Thesis

Studies on Microbial Diversity and Antimicrobial Resistance Genes in Sea Ice and Antarctic Ice Cores

（海氷および南極アイスコアにおける微生物叢の解析と
抗菌薬耐性遺伝子の検出に関する研究）

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ABBREVIATIONS

Antimicrobials

- ABPC: ampicillin
- DSM: dihydrostreptomycin
- GM: gentamicin
- KM: kanamycin
- SMX: sulfamethoxazole

ARB: antimicrobial resistant bacteria

ARG: antimicrobial resistance gene

BLAST: Basic Local Alignment Search Tool

CLSI: Clinical and Laboratory Standards Institute

bp: base pairs

DDBJ: DNA Data Bank of Japan

ICE: integrative conjugation element

MIC: minimum inhibitory concentration

NCBI: National Center for Biotechnology Information

ybp: years before present

PREFACE

The discovery of antimicrobial agents is one of the greatest medical advances of the 20th century. While the therapeutic usage of antimicrobials has saved many people and animals from bacterial infection, the emergence of antimicrobial resistant bacteria (ARB) has became a global healthcare concern [61]. The World Health Organization mentioned that the global spread of ARB is currently one of the biggest public health threats [114]. ARB poses significant health and socio-economic burdens as it leads to an increased mortality rate or prolonged illness, and costs economies several billion dollars [69, 114]. In addition, ARB has raised healthcare and economic problems in livestock and companion animals [41, 67]. Nowadays, multidrug resistant bacteria such as extended-spectrum β-lactamase-producing *Enterobacteriaceae*, carbapenem-resistant *Enterobacteriaceae*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant Enterococci have been isolated from both human and animal sources in many countries [3, 18, 45, 84, 100].

Several mechanisms of antimicrobial resistance have been identified in bacteria: 1) the inactivation of the antibiotics by drug-degrading and/or drug-modifying enzymes such as β-lactamase and aminoglycoside phosphotransferase, 2) the alteration of the drug target by methylases and/or the acquisition of genetic mutations, 3) the protection of the drug target site by enzymes such as ribosomal protection proteins, 4) the acquisition and/or overexpression of efflux pumps, 5) the reduction of cell permeability by loss of porin channels on the outer membrane, and 6) the production of alternative, drug resistant enzymes such as dihydropteroate synthase and/or dihydrofolate reductase [61]. Because most of the antimicrobial agents are produced by

antibiotic-producing environmental microorganisms such as fungi and *Actinomycetes*, various bacteria in environment ecosystems harbor antimicrobial resistance genes (ARGs) as part of their survival strategy against antibiotics produced by competing microorganisms [4]. The antibiotic-producing microorganisms also possess ARGs to protect themselves from self-produced antibiotics [32]. Therefore, it has been suggested that microbiota in the natural environment is a source and/or reservoir of ARGs, called the "resistome" [4, 25]. Because ARGs found in non-pathogenic, environmental bacteria are homologous to those identified in pathogenic bacteria in clinical conditions, it is believed that ARGs have been passed from environmental bacteria to pathogenic bacteria via horizontal gene transfer [6, 29]. To date, the wild species origin of several ARGs has been tracked; e.g., CTX-M-type β-lactamase genes ($bla_{\text{CTX-M}}$) originated from *Kluyvera* spp., plasmid-mediated AmpC-type β-lactamase genes (bla_{AmpC}) from *Citrobacter* spp. and *Aeromonas* spp., and aminoglycoside N-acetyl-transferase genes from *Streptomyces* spp. [11, 88, 91]. Surprisingly, some environmental bacteria harbor chromosomal ARGs against systemic antibiotics such as fluoroquinolones without any antibiotic selective pressure [102]. The presence of ARGs in environmental bacteria indicates that probably, no antimicrobial agents are free from the emergence of ARB, regardless of whether they are natural or synthetic products.

History shows that ARB emerged soon after the adoption of antimicrobials [2, 13, 106]. Indeed, ARB and ARGs have been isolated from permafrost samples containing ancient microbiota, called the samples of "pre-antibiotic era" [26, 71]. These studies showed that ARB and ARGs predate the use of antimicrobial agents. The study of ARB and ARGs in pristine conditions that were isolated from anthropogenic

influences is important to increase our understanding of the dissemination of ARB in the modern world.

In this thesis, we attempted to detect ARGs in samples derived from one of the most pristine habitats in the world: the ice sheets of Antarctica. Since ice sheets were formed by accumulated snow, they have contained and preserved dust and particles deposited over time [5, 111]. The low temperature and isolated location of the ice sheets are favorable preservation method of the ancient microbial environment [54, 72]. First, we established a safe decontamination method to exclude contaminations from shipping and handling for ice samples derived from the Southern Ocean and the Sea of Okhotsk (Chapter 1). The cleaning step was important because ice samples used in this study were transported from the sampling site and stored in freezers for a long time. In parallel with this, we examined bacterial diversity in these samples by 16S rDNA profiling (Chapter 1). Next, we performed a comprehensive analysis of the microbial community in Antarctic ice core samples from Dome Fuji Station (Chapter 2). Finally, we investigated the presence of ARG in sea ice and Antarctic ice core samples (Chapter 3). The goal of this study was to assess the existence and prevalence of ARGs in pristine environments.

CHAPTER 1

Microbial diversity in sea ice of the Southern Ocean and the Sea of Okhotsk

1.1. Introduction

The occurrence of ice is one of the distinctive features of the oceans in polar and sub-polar regions. Seawater in these regions becomes cold enough to freeze (below -1.9°C) during the winter season. Despite low temperatures, reduced water activity for organisms, and transience, it has been reported that sea ice contains many types of microorganisms such as diatoms, protozoa, bacteria, archaea, and viruses [24, 65, 109]. Among them, bacteria have been reported to reside in concentrated brine pockets and remain active in sub-zero temperature [49]. Therefore, they are considered a good model for studying the adaptation mechanisms of microorganisms to extreme conditions for survival [75].

Several studies have reported a rich bacterial diversity in sea ice and noted a predominance of gram-negative and psychrophilic or psychrotolerant bacteria in these habitats [12, 14, 15]. Although some of the bacteria are cultivatable under artificial conditions, culture-independent methods such as 16S rDNA-based analyses have been mainly utilized for the comprehensive identification of the bacterial diversity in sea ice [7, 12, 14, 15, 24]. These studies reported an abundance of phylum *Proteobacteria* (particularly class *Alphaproteobacteria* and *Gammaproteobacteria*) and *Bacteroidetes* (reported as *Cytophaga-Flavobacterium-Bacteroidetes* group) in sea ice bacterial communities in both the Arctic and Antarctic regions. At the genus level, *Roseobacter* (*Alphaproteobacteria*), *Colwellia*, *Glaciecola*, *Marinobacter* (*Gammaproteobacteria*), and *Polaribacter* (*Bacteroidetes*) were reported as predominant [14, 99]. However, the majority of those studies used sea ice samples that were collected from the Indian side of the Southern Ocean [12, 14], the Arctic Ocean [14, 109], and the Baltic Sea [74].

Therefore, the information about sea ice microbiota in other frozen seas such as the Australian side of the Southern Ocean and the Sea of Okhotsk, is limited.

In this study, we established a reliable method for cleaning the surface of ice core samples and for nucleotide extraction from these samples, which were subsequently used for further experiments. In addition, we assessed the bacterial diversity in the sea ice of the Southern Ocean and the Sea of Okhotsk, and compared it to previous reports to gain more insight in the composition of bacterial communities in sea ice.

1.2. Materials and Methods

1.2.1. Sampling and sample preparation

Drifting sea ice of the Southern Ocean was collected in Dumont d'Urville Sea during the 24th cruise of the training ship "Umitaka-maru" (Tokyo University of Marine Science and Technology, Tokyo, Japan) in February 2008. Three blocks of clear ice (each approximately 10.0 × 5.0 × 4.0 cm large) were used in this study as "Antarctic sea ice". An ice floe of the Sea of Okhotsk was collected on the shore of Monbetsu City, Hokkaido, Japan (44º 31′ N, 143º 39′ E) in March 2008. An apparently clean ice block (approximately 10.0 × 10.0 × 15.0 cm large) that was cut off from the entire ice floe was used in this study as "Okhotsk sea ice". Both ice samples were maintained at below -20ºC for approximately 2 years until analysis. We could not determine the date of the ice floes, but taking the transience of ice floes of the sampling areas into consideration, both appeared to be first-year ice [14, 75, 76, 98]. Prior to melting, the ice samples were soaked for 1 min in ice-cold 99.5% ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 3 times and carefully rinsed 3 times with deionized, distilled water to remove contaminants from the surface layer [21]. Approximately 1 cm of surface layer was removed by this step. At different locations within sea ice samples, typical microbiota communities such as bottom, surface, and internal communities have been observed [98]. However, we studied the internal community in further examination using only clear core of the ice samples. After cleaning of the surface, the ice cores were placed in sterilized metal trays and allowed to thaw at room temperature. As a control, distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was frozen and

decontaminated using the same procedure described above. All operations were performed on clean bench that had been sterilized by ultraviolet ray at least 12 hours.

1.2.2. DNA extraction from sea ice samples

The thawed ice were filtered through 3.0-μm pore-size Isopore Track-Etched Membrane Filter made of polycarbonate (Merck Millipore, Billerica, MA, USA) to remove coarse particles, and subsequently through 0.22-μm pore-size Millipore Express PLUS Membrane Filter made of polyethersulfone (Merck Millipore) to collect the bacterial cells. The 0.22-μm filters were washed in 2 mL of phosphate-buffered saline of 0.05% Tween-80 (Wako) and shook for 1 min. The suspension of liquids were centrifuged at 22,000 × *g* for 5 min and the supernatants were discarded, then the pellets were used for DNA extraction.

Bacterial DNA was extracted using an InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions for extraction of DNA from general bacteria. To allow the extraction of DNA from bacteria with thick, rigid cell walls such as *Mycobacteria*, the pellets were resuspended in 25 μL of bacteriolysis buffer from EXTRAGEN MB mycobacterial DNA extraction kit (Tosoh Corporation, Tokyo, Japan). The DNA from each ice sample was combined and the total DNA concentration was determined by measuring the absorbance at 260 nm using a Gene Quant pro RNA/DNA Calculator spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK).

1.2.3. Cloning and sequencing of the 16S rDNA

The bacterial 16S rRNA gene was amplified by PCR with universal primers for the V3-V4 region, producing 527 bp amplicon: forward primer 5'-GAGAGTTTGATCMTGGCTCAG-3' and reverse primer 5'-ATTACCGCGGCTGCTGGCAC-3' [80, 121]. After electrophoresis, we extracted the amplified 16S rDNA product from the agarose gel using a Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). Cloning was performed using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transformants were selected on LB agar (Invitrogen) containing 50 mg/L kanamycin (Sigma-Aldrich, St. Louis, MO, USA). One hundred twenty colonies from each of the Antarctic and Okhotsk sea ice samples and 60 colonies from the control ice sample were selected and preserved in glycerol stocks at -80°C. We conducted colony-direct PCR for 16S rRNA genes with these 300 transformants using the M13 primers provided in the TOPO TA cloning kit. The amplicons were purified using a High Pure PCR Cleanup Micro Kit (Roche Diagnostics, Basel, Switzerland) and analyzed by direct sequencing at Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan).

