_133	98	Arctic Ocean	+	+	+	+
C5	CFB	AY167338 *Bacteroides* bacterium ARK10264	99	Arctic pack ice	−	−	−	+
C6	CFB	AY921684 clone AKYH1027	87	Soil near to a silage storage	−	−	+	+
C7	CFB	DQ129648 clone AKIW814	95	Urban aerosol	+	+	+	+
C8	γ-proteobacteria	AF468287 clone ARKIA-103	95	Arctic pack ice	−	−	+	+
C9	γ-proteobacteria	AY771728 *Pseudoalteromonas nigrifaciens* S3-28	98	Arctic bacteria	−	−	+	+
C10	γ-proteobacteria	AF539778 *Pseudoalteromonas* sp. RE2-11	99	Biofilms of the marine alga	−	−	−	+
C11	γ-proteobacteria	DQ537521 *Pseudoalteromonas* sp. BSi20629	98	Arctic Sea ice	−	−	+	+
C12	γ-proteobacteria	DQ517877 *Pseudoalteromonas* sp. BSi20590	99	Arctic sea ice	−	−	−	+
C13	Firmicutes	DQ239694 *Enterococcus faecalis* strain D3	99	/	−	−	−	+
C14	γ-proteobacteria	AF469307 clone 33-PA60B00	100	Subseafloor habitat	−	+	+	+
C15	γ-proteobacteria	AY771728 *Pseudoalteromonas nigrifaciens* S3-28	99	Arctic bacteria	+	+	+	+
C17	CFB	AY238335 *Flexibacter* sp. MDA2495	95	A patient's sputum	+	+	+	+
C18	γ-proteobacteria	DQ517880 *Pseudoalteromonas* sp. BSi20673	100	Arctic sea ice	+	+	+	+
C19	γ-proteobacteria	AY167267 *Marinobacter* sp. ANT8277	100	Antarctic pack ice	−	+	+	+
C20	CFB	AY258121 bacterium DG887	99	Associated with the shellfish	−	+	+	+
C21	CFB	AY167338 *Bacteroides* bacterium ARK10264	99	Arctic pack ice	−	+	+	+
C22	γ-proteobacteria	AY167267 *Marinobacter* sp. ANT8277	98	Antarctic pack ice	+	−	−	−
C23	Actinobacteria	DQ449553 clone Ice-10K-112	99	Arctic fjord ice	−	+	+	+
C24	γ-proteobacteria	DQ060402 *Marinobacter* sp. BSi20041	98	Arctic Sea Ice	+	−	−	−

3.2 *Phylogenetic analysis*

Twenty-three individual bands were sequenced from the DGGE gels (Fig. 1). The lengths of the resolved partial 16S rDNA sequences varied from 174 to 194 nucleotides. After a BLAST search, the phylogenetic affiliations of the sequences obtained in this study with their similarities (87 to 100%) to their closely related known sequences were shown in Table 2. Eighteen sequences showed the highest similarities to GenBank sequences of bacteria originating from marine environment, especially the Antarctic and Arctic sea ice. All 16S rDNA sequences derived from samples were clustered into four phylogenetic groups including γ-proteobacteria, CFB group, Firmicutes and Actinobacteria (Table 2). Fig. 2 showed the phylogenetic relationships of the sequences isolated in this study with those retrieved from database.

A total of 14 sequences were grouped into γ-proteobacteria. Most of the sequences were closely related to species previously isolated from Antarctic and/or Arctic sea ice. These sequences were found to belong to 3 known genera including *Pseudoalteromonas*, *Marinomonas* and *Stenotrophomonas* (Table 2 and Fig. 2). The sequences of the genera *Pseudoalteromonas* were dominant, which include sequences C1, C3, C9, C10, C11, C12, C14, C15 and C18. The band/sequence C1, which was present in four brine samples (Fig. 1), had 99% similarity to *Pseudomonas* sp. ANT92 isolated from Antarctic pack ice (Brinkmeyer *et al.* 2003). Sequences C3, C9 and C15 were closely related to *Pseudoalter-*

amonas nigrifaciens S3-28 isolated from deep-sea sediment of the east Pacific Ocean. Sequences C11, C12 and C18 clustered with genera *Pseudoalteramonas* were highly similar to sequences of bacterial strains isolated from Arctic sea ice by us. The sequence C10 was only present in sea ice brine 8, with 99% similarity to *Pseudoalteramonas* sp. RE2-11 isolated from the biofilms of marine alga. Sequence C14 showing 100% similarity to uncultured bacterial clone from subseafloor habitat, Axial Volcano. Three sequences (C19, C22 and C24) were related to *Marinamonas* sp. from Arctic and Antarctic sea ice with similarities ranging from 98% to 100%. Only one sequence (C2), which was detected in all lanes, was the closest relative of *Stenotrophamonas* sp. 7022 isolated from the deep-sea sediment of east Pacific Ocean. In addition to the sequences mentioned above, band C8, which was clustered into γ-proteobacteria but could not be affiliated to known genera, showing 95% similarity to uncultured bacterial clone AKIWA-103 obtained from Arctic pack ice (Brinkmeyer *et al*. 2003).