The obtained nucleotide sequences were compared to sequences deposited in GenBank using the Basic Local Alignment Search Tool (BLAST) search application version 2.2.18 (http://blast.ddbj.nig.ac.jp/). Based on > 98% nucleotide identity with sequences in the database, clones were identified to the genus level. Evolutionary distances were estimated using the Jukes-Cantor method and phylogenetic trees were constructed by the neighbor-joining method with 1,000 bootstrap replicates using the MEGA software version 5.2. The 16S rDNA sequences were deposited at the DNA

Data Bank of Japan (http://www.ddbj.nig.ac.jp/index-e.html) under the GenBank accession numbers LC003653-LC003873. Rarefaction curves were drawn by statitstic analysis software R-3.1.2 to compare bacterial diversity between 2 samples [40].

1.3. Results

1.3.1. Melting and DNA extraction

We obtained 257 mL of melted ice sample from the Antarctic sea ice, 400 mL from the Okhotsk sea ice, and 160 mL from the control ice. The total DNA concentration of each sample was as follows: Antarctic sea ice, 380.86 pg/μL; Okhotsk sea ice, 247.01 pg/μL; control ice, 0.20 pg/μL.

1.3.2. Phylogenetic analysis

Bacterial 16S rRNA gene libraries were successfully constructed from each sample. We obtained 300 clones including 120 clones from the Antarctic sea ice, 120 from the Okhotsk sea ice, and 60 from the control ice for identification of the bacteria to the genus level. Among the 60 clones derived from the control ice, genus *Bradyrhizobium* (n = 11), *Caulobacter* (n = 13), *Escherichia* (n = 1), *Moraxella* (n = 1), and unclassified *Actinobacteria* (n = 3) were found exclusively in the control. Genus *Sphingomonas*, *Novosphingobium*, and unidentified *Betaproteobacteria* were found in both the Antarctic sea ice and the control ice (n = 3 and 10, n = 3 and 20, and n = 2 and 1, respectively). Because they were most likely contaminants that were introduced at some point during the handling of the samples, we excluded them from further analysis. In addition, the 3 clones from the Antarctic sea ice and the 8 from the Okhotsk sea ice samples that were insufficiently amplified by the M13 primers were excluded from the study. The remaining 221 clones, 109 from the Antarctic sea ice and 112 from the Okhotsk sea ice, were used for further analysis.

The taxonomic distribution patterns of 16S rRNA gene clones from the 2 sea ice samples are shown in Table 1 and Fig. 1. Class *Alphaproteobacteria* and *Gammaproteobacteria* of phylum *Proteobacteria* were more frequently detected in the Antarctic sea ice than in the Okhotsk sea ice (26.6% vs. 10.7% and 71.6% vs. 48.2%, respectively) (Fig. 1). Phylum *Bacteroidetes* was the second-largest group in the Okhotsk sea ice (n = 43, 38.4%), whereas no *Bacteroidetes* were detected in the Antarctic sea ice. Only few clones of class *Deltaproteobacteria*, *Epsilonproteobacteria* and phylum *Planctomycetes* were detected in both samples. The gram-positive bacteria such as phylum *Firmicutes* and *Actinobacteria* were not detected in both samples.

In the Antarctic sea ice sample, class *Gammaproteobacteria* was most frequently detected (n = 78, 71.6%), followed by *Alphaproteobacteria* (n = 29, 26.6%) (Fig. 1). Within class *Gammaproteobacteria*, genus *Psychrobacter* was predominant (n = 41), followed by *Halomonas* (n = 10) and *Pseudoalteromonas* (n = 5) (Table 1). Among class *Alphaproteobacteria*, *Sulfitobacter* (n = 13) and *Roseobacter* (n = 8) of family *Rhodobacteraceae* were commonly observed (Table 1).

In the Okhotsk sea ice sample, class *Gammaproteobacteria* was similarly predominant (n = 54, 48.2%), while the second most frequent group was phylum *Bacteroidetes* (n = 43, 38.4%) (Fig. 1). Classification at the genus level revealed that *Polaribacter* (phylum *Bacteroidetes*) was the most frequent genus in the Okhotsk sea ice (n = 43) (Table 1). Among *Gammaproteobacteria*, genus *Colwellia* was the most common (n = 27), followed by *Psychromonas* (n = 12) and *Glaciecola* (n = 10).

Rarefaction curve showed that bacterial community in Antarctic sea ice was more divergent than the Okhotsk sea ice sample at genus level (Fig. 2). It also showed that the bacterial populations we detected were seemed to be represent the whole

bacterial community in the samples because the rarefaction exhibitied decelerating slopes at the right end of curves. Phylogenetic trees of the evolutionary relationship of 16S rDNA are shown in Fig. 3A and 3B. Although class *Alphaproteobacteri*a and *Gammaproteobacteria* were abundant in both sea ice samples, there were no bacteria in common at the genus level.

Table 1. Bacteria identified in sea ice samples by 16S rRNA gene sequencing. The numbers indicate acquired 16S rRNA gene clones.

Phylum	Class	Order	Family	Genus	Antarctic sea ice	Okhotsk sea ice	Total
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Roseobacter*	8	0	8
				Sulfitobacter	13	1	14
				Thioclava	1	0	1
				Octadecabacter	0	5	5
		Rhodospirillales	*Rhodospirillaceae*	*Thalassospira*	4	0	4
			Acetobacteraceae	*Roseomonas*	0	1	1
			Erythrobacteraceae	*Alterierythrobacter*	2	0	2
				Erythrobacter	1	0	1
		Unclassified *Alphaproteobacteria*		*Pelagibacter ubique*	0	1	1
				Alphaproteobacterium SCGC AAA298-C11	0	1	1
		Unidentified			0	3	3
	Gammaproteobacteria	*Alteromonadales*	*Alteromonadaceae*	*Marinobacter*	1	0	1
				Glaciecola	0	10	10
			Pseudoalteromonadaceae	*Pseudoalteromonas*	5	0	5
			Colwelliaceae	*Colwellia*	0	27	27
			Psychromonadaceae	*Psychromonas*	0	12	12
		Enterobacteriales	*Enterobacteriaceae*	*Klebsiella*	1	0	1
				Pantoea	1	0	1
		Pseudomonadales	*Moraxellaceae*	*Acinetobacter*	2	0	2
				Psychrobacter	41	0	41
			Pseudomonadaceae	*Pseudomonas*	2	0	2
		Oceanospirillales	*Halomonadaceae*	*Halomonas*	10	0	10
				Chromohalobacter	2	0	2
		Thiotrichales	*Piscirickettsiaceae*	*Methylophaga*	4	0	4
		Unclassified *Gammaproteobacteria*			0	1	1
		Unidentified			9	4	13
	Deltaproteobacteria	Unidentified			2	0	2
	Epsilonproteobacteria	Unidentified			0	1	1
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Polaribacter*	0	43	43
Planctomycetes	*Planctomycetacia*	*Planctomycetaceae*	*Planctomycetaceae*	Unidentified	0	2	2
Total					109	112	221

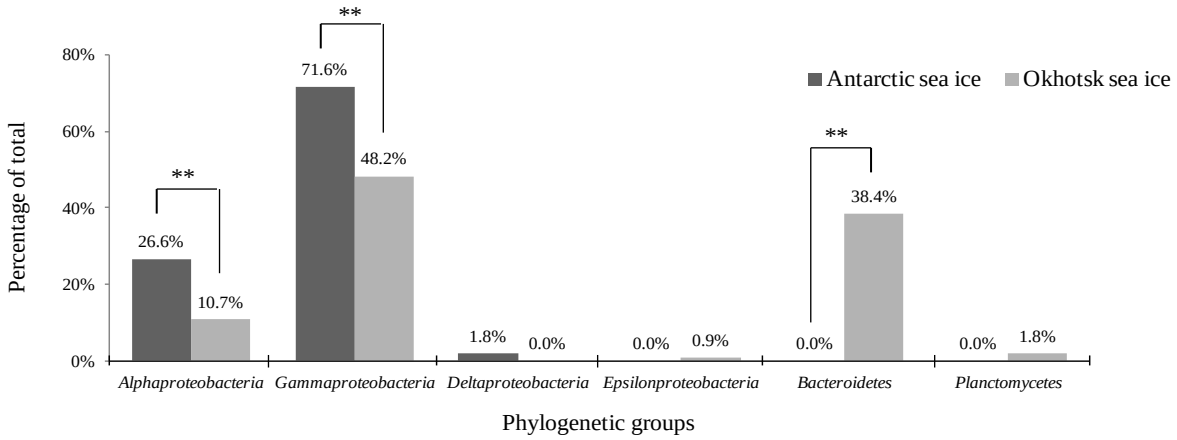


Fig. 1. Phylogenetic distribution of 16S rRNA gene clones from Antarctic sea and Okhotsk sea ice samples. **, $p < 0.01$ by chi-squared test.

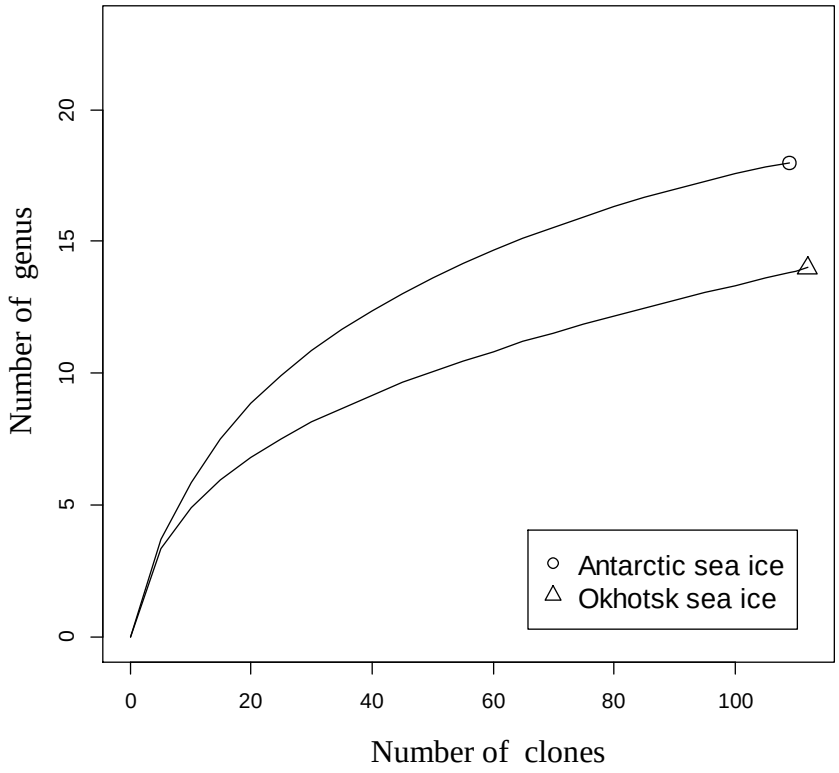
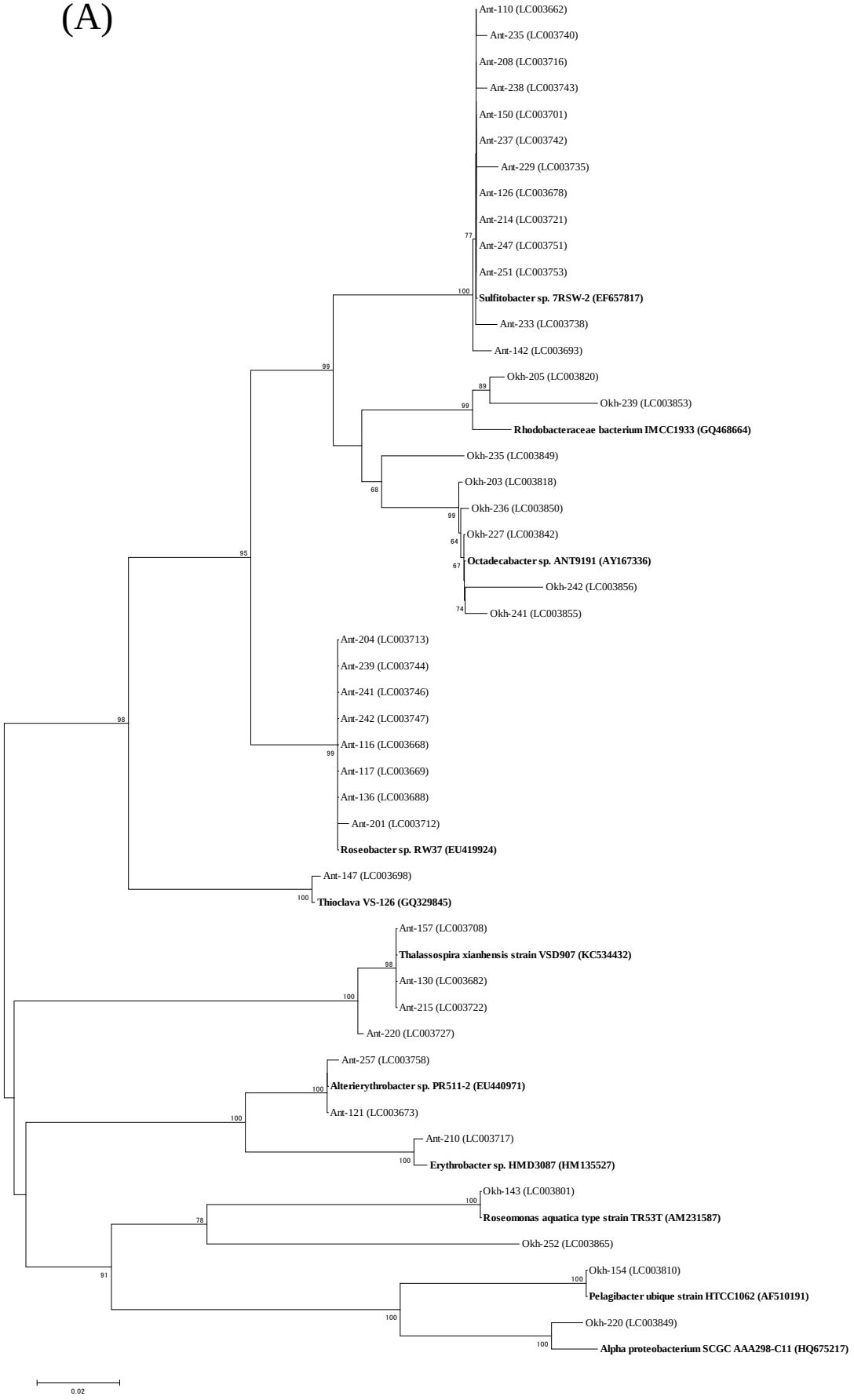


Fig. 2. Sample-based rarefaction curves based on the number of clones and the number of genus that were identified by BLAST analysis of cloned 16S rDNA. Blank circle represents the genus richness of Antarctic sea ice and blank triangle represents that of Okhotsk sea ice. Calculation was conducted by statistic analysis software R-3.1.2.

Fig. 3. Phylogenetic trees showing the evolutionary relationship of 16S rDNA sequences from sea ice samples. Clones derived from the Antarctic sea ice are indicated as “Ant” and those from the Okhotsk sea ice as “Okh”. Codes in brackets following the entry name indicate the GenBank accession number. Numbers at branch nodes are bootstrap values (only values of more than 60% are shown). Scale bars indicate Jukes-Cantor distances. (A) Class *Alphaproteobacteria*; (B) Class *Gammaproteobacteria*.

(A)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Thioclava VS-126 (GQ329845)
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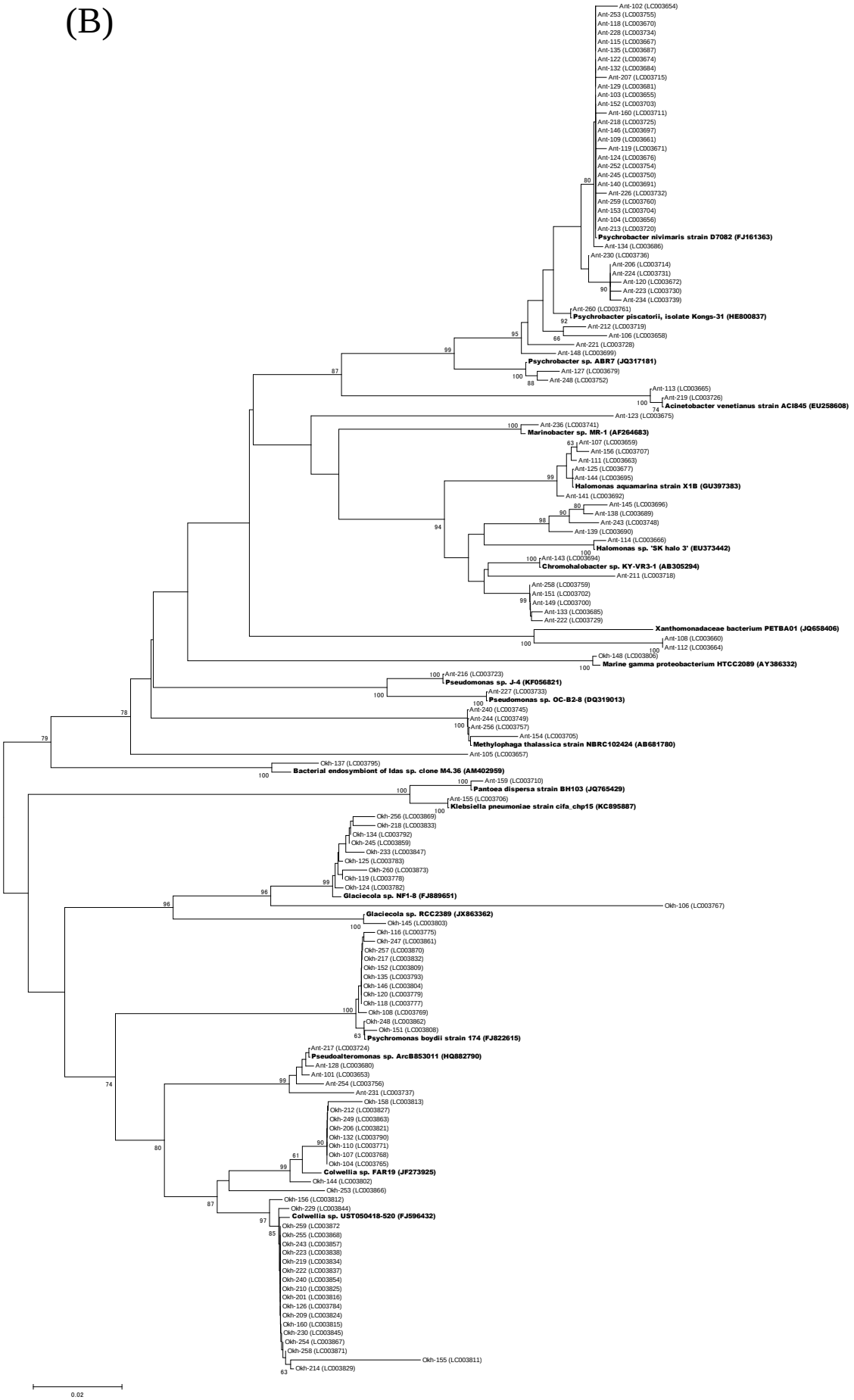
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0.02

1.4. Discussion

We cleaned the surface of the ice samples and obtained enough thawed ice fluid for DNA extraction. Compared to the control ice, large amounts of DNA were obtained from the 2 sea ice samples, indicating that: 1) DNA was extracted efficiently from the sea ice samples, and 2) contamination during the melting and DNA extraction steps was minimized although a few possible contaminants were detected by 16S rDNA analysis. Therefore, we considered our cleaning and DNA extraction procedures appropriate for treating ice samples.

The observed abundance of phylum *Proteobacteria* (particularly class *Alphaproteobacteria* and *Gammaproteobacteria*) in the Antarctic sea ice was consistent with previous studies on sea ice derived from other frozen oceans [14, 15, 78]. However, the finding that, within class *Gammaproteobacteria*, the predominance of genus *Psychrobacter* was differed from previous reports that genus *Colwellia*, *Glaciecola*, and *Marinobacter* (class *Gammaproteobacteria*) were the major groups among bacterial communities in sea ice of the Southern Ocean [14, 78]. In addition, phylum *Bacteroidetes*, which was previously reported to be one of the major bacterial groups in sea ice [14, 99], was not detected in the Antarctic sea ice sample. The reason for this difference in composition of the bacterial population is unclear, but the difference of sampling site may provide an explanation. We utilized the ice floes collected in Dumont d'Urville Sea, the Australian side of the Southern Ocean, whereas most previously reported bacterial communities associated with Antarctic seawater or ice were sampled from the Indian side or South American side of the Southern Ocean [14, 12, 70, 78]. Delille *et al.* [31] reported a possible anthropogenic influence on the marine bacterial

community of the Dumont d'Urville Sea, near the French Base station of the c Land area, as revealed by the presence of enteric bacteria in the seawater, but no information on the biodiversity of the microorganisms in this area was reported. We considered that the abundance of class *Gammaproteobacteria* and the absence of phylum *Bacteroidetes* were distinctive features of bacterial population in the Antarctic sample of this region. These data provide new information about the bacterial community in this area and can help elucidating the characteristics of bacterial ecosystems in the Antarctic region.