Seven sequences (30%) were clustered into CFB group (Table 2 and Fig. 2). Four sequences (C4, C5, C20 and C21) were closely related to species previously isolated from Arctic sea ice and other marine habitats. It was surprising that other sequences were closely related to those retrieved from soil, urban aerosol and patient's sputum. These sequences were found to belong to 3 known genera including *Polaribacter*, *Bacteroides* and *Flexibacter*. Four sequences (C6, C7, C17 and C20) related to clones or bacteria from non-polar environment clustered with *Flexibacter*, implying that this genus was dominant in the CFB group. Sequence C6 was 87% similar to an uncultured bacterium clone AKYH1027 obtained from soil adjacent to a silage storage bunker. Band C7 was closely related to the clone AKIW814 of the urban aerosol of two cities in Texas with 95% similarity. While sequence C17 was the closest relative of *Flexibacter* sp. MDA2495 isolated from a patient's sputum; sequence C20 showed 99% similarity to bacterium DG887 which associated with shellfish. Sequences C5 and C21 were 99% similar to *Bacteroides* bacterium from Arctic pack ice. Sequence C4 was 98% similar to uncultured *Polaribacter* sp. clone from Arctic Ocean.

Sequence C13, which is related to *Enterococcus faecalis* strain D3, was found to belong to Firmicutes. This sequence was only retrieved from and present in sea ice brine sample 8, and it was only one low G + C Gram-positive bacterial sequence detected in this study. Band C23 presented in three sea ice brine samples was closely related to the clone Ice-10K-112 from Arctic fjord ice, which was grouped to Actinobacteria (Gram-positive bacteria with a high GC content of DNA) with a 99% similarity.

3.3 Detection of bands in sea ice brine samples

Bands C1, C2, C4, C7, C15, C17 and C18 were detected in all studied sea ice brine samples (Table 2), suggesting the members of *Pseudamonas*, *Stenotraphamonas*, *Polaribacter*, *Flexibacter* and *Pseudoalteramonas* were presented in all sea ice brine sample. Sequences corresponding to bands C14, C19, C20, C21 and C23, which were clustered to *Pseudoalteramonas*, *Marinobater*, *Flexibacter*, *Bacteroides* and Actinobacteria respectively, were detected in all sea ice brine samples except 45. The results indicated that phylotypes

of *Pseudoalteromonas*, *Stenotraphomonas*, *Polaribacter*, *Marinobater*, *Flexibacter*, *Bacteroides* and Actinobacteria were rich in studied sea ice brine.