The results regarding the bacterial diversity in the Okhotsk sea ice were consistent with previous reports on the predominance of phylum *Proteobacteria* and *Bacteroidetes* in bacterial communities in Arctic sea ice [7, 14, 24]. Given the fact that the Sea of Okhotsk is located in the sub-Arctic region rather than the Arctic region, these results suggest that sea ice bacterial communities of the Northern Hemisphere may share similar characteristics. To the best of our knowledge, this was the first study on the phylogenetic analysis of bacteria in ice floes from the Sea of Okhotsk area.

Except for the fact that we did not observe phylum *Bacteroidetes* in the Antarctic sea ice, our results were consistent with previously reported results obtained by culture-dependent or culture-independent assays at the phylum and class level [14, 15, 78]. This suggests that the bacterial communities in sea ice are similar to each other. However, at the genus level, *Sulfitobacter* (n = 13 in the Antarctic sea ice and n = 1 in the Okhotsk sea ice) was the only genus detected in both of our ice samples (Table 1). The other genera were distinctly different between the 2 sea ice samples as demonstrated by the phylogenetic trees (Fig. 2). A review article on the bacterial diversity in sea ice described that no cosmopolitan bacterial species could be isolated from both the Arctic and the Antarctic sea ice despite of the environmental similarity in

coldness of these regions [99]. Our results were in agreement with this finding and confirmed that the microbial populations in sea ice are endemic.

In this chapter, we outlined effective decontamination and DNA extraction methods for ice samples by comparing the amount of DNA contained in sea ice samples and the control. This allowed us to examine Antarctic ice cores as described in the next chapters. In addition, we also identified and compared the bacterial population in sea ice samples of the Southern Ocean and the Sea of Okhotsk. Bacterial communities in both sea ice samples resembled each other at the phylum and class levels but differed at the genus level. Although our sample size was not sufficiently large to draw definite and comprehensive conclusions, our findings indicated that the characteristics of bacterial communities in sea ice are intrinsic.

1.5. Summary of Chapter 1

In order to reveal the diversity of sea ice bacterial communities in polar and sub-polar regions, we investigated 2 drifting ice floes, one from the Australian side of the Southern Ocean named and the other from the Sea of Okhotsk. We established a decontamination method for ice samples by cleaning the surface by ethanol. We also extracted bacterial DNA from the sea ice samples and constructed total 221 16S rDNA clone libraries including 109 clones from the Antarctic sea ice and 112 from the Okhotsk sea ice. The phylogenetic analysis of 16S rDNA sequences showed that genus *Roseobacter* and *Sulfitobacter* (phylum *Alphaproteobacteria*), *Psychrobacter*, *Halomonas*, and *Pseudoalteromonas* (*Gammaproteobacteria*) were frequent in the Antarctic sea ice, whereas *Colwellia*, *Psychromonas*, and *Glaciecola* (*Gammaproteobacteria*) and *Polaribacter* (*Bacteroidetes*) were major genera in the Okhotsk sea ice. While class *Alphaproteobacteria* and *Gammaproteobacteria* were abundant in both samples, phylum *Bacteroidetes* was detected only in the Okhotsk sea ice. Comparing the bacterial diversity of our samples with that of other studies, bacterial communities in sea ice were similar to each other at the phylum and class level but quite different at the genus level. Our results about bacterial populations provide additional information about the bacterial diversity in sea ice.

CHAPTER 2

Microbial diversity in ice cores from the Dome Fuji Station in East Antarctica

2.1. Introduction

Ice cores, which are obtained by vertical drilling into ice sheets or glaciers, are valuable sources of information on embedded dust particles, atmospheric gases, non-sea-salt sulfate as a volcanic signal, and cosmogenic nuclides from the past [46, 101, 113]. Therefore, the materials in ice cores have been used to reveal past environmental conditions in geophysical and environmental studies [5, 111]. Some ice cores provided records of the ancient environmental conditions of hundred thousand years before present (ybp) as reported by the Greenland Ice Sheet Project, the European Project for Ice Coring in Antarctica, and the examination of ice cores from Dome Fuji Station [28, 51, 59].

Previously, Christner *et al.* [22] reported that biogenic substances have played major role as ice nucleators in snowfall. Since ice cores have been formed by the deposition of snow, airborne biological materials such as bacterial cells, fungal spores, plant debris, and pollens, as well as inorganic materials that were trapped in ice sheets have been hypothesized to reflect climatic and environmental factors of the deposited period [72, 112, 115, 117]. These approaches with microbiological information from ice core samples, together with other physical and chemical parameters, are supposed to provide new insight for a better understanding of the past climatic and environmental changes.

Previous studies on the genetic analysis of bacterial populations in ice cores showed divergent results. In Greenland ice core samples, phylum *Firmicutes* was the most prevalent in a 57,000 ybp sample whereas phylum *Actinobacteria* dominated in an sample of 68,000 ybp [73]. Studies of bacterial phylogenetic patterns in mountain

glacial ice cores showed the abundance of phylum *Proteobacteria* and phylum *Bacteroidetes* in these samples [20, 115]. Genetic studies on fungal diversity have also reported varying results. A significant number of fungal sequences related to genus *Acremonium* were detected in a Greenland ice core of 63,200 ybp, whereas genus *Cladosporium*, *Phaeosphaeria*, *Torulaspora*, and *Tricholoma*, along with genus *Acremonium*, were detected in a Greenland ice core of 140,000 ybp [63, 73]. This diversity in microflora suggests that additional studies on the microbial variety in ice cores collected from different locations and periods are needed to reveal a full picture of the past environmental conditions.

In this study, we extracted microbial RNA from 2 ice cores, approximately 1,670 ybp and 2,860 ybp, sampled at Dome Fuji Station in East Antarctica. We analyzed the microbial populations in our samples using metagenomic analysis to obtain comprehensive genomic information, and compared the results to those reported for other ice core studies. Our aim was to increase the current knowledge on the ancient microbiomes preserved in the ice sheets of Antarctica.

2.2. Materials and Methods

2.2.1. Sample preparation

We examined 2 ice core samples that were drilled at 72-73 m and 107-108 m depth at Dome Fuji Station in East Antarctica (39˚ 42′ E, 77˚ 19′ S) within The Second Deep Ice Coring Project during 2003 to 2007 [77]. The ages of the ice cores were determined as 1,667 ± 17 and 2,863 ± 16 years by rough estimation from volcanic chronology analysis data [111]. We named them the "1,670 ybp" and the "2,860 ybp" ice cores in this study. Both samples were maintained at below -30°C until the melting. Prior to thawing, the samples were decontaminated as described in Chapter 1. The core of the samples was placed in glass beakers to melt completely at room temperature. The obtained water samples were transferred into sterile plastic bottles for cell culture (Corning Incorporated, Corning, NY, USA) and stored at 4°C until further use. As a control, Otsuka distilled water (Otsuka) was frozen at -20°C to make a blank ice core and treated as described in Chapter 1. All the procedures were conducted on a clean bench and all instruments such as glass beakers and forceps were sterilized by ultraviolet ray for at least 12 hours beforehand.

2.2.2. RNA extraction from the ice cores and cDNA synthesis

To enhance the chances of detecting organisms in the ice core samples, we used RNA for phylogenetic analysis because multiple copies of rRNA are present in a cell, whereas there is only one copy of genomic DNA [39]. Because RNA molecules remain preserved in ice up to several hundred thousand years, ice core samples contain enough RNA molecules for metagenomic analysis [112]. In addition, the use of RNA

allowed us to detect RNA viruses in the ice samples because it was suggested that ice can become a reservoir of pathogenic viruses [97]. Fifty-four milliliters of melted water from each ice sample were centrifuged at 25,000 rpm (approximately 100,000 *g*) for 2 hours under cooling and total RNA was extracted from the pellets using a QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, without adding carrier RNA to the reagent mixture. Extracted RNA was reverse-transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche) as previously described [79].

2.2.3. Metagenomic analysis of RNA from the ice cores

The constructed cDNA libraries were analyzed by 454 pyrosequencing on the GS Junior platform (Roche) at Research Institute for Microbial Diseases, Osaka University (Suita, Osaka, Japan). The obtained sequences were compared to those of a database of The Research Foundation for Microbial Diseases of Osaka University. For identification of the organisms, the sequences were also compared to those deposited in GenBank by using BLAST and to The Taxonomy Database (National Center for Biotechnology Information, MD, USA). We used the top BLAST hit to identify the organisms at the kingdom level (*Animalia*, *Plantae*, *Fungi*, *Protista* and *Bacteria*) and viruses. The sequences of kingdom *Bacteria* and *Fungi* were further identified to the family level. The negative control (distilled water) genome data were subtracted from the data to eliminate putative contamination.

2.3. Results

2.3.1. Organisms detected in the Antarctic ice core samples

In total, 25,857 reads were obtained; 10,152 reads from the 1,670 ybp ice core, 6,904 from the 2,860 ybp ice core, and 8,801 from the control sample. The latter were excluded from further analysis, and 10,041 reads from the 1,670 ybp core and 5,972 from 2,873 ybp core that were detected in both ice cores and in the control ice were excluded because we considered them as contaminants that may have been introduced by the handling procedure and/or were present in distilled water itself [54]. The remaining 1,043 reads, 111 from the 1,670 ybp and 932 from the 2,860 ybp sample, were classified to the kingdom level or as virus (Fig. 4). Thirty-six reads of kingdom *Animalia*, 2 of *Plantae*, 22 of *Fungi*, 35 of *Bacteria*, and 16 of viral origin were detected in the 1,670 ybp ice core while 43 of *Animalia*, 16 of *Plantae*, 304 of *Fungi*, 7 of *Protista*, and 562 of *Bacteria* were detected in the 2,860 ybp ice core.

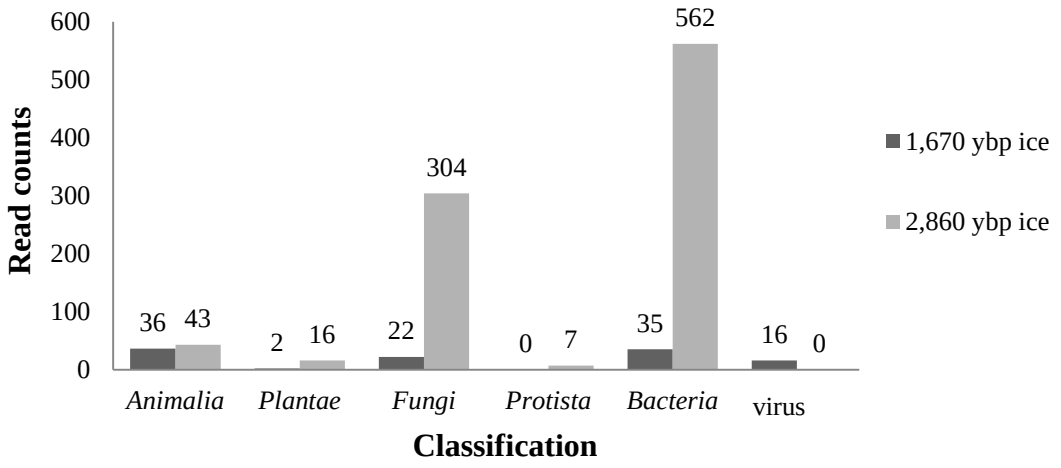


Fig. 4. Classification and read counts of the organisms detected by the metagenomic analysis. Dark and light gray bars represent the read counts of RNA sequences from the 1,670 ybp ice core and the 2,860 ybp ice core, respectively.