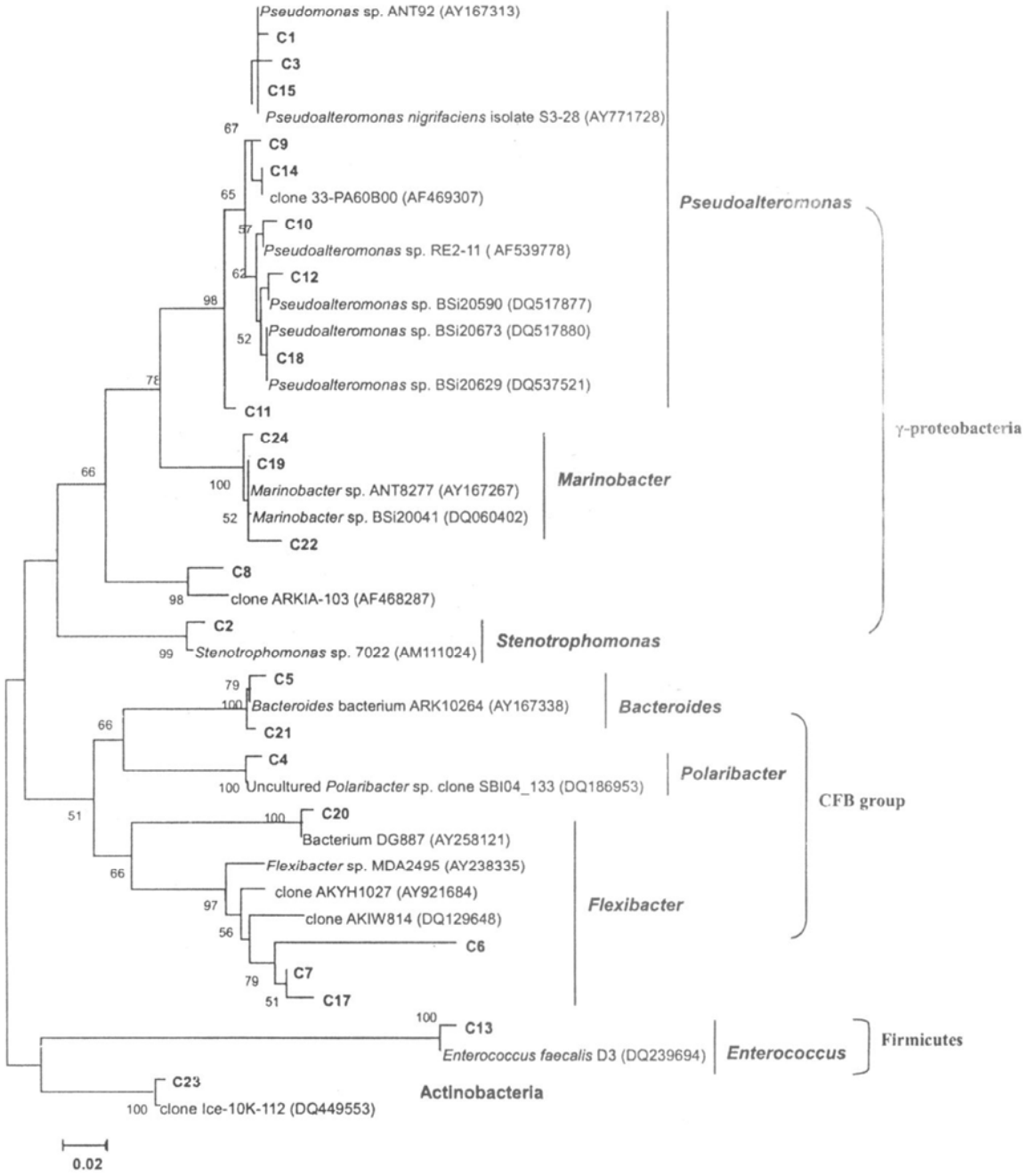

Fig 2 Phylogenetic relationships of bacterial 16S rDNA sequences from sea ice brine samples to closely related sequences retrieved from GenBank. The tree was constructed based on Kimura (two-parameter correction) distances using neighbor-joining method. Sequences from this study are in boldface type. The numbers in parentheses are accession numbers of sequences. Bootstrap values > 50% (1000 iterations) are shown. Scale bar indicates 0.02 nucleotide substitution per sequence position.

4 Discussion

Phylogenetic analysis of 16S rDNA sequences of bacteria from the sea ice brine samples collected from the Canada Basin revealed rich genetic diversity. These sequences are highly similar to those reported before with similarities ranging from 87% to 100%. In general, the most closely related sequences were retrieved from sea ice. All sequences derived

from sea ice brine samples fell into four phylogenetic groups including γ-proteobacteria, CFB group, Firmicutes and Actinobacteria. Similar to our previous results (Li *et al.* 2005), sequences fell into γ-proteobacteria were found to be predominant and members of CFB group were highly abundant in this study. Beside the cultivation of sea ice bacteria, Brinkmeyer and his colleagues (2003) constructed also 16S rDNA libraries from Arctic and Antarctic pack ice samples. They found that γ-proteobacteria and CFB group were the dominant taxonomic groups at both poles. A similar predominance of phylotypes was also found in sea ice microbial communities analyzed by Brown and Bowman (2001).

Many sequences obtained in this study were the closest relatives of species originally isolated from Arctic and Antarctic sea ice and some other marine habitats. According to phylogenetic inferences, most of the phylotypes were known to be heterotrophic bacterial genera. From the results of our and previous studies (Bowman *et al.* 1997; Gosink *et al.* 1998; Brown and Bowman 2001; Junge *et al.* 2002; Brinkmeyer *et al.* 2003), it could be suggested that heterotrophic bacteria were the major prokaryotes in sea ice and most sea ice bacterial strains could be cold adaptive and halotolerant. The studies of Brown and Bowman (2001) and Brinkmeyer *et al.* (2003) showed that 16S rDNA sequences of the cultivated bacteria and uncultured clones from sea ice were overlapped strongly. These results indicated that only metabolically active and cultivatable species were able to significantly compete and successfully colonize sea ice during sea ice formation.

However, the diversity in sea ice based on cultivated phylotypes and 16S rDNA clone-libraries seemed to be reduced in Arctic microbial communities (Mock and Thomas 2005). In our study, the diversity in sea ice brine samples detected is less than that in Antarctic sea ice. Brown and Bowman (2001) analysed 16S rDNA sequences data from sea ice, mainly in the Antarctic, and found that sea ice bacterial phylotypes fell into α- and γ-proteobacteria, CFB group, Gram-positive bacteria and the orders of Chlamydiales and Verrucomicrobiales. In contrast, only sequences of γ-proteobacteria, CFB group and Gram-positive bacteria were presented in our study. Junge *et al.* (2002) had proposed that considering the years of sea ice habitat formed on Earth was geologically young, relatively little time had been available for the evolution of highly diverse sea ice bacteria.

Beside sequences closely related to species previously isolated from Arctic sea ice and other marine habitats, some were the closest relatives of bacterial strains originating from soil, urban aerosol and patient's sputum. In contrast to the Southern Ocean, the Arctic Ocean was strongly influenced by high terrestrial input. The sea ice clone sequences related to β-proteobacteria, which was characteristic of freshwater habitats, have been reported in the study of Brinkmeyer *et al.* (2004). Moreover, our results may indicated that aerosol could be considered to be one source of sea ice bacterial communities.

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