2.3.2. Bacterial diversity in the ice cores

We classified the 597 reads of kingdom *Bacteria* (n = 35 from the 1,670 ybp ice and n = 562 from the 2,860 ybp ice) into 6 phyla, 10 classes, and 22 families (Table 2, Fig. 5). Family *Pasteurellaceae* (class *Gammaproteobacteria*) and *Microchaetaceae* (phylum *Cyanobacteria*) were the major families in the 1,670 ybp ice core (n = 13 [37.1%] and n = 12 [34.3%], respectively), followed by *Pelobacteraceae* (class *Deltaproteobacteria*) (n = 6 [17.1%]). In the 2,860 ybp ice core, family *Deinococcaceae* and *Trueperaceae* (phylum *Deinococcus-Thermus*) were predominantly represented (n = 353 [62.8%] and n = 184 [32.7%], respectively, and a total of 537 reads as phylum *Deinococcus-Thermus*). The 2 samples had no bacterial reads in common.

Table 2. Bacterial populations in the Antarctic ice cores. The numbers represent read counts of the 16S rRNA sequences detected by metagenomic analysis.

Phylum	Class	Family	Age of ice core		Total
			1,670 ybp	2,860 ybp	
Deinococcus-Thermus	*Deinococci*	*Deinococcaceae*	0	353	353
		Trueperaceae	0	184	184
Actinobacteria	*Actinobacteria*	*Cellulomonadaceae*	2	0	2
		Kineosporiaceae	0	4	4
		Micrococcaceae	0	4	4
		Nakamurellaceae	0	3	3
		Promicromonosporaceae	0	1	1
		Thermomonosporaceae	0	1	1
Proteobacteria	*Alphaproteobacteria*	*Beijerinckiaceae*	0	1	1
		Phyllobacteriaceae	1	0	1
	Gammaproteobacteria	*Pasteurellaceae*	13	0	13
		Ectothiorhodospiriaceae	0	1	1
		Halomonadaceae	0	1	1
		Syntrophaceae	0	1	1
	Deltaproteobacteria	*Pelobacteraceae*	6	0	6
Firmicutes	*Bacilli*	*Enterococcaceae*	1	0	1
		Leuconostocaceae	0	2	2
		Listeriaceae	0	1	1
		Planococcaceae	0	1	1
	Clostridia	*Clostridiaceae*	0	1	1
Bacteroidetes	*Bacteroidetes*	*Bacteroidaceae*	0	2	2
	Sphingobacteria	*Sphingobacteriaceae*	0	1	1
Cyanobacteria	*Cyanophyceae*	*Microchaetaceae*	12	0	12
Total			35	562	597

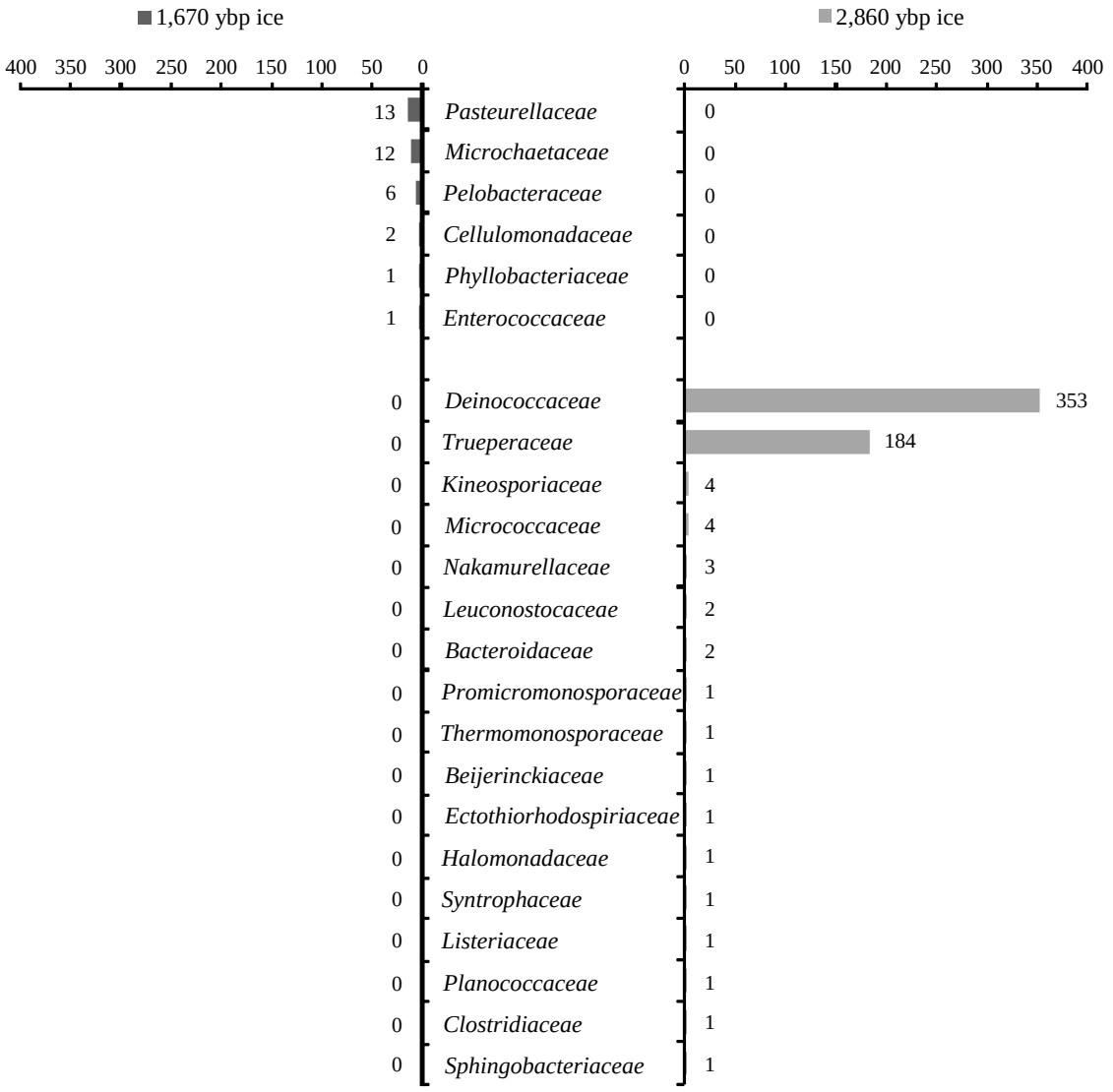


Fig. 5. Bacterial populations in the Antarctic ice cores arranged in descending order of read counts. Dark and light gray bars represent the read counts of the RNA sequence in the 1,670 ybp and in the 2,860 ybp ice core, respectively.

2.3.3. Fungal diversity in the ice cores

The reads of kingdom *Fungi* (n = 22 from the 1,670 ybp and n = 304 from the 2,860 ybp ice core) were classified at the family level (Table 3, Fig. 6). Overall, 3 phyla, 13 classes, and 40 families were identified. The most common family in the 1,670 ybp ice core was *Pucciniaceae* although only 13 reads represented in the group (59.1%). In the 2,860 ybp ice core, family *Sphinctrinaceae*, *Trichocomaceae*, and *Dactylosporaceae*

were frequently detected (n = 77 [25.3%], 46 [15.1%], and 39 [12.8%], respectively). Similar to the bacterial reads, the 2 samples had no fungal reads in common.

Table 3. Fungal populations in the Antarctic ice cores. The numbers represent read counts of the 18S rRNA sequences detected by metagenomic analysis.

Phylum	Class	Family	Age of ice core		Total
			1,670 ybp	2,860 ybp	
Ascomycota	*Dothideomycetes*	*Capnodiaceae*	0	3	3
		Davidiellaceae	0	1	1
		Mycosphaerellaceae	0	15	15
		Dothioraceae	0	2	2
		Elsinoaceae	0	5	5
		Leptosphaeriaceae	0	1	1
		Pleosporaceae	4	0	4
		Tubeufiaceae	0	5	5
		Botryosphaeriaceae	0	2	2
	Eurotiomycetes	*Herpotrichiellaceae*	0	16	16
		Verrucariaceae	0	8	8
		Trichocomaceae	0	46	46
		Ajellomycetaceae	0	8	8
		Sphinctrinaceae	0	77	77
	Geoglossomycetes	*Geoglossaceae*	0	7	7
	Lecanoromycetes	*Acarosporaceae*	0	1	1
		Gyalectaceae	0	1	1
		Icmadophilaceae	0	3	3
		Pertusariaceae	0	1	1
		Dactylosporaceae	0	39	39
		Lecanoraceae	0	4	4
		Rhizocarpaceae	0	3	3
		Collemataceae	0	1	1
		Coccodiniaceae	0	8	8
		Umbilicariaceae	0	6	6
	Leotiomycetes	*Helotiaceae*	0	4	4
		Sclerotiniaceae	0	1	1
	Orbiliomycetes	*Orbiliaceae*	0	7	7
	Pezizomycetes	*Sarcosomataceae*	0	1	1
	Sordariomycetes	*Ophiocordycipitaceae*	0	3	3
		Melanosporaceae	1	0	0
		Halosphaeriaceae	0	1	1
		Microascaceae	0	7	7
		Magnaporthaceae	0	2	2
		Thyridiaceae	2	0	0
	Pneumocystidomycetes	*Pneumocystidaceae*	0	10	10
Basidiomycota	*Agaricomycetes*	*Agaricaceae*	0	4	4
	Pucciniomycetes	*Pucciniaceae*	13	0	0
	Ustilaginomycetes	*Ustilaginaceae*	2	0	0
Glomeromycota	*Glomeromycetes*	*Glomeraceae*	0	1	1
Total			22	304	326

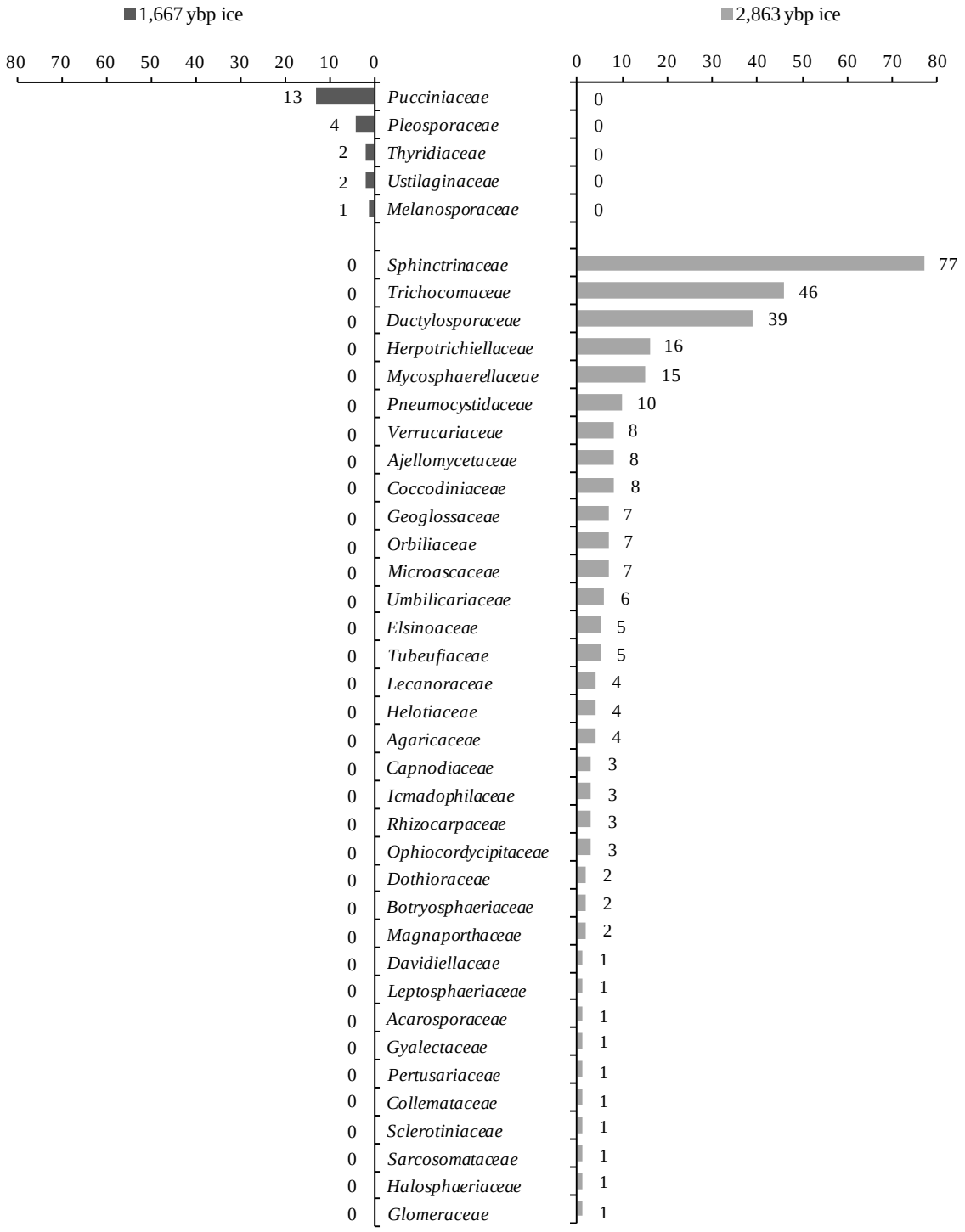


Fig. 6. Fungal populations in the Antarctic ice core arranged in descending order of read counts. Dark and light gray bars represent the read counts of the RNA sequence in the 1,670 ybp and in the 2,860 ybp ice core, respectively.

2.4. Discussion

We examined RNA sequences from Dome Fuji ice cores by metagenomic analysis. Although there were some differences in the methods used, the number of reads obtained in the present study was much lower than previously reported in metagenomic studies using ice core samples of Alpine glacier (1,076,539 reads), Alaskan glacier (136,579 reads), or Greenland ice sheet (107,700 reads) [20, 54, 95]. This may be explained by the remote location of the station and the harsh conditions at the location. Dome Fuji Station is located approximately 1,000 km inland from the nearest seashore thus only a small number of airborne biogenic particles can reach there [77]. In addition, it has previously been reported that bacterial activity in the Antarctic snow is very limited because of the lack of liquid water [110]. Therefore, we speculated that all of the RNA detected in the ice cores was derived from airborne microbes. As studies on ice core contents of the Antarctica revealed that the Australian continent has probably been the major dust source for the East Antarctic plateau over the last 170,000 years [30, 92], it is plausible that RNA we detected were transported by wind from the Australian continent and subsequently entrapped in the Antarctic ice sheet.

Among the bacteria detected in the 2,860 ybp ice core, of the largest part belonged to phylum *Deinococcus-Thermus*. Bacterial species in this group are known for its extraordinary ability to resist ionizing and ultraviolet radiation, desiccation, and oxidizing agents [8]. Because they have isolated from various habitats including lake sediments in South Australia, which were suspected to be one of the potential sources of airborne dust, we considered that *Deinococcus-Thermus* in the 2,860 ybp ice core may be transported from the Australian continent, the major source of particles in ice sheets

of East Antarctica as described above [1, 90, 120]. This hypothesis is supported by a previous report that 16S rRNA gene of *Deinococcus* spp. was detected in Antarctic surface snow [16]. However, a previous metagenomic analysis of Greenland ice cores revealed that phylum *Actinobacteria*, *Cyanobacteria*, and *Firmicutes* were frequently found while *Deinococcus-Thermus* were not detected [54]. In addition, a 16S rDNA-based study on Alpine glacier ice was reported in which class *Betaproteobacteria*, phylum *Bacteroidetes*, and *Actinobacteria* were found to be the predominant phylogenetic groups, while only 2 sequences out of 338 reads were identified as *Deinococcus-Thermus* [95]. Therefore, we believe that the existence of *Deinococcus-Thermus* might be one of the characteristics features of the bacterial populations in the Antarctic 2,860 ybp ice core. In contrast, the 1,670 ybp ice core contained much small number of bacterial RNA. The predominant groups in the 1,670 ybp ice core were family *Pasteurellaceae* (class *Gammaproteobacteria*) and *Microchaetaceae* (class *Cyanophyceae*) which were also detected in other studies as described above, suggesting that bacteria of these groups were common in deposited bacterial population in ice core samples.

The number of reads of fungi was higher in the 2,860 ybp ice core than in the 1,670 ybp ice core. Fungal species from family *Sphinctrinaceae*, *Trichocomaceae* (class *Eurotiomycetes*), and *Dactylosporaceae* (class *Lecanoromycetes*), which were abundant in 2,860 ybp ice core, have been reported to be cosmopolitans and considered airborne [47, 85, 108]. This suggests that the fungal RNA in our samples was transported by wind, similar to that of bacteria. Previous studies on fungi detected in Greenland ice cores reported that genus *Acremonium* and class *Sordariomycetes* (phylum *Ascomycota*) were found in multiple samples of different periods [63, 73]. Another study showed that

genus *Cladosporium* and *Alternaria* (phylum *Ascomycota* and class *Dothideomycetes*) were detected in ice from Vostok Station, Antarctica [54]. As these classes differed from those in these reports, it seems that fungal populations in Antarctic ice cores are more divergent than bacterial populations.

The 2,860 ybp ice core yielded substantially more microbial RNA than the 1,670 ybp ice core. The cause of this difference is not clear, but one possible explanation is that the 2 samples were deposited under different climatic conditions. It has been reported that more microorganisms can be detected in ice cores deposited in a cold climate and more frequent dust fall [115]. Another possibility is that the preservation of the RNA differed between the 2 samples; Knowlton *et al.* [54] reported that low temperature, low atmospheric CO_2, and high dust levels were related to the better conservation of nucleic acids in ice sheets. Although we did not analyze these factors, it can be suspected that the climate conditions in which the 2,860 ybp ice core formed were colder and/or more dust-rich condition than those in which the 1,670 ybp ice core did, which might affect microbial patterns in the 2 ice cores.

To the best of our knowledge, this is the first report on microbial diversity in the Antarctic ice using metagenomic analysis. Although our analysis was limited to a single location and 2 different periods, we can say that these results present a metagenomic snapshot. Additional Dome Fuji ice core samples of different depths and ice cores from different drilling stations may provide further information about the biodiversity and climate conditions in ancient times in this environment.

2.5. Summary of Chapter 2

Microbial population embedded in ice sheets is considered as a parameter of paleoclimate condition because they were reported to reflect the environmental status at the deposited period. To obtain insight into microbial patterns in ice sheets, we studied the 2 ice core samples, the 1,670 years before present (ybp) and the 2,860 ybp ice core sampled from Dome Fuji Station in East Antarctica. We extracted RNA from the samples and determined the phylogenetic diversities of organisms entrapped in the samples by metagenomic analysis. Bacterial and fungal populations were further classified at the family level. Family *Pasteurellaceae* and *Microchaetaceae* were major bacterial group in 1,670 ybp ice core whereas family *Deinococcaceae* and *Trueperaceae* were predominantly detected in 2,860 ybp ice core. The 2,860 ybp ice also contained abundant fungal sequences of family *Sphinctrinaceae*, *Trichocomaceae*, and *Dactylosporaceae*. These differences in microbial patterns suggest that microflora in ice sheets were distinct between different time scales, and that they might be highly influenced by environmental condition at deposition. Our results exhibited a metagenomic snapshot in microbial populations in the Antarctic ice sheets.

CHAPTER 3

Antimicrobial resistance genes in sea ice and Antarctic ice core

3.1. Introduction

Antimicrobial resistant bacteria (ARB), which show reduced susceptibility to antimicrobials, have been disseminated around the world [114]. It has been suggested that there are 2 main mechanisms of dissemination of ARB: clonal spread of the ARB and horizontal transfer of the antimicrobial resistance genes (ARGs) between bacteria [61]. Particularly, horizontal gene transfer via plasmids or integrative conjugation elements (ICEs) can cause drastic dissemination of ARGs because these elements can easily transferred between bacteria [9]. In addition, ARGs from non-pathogenic environmental bacteria can be transferred to pathogenic bacteria [6]. Because environmental bacteria was reported to have ARGs with high sequence identity to those of pathogenic bacteria, the gene transfer from environmental to pathogenic bacteria is now of major concern [37].

ARB and ARGs have been detected in various natural sources including feces of wild animals, soil, river water, sea water, snow, and glacier ice [4, 33, 68, 89]. Furthermore, ARB and ARGs were detected in pristine conditions such as permafrost sediments, microbiome in deep cave sediments, and the surface snow of the Antarctica [10, 26, 71, 94]. Because these samples were extremely isolated from anthropogenic influences, it is suggested that emergence of ARGs predated the discovery of antimicrobial agents. The period before that the antimicrobials had been discovered is called as “pre-antibiotic era” [26]. Indeed, it is suggested that most of ARGs were originated from chromosomal DNA which had been encoded by some environmental bacteria [4]. However, information about the existence of ARGs in pristine conditions is still limited because of the difficulty in collecting appropriate samples. To study ARB

and ARGs in pristine habitats is important for a better understanding of the dissemination of antimicrobial resistance in the modern world, as it has been postulated that the emergence of ARGs is caused by the selective pressure of clinical and agricultural antibiotic use on environmental bacteria, which harbored ARGs as part of their survival strategy against antibiotic-producing microorganisms [4].

In this study, we attempted to detect and identify ARGs in sea ice and Antarctic ice core samples to assess the distribution of ARGs in pristine environment, which is devoid of anthropogenic influences. We detected the *sul2-strA-strB* gene cluster in one sample, and compared its sequence with that of modern bacteria. In addition, we examined the expression of the ARGs in laboratory strain of modern bacteria.

3.2. Materials and Methods

3.2.1. Samples

The ice samples used in this study are summarized in Table 4. The sea ice samples of the Southern Ocean and the Sea of Okhotsk were the same as described in Chapter 1. Because the entire Antarctic ice core samples described in Chapter 2 was used for metagenomic analysis, we used other Antarctic ice cores derived from Dome Fuji Station for this study. The age of the 3 ice cores was determined in the same method described in Chapter 2. The preparation of the control ice and decontamination process of samples were performed as described in Chapter 1.

Table 4. Ice samples used in this study.

Origin	Sample name	Location	Depth (m)	Estimated age of ice
Sea ice	Ant	the Southern Ocean	------	First-year ice
	Okh	the Sea of Okhotsk	------	First-year ice
Antarctic ice core	DF-63.5	Dome Fuji Station	63.57	1,200-1,400 ybp
	DF-85.3	Dome Fuji Station	85.34	1,700-2,100 ybp
	DF-107.8	Dome Fuji Station	107.86	2,200-2,800 ybp
Otsuka distilled water	Control	------	------	------

3.2.2. DNA extraction

For the sea ice samples, we used the DNA extraction method described in Chapter 1. DNA from the Antarctic ice cores was extracted using the InstaGene Matrix kit (Bio-Rad) and EXTRAGEN MB mycobacterial DNA extraction kit (Tosoh) according to the manufacturers' instructions.

3.2.3. Cloning and characterization of ARGs

We expected that ARGs, if present in the melted samples, would be in concentrations below the detection limit of PCR. Therefore, whole-genome amplification was conducted using an illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Japan, Tokyo, Japan) according to manufacturer's instructions. We amplified the following ARGs by PCR: *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{MOX}$, *bla*$_{CIT}$, *bla*$_{DHA}$, *bla*$_{ACC}$, *bla*$_{EBC}$, *bla*$_{FOX}$, *aac(3)-Ia*, *ant(2")-Ia*, *ant(3")-Ia*, *aph(3')-Ia*, *aph(3')-VIa*, *strA* (synonym *aph(3")-Ib)*, *strB* (synonym *aph(6)-Id*), *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(M), *tet*(S), *ermA*, *ermB*, *mefA/E*, *cat* I, *cat* II, *cat* III, *cat* IV, *qnrA*, *qnrB*, *qnrS*, *vanA*, and *vanB* [17, 35, 44, 48, 53, 55, 56, 60, 64, 86, 116, 119]. Genes for heavy metal tolerance (*merA*, *merB*, *tcrB*, and *cadA*) were also screened by PCR [42, 62, 82]. The primer sequences used in this study are listed in Table 5. All reactions were performed using TaKaRa ExTaq polymerase (Takara Bio Inc., Shiga, Japan) and KOD FX Neo polymerase (Toyobo Co., Ltd., Life Science Department, Osaka, Japan) for duplicate reactions, according to the manufacturers' instructions. PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) and analyzed by direct sequencing at Hokkaido System Science. Obtained nucleotide sequences were deposited at the DNA Data Bank of Japan (GenBank accession number: LC010221). PCR products that were amplified with KOD FX Neo polymerase were cloned into pTA2 vector using a TArget Clone -Plus- kit (Toyobo) and then transformed into *Escherichia coli* using a Competent high DH5α Competent Cell kit (Toyobo) according to the manufacturer's protocol.

Table 5. Primer sequences used in this study.

Primer name	Forward (5′ - 3′)	Reverse (5′ - 3′)	Ref.
bla$_{\text{TEM}}$	ATGAGTATTCAACATTTTCG	TTACCAATGCTTAATCAGTG	[55]
bla$_{\text{SHV}}$	ATGCGTTATATTCGCCTGTG	TTAGCGTTGCCAGTGCTCGA	[55]
bla$_{\text{CTX-M-1 group}}$	GCGTGATACCACTTCACCTC	TGAAGTAAGTGACCAGAATC	[116]
bla$_{\text{CTX-M-2 group}}$	TGATACCACCACGCCGCTC	TATTGCATCAGAAACCGTGGG	[116]
bla$_{\text{CTX-M-8 group}}$	CAATCTGACGTTGGGCAATG	ATAACCGTCGGTGACAATT	[116]
bla$_{\text{CTX-M-9 group}}$	ATCAAGCCTGCCGATCTGGTTA	GTAAGCTGACGCAACGTCTGC	[116]
bla$_{\text{MOX group}}$	GCTGCTCAAGGAGCACAGGAT	CACATTGACATAGGTGTGGTGC	[86]
bla$_{\text{CIT group}}$	TGGCCAGAACTGACAGGCAAA	TTTCTCCTGAACGTGGCTGGC	[86]
bla$_{\text{DHA group}}$	AACTTTCACAGGTGTGCTGGGT	CCGTACGCATACTGGCTTTGC	[86]
bla$_{\text{ACC group}}$	AACAGCCTCAGCAGCCGGTTA	TTCGCCGCAATCATCCCTAGC	[86]
bla$_{\text{EBC group}}$	TCGGTAAAGCCGATGTTGCGG	CTTCCACTGCGGCTGCCAGTT	[86]
bla$_{\text{FOX group}}$	AACATGGGGTATCAGGCAGATG	CAAAGCGCGTAACCGGATTGG	[86]
aac(3)-Ia	ATGGGCATCATTCGCACATGTAGG	TTAGGTGGCGGTACTTGGGTC	[44]
aac(3)-IIa,b,c,	GCGCACCCCGATGCMTCSATGG	GGCAACGGCCTCGGCGTARTGSA	[43]
aac(3)-IVa	GCCCATCCCGACGCATCSATGG	CGCCACCGCTTCGGCATARTGSA	
aac(6′)-Ib	TGACCTTGCGATGCTCTATG	CGGTACCTTGCCTCTCAAAC	[81]
ant(2″)-Ia	GAGGAGTTGGACTATGGATT	CTTCATCGGCATAGTAAAAG	[56]
ant(3″)-Ia	ATGAGGGAAGCGGTGATCG	TTATTTGCCGACTACCTTGGTG	[44]
aph(3′)-Ia	ATGGGCTCGCGATAATGTC	CTCACCGAGGCAGTTCCAT	[44]
aph(3′)-VIa	ATGGAATTGCCCAATATTATTC	TCAATTCAATTCATCAAGTTTTA	[44]
strA	CCTGGTGATAACGGCAATTC	CCAATCGCAGATAGAAGGC	[60]
strB	ATCGTCAAGGGATTGAAACC	GGATCGTAGAACATATTGGC	[60]
strA-2	GGTTGCCTGTCAGAGGCGG	GTCAGAGGGTCCAATCGC	[71]
strB-2	CTGCTCATTGGCACGTTTCG	ACGTCTGTCGCACCTGCTTG	This study
tet(A)	GCGCTNTATGCGTTGATGCA	ACAGCCCGTCAGGAAATT	[48]
tet(B)	GCGCTNTATGCGTTGATGCA	TGAAAGCAAACGGCCTAA	[48]
tet(C)	GCGCTNTATGCGTTGATGCA	CGTGCAAGATTCCGAATA	[48]
tet(D)	GCGCTNTATGCGTTGATGCA	CCAGAGGTTTAAGCAGTGT	[48]
tet(E)	GCGCTNTATGCGTTGATGCA	ATGTGTCCTGGATTCCT	[48]
tet(G)	GCGCTNTATGCGTTGATGCA	ATGCCAACACCCCCGGCG	[48]
tet(M)	GTTAAATAGTGTTCTTGGAG	CTAAGATATGGCTCTAACAA	[53]
tet(S)	CATAGACAAGCCGTTGACC	ATGTTTTTGGAACGACAGAG	[53]
ermA	CCCGAAAAATACGCAAAATTTCAT	CCCTGTTTACCCATTTATAAACG	[64]
ermB	TGGTATTCCAAATGCGTAATG	CTGTGGTATGGCGGGTAAGT	[64]
mefA/E	CAATATGGGCAGGGCAAG	AAGCTGTTCCAATGCTACGG	[64]
cat I	CCATCACATACTGCATGATG	GGTGATATGGGATAGTGTT	[119]
cat II	GATTGACCTGAATACCTGGAA	GGTGATATGGGATAGTGTT	[119]
cat III	CCATACTCATCCGATATTGA	GGTGATATGGGATAGTGTT	[119]
cat IV	CCGGTAAAGCGAAATTGTAT	GGTGATATGGGATAGTGTT	[119]
qnrA	AGAGGATTTCTCACGCCAGG	TGCCAGGCACAGATCTTGAC	[17]
qnrB	GGMATHGAAATTCGCCACTG	TTTGCYGYYCGCCAGTCGAA	[17]
qnrS	GCAAGTTCATTGAACAGGGT	TCTAAACCGTCGAGTTCGGCG	[17]
vanA	GGGAAAACGACAATTGC	GTACAATGCGGCCGTTA	[35]
vanB	ATGGGAAGCCGATAGTC	GATTTCGTTCCTCGACC	[35]
merA	ACCATCGGCGGCACCTGCGT	ACCATCGTCAGGTAGGGGAACAA	[62]
merB	TCGCCCCATATATTTTAGAAC	GTCGGGACAGATGCAAAGAAA	[62]
tcrB	CATCACGGTAGCTTTAAGGAGATTTTC	ATAGAGGACTCCGCCACCATTG	[42]
cadA	AACGAGCTCCTGCACAAG	GTCAGTACCGAAATACATG	[82]

Table 5 (continued).

Primer name	Forward (5′ - 3′)	Reverse (5′ - 3′)	Ref.
sul2	ATTCCCTTTCGACCCGAGCATC	GGTGAATCGCATTCTGACTGGTTG	This study
sul2-strA	AGCTGACTTCATCCGCACACAC	CATTGACGGCCACGCTTTGAG	This study
strA-strB	TGCTAACGCCGAAGAGAACTGG	CGCGCAGTTCATCAGCAATGTC	This study
Lower *strB*	TACGCTTATGGGTGCCTTTC	AGTGCCGCCTTGCTGGTTGATG	This study
RSF1010	GGTACTGGAAGCTATGG	AGGTCAAACTCGCTGAGGTCG	[83]
Tn5393-1	GATGTGTTCCCATCCG	ACGTTATCTTGGCGGAAGGG	[87]
Tn5393-2	TGTCGAACGGACAATATC	GAGCCAAAAGATCGA	[87]

3.2.4. *strA* sequence analysis

As nucleotide sequence polymorphism at position 1, 69, 81, 204, 467, 471, and 593 in *strA* gene have been reported for bacteria from different origins [104], we compared the *strA* sequence found in DF-63.5 with other *strA* sequences deposited at the National Center for Biotechnology Information (NCBI) database using BioEdit Sequence Alignment Editor software version 7.0.5.3.

3.2.5. Detection of the genetic elements surrounding the ARG cluster

We designed primers for the sulfonamide resistance gene *sul2* using ApE - A plasmid Editor software version 1.17 because *sul2* has often been detected in the upstream region of *strA-strB* gene cluster (Table 5) [27, 83, 118]. In addition, to analyze complete nucleotide sequence of this ARG cluster, we also designed primers for the intermediate region between *sul2-strA*, *strA-strB*, and the lower region of *strB* (Table 5). Furthermore, as the *strA-strB* gene cluster has been reported to be frequently located on the broad host range plasmid RSF1010 or on the transposon Tn*5393* [83, 87, 103, 105], we amplified this cluster in DF-63.5 by PCR. Primers for the *mobB-repB* region in RSF1010, for *tnpA* in Tn*5393* (primer Tn5393-1), and for the *tnpR-strA* region in Tn*5393* (primer Tn5393-2) are listed in Table 5.

3.2.6. Antimicrobial susceptibility test

We tested whether the ARGs identified in this study could confer heterogonous resistance to *E. coli* by comparing the antimicrobial susceptibility of the untransformed competent *E. coli* strain DH5α with that of the transformed DH5α strain DH5α/pTA2[*sul2-strA-strB*]. The minimum inhibitory concentration (MIC) was determined by using the agar-dilution method according to the protocol recommended by the Clinical and Laboratory Standards Institute (CLSI) [23] for the following antimicrobials; ampicillin (ABPC), dihydrostreptomycin (DSM), kanamycin (KM), gentamicin (GM), and sulfamethoxazole (SMX). Mueller-Hinton agar (Oxoid, Hampshire, UK) was used for the susceptibility tests following the CLSI recommendation. All antimicrobial agents were purchased from Sigma-Aldrich. *Staphylococcus aureus* ATCC 29123 and *E. coli* ATCC 25922 were used as quality control strains for MIC tests.

3.3. Results

3.3.1. Detection of the ARGs

The aminoglycoside phosphotransferase genes *strA* (*aph(3")-Ib*) and *strB* (*aph(6)-Id*) were detected in the Antarctic 1,400 ybp ice core (sample DF-63.5) alone. In addition, we successfully amplified the entire *strA-strB* gene cluster from this sample using the *strA* forward and the *strB* reverse primer, as confirmed by direct sequencing. Amplification of these genes using the alternate primers strA-2 and strB-2 was also successful. No significant amplicons were obtained by PCR using other primers.

3.3.2. Analysis of the sequence polymorphisms in *strA*

The *strA* nucleotide sequence of sample DF-63.5 was compared with well-characterized *strA* sequences including 5 RSF1010 variants, 3 Tn*5393* variants, and 1 ICE to detect putative polymorphisms at the 7 selected, variable positions (Table 6). The sequence obtained from DF-63.5 was identical to that of RSF1010 variants pASL01a, pYT3, and pAB5S9 and to that of ICEVchind4. Compared with RSF1010, the DF-63.5 *strA* sequence contained 2 polymorphisms at position 467 (T in RSF1010 to A in DF-63.5) and position 470 (A in RSF1010 to T in DF-63.5). pLS88, a plasmid that is widely used as cloning vector, also showed 2 polymorphisms at position 69 (C in pLS88 to T in DF-63.5) and position 204 (G in pLS88 to C in DF-63.5). In comparison to the 3 Tn*5393* variants, pEa23, pRAS2, and pI-35, *strA* in DF-63.5 showed 1 transversion at position 204 (G in Tn*5393* variants to C in DF-63.5).

Table 6. Comparison of the polymorphisms in the *strA* nucleotide sequence.

Genetic name	Host bacteria	Source of isolation	Polymorphism in *strA* sequence							Accession Number	Ref.
			1	69	80	204	467	470	593		
***sul2-strA-strB* gene cluster in DF-63.5**		Ice core	T	T	T	C	A	T	A	LC010221	This study
RSF1010 variants											
RSF1010	*E. coli* and other G(-) bacteria	Various	T	T	T	C	T	A	A	M28829	[]93]
pASL01a	*E. coli*	Clinical	T	T	T	C	A	T	A	JQ480155	[57]
pYT3	*Salmonella* Typhimurium	Cattle	T	T	T	C	A	T	A	AB591424	[107]
pAb5S9	*Aeromonas bestiarum*	River	T	T	T	C	A	T	A	EF495198	[38]
pLS88	*Haemophilus ducreyi* and cloning vector	Vector	T	C	T	G	A	T	A	L23118	[34]
Tn*5393* variants											
pEa34	*Erwinia amylovora*	Plant	T	T	T	G	A	T	A	M96392	[19]
pRAS2	*Aeromonas salmonicida* subsp. *salmonicida*	Fish	T	T	T	G	A	T	A	AF262622	[58]
pI-35	*Pseudomona*s sp.	Permafrost	T	T	T	G	A	T	A	not available	[87]
ICE											
ICEVchind4	*Vibrio cholerae*	Clinical	T	T	T	C	A	T	A	GQ463141	[66]

3.3.3. The genetic elements surrounding the *sul2-strA-strB* gene cluster in DF-63.5

We successfully amplified the *sul2* gene from DF-63.5. In addition, the *sul2-strA* gene cluster and the lower region of *strB* were successfully amplified using the *sul2* forward and *strA* reverse primers, and the lower *strB* primers, respectively (Table 5). Sequencing of the PCR products showed that these clusters were located within the 2,764 bp gene *sul2-strA-strB* gene cluster (Fig. 7).

BLAST analysis revealed the high sequence similarity of the *sul2-strA-strB* cluster in DF-63.5 to various sequences from GenBank (Fig. 8). The sequence of *sul2-strA-strB* gene cluster in DF-63.5 showed 100% (2,764/2,764 bp) homology to that of ICEVchind4 (Fig. 7 and 8). In addition, it also showed high homology to the sequence of RSF1010, pASL01a, pYT3, and pAb5S9. Despite repetitions of amplification attempts, the genetic elements of RSF1010 and Tn*5393* were not detected in DF-63.5.

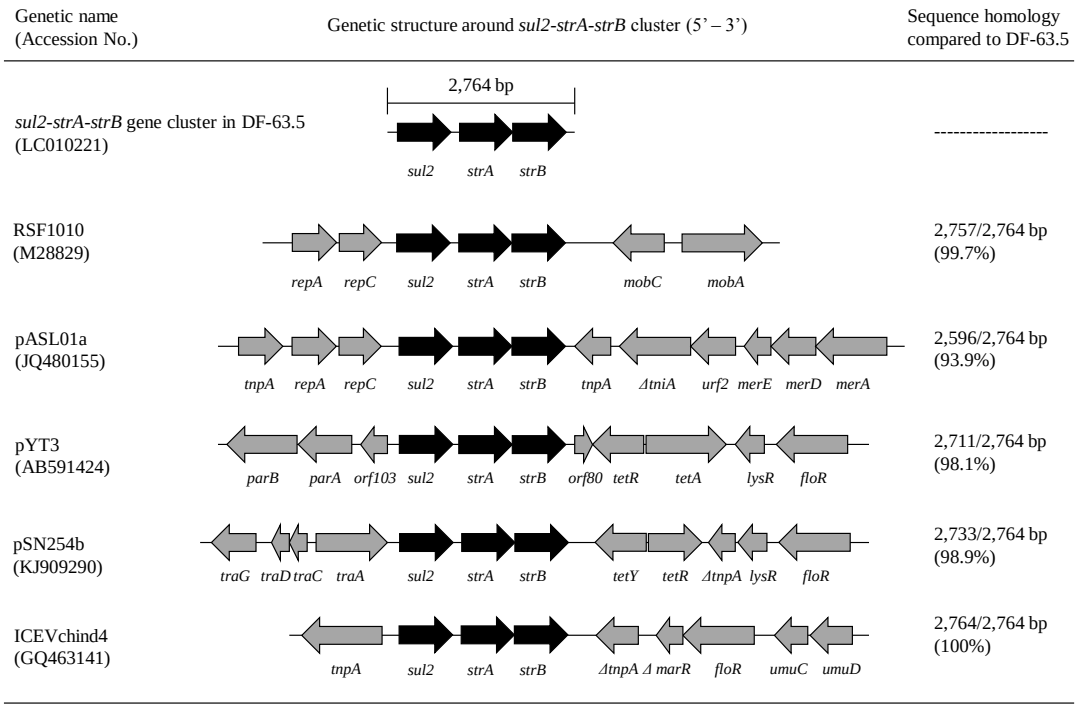
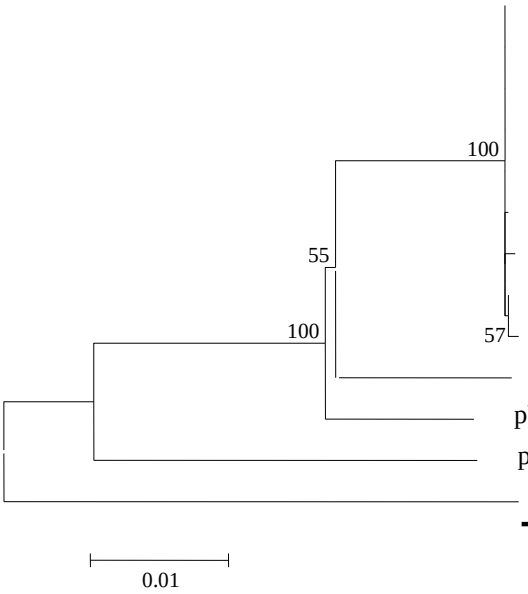


Fig. 7. The genetic structure of the *sul2-strA-strB* gene cluster and its surrounding genes. The arrow indicates the direction of transcription of the gene.



Name	Host	Accession No.
ICEVchind4	*Vibrio cholerae*	GQ463141
p9123	*Escherichia coli*	AY360321
pSFxv_3	*Shigella flexneri*	CP001386
pBK30683	*Klebsiella pneumoniae*	KF954760
sul2-strA-strB	**gene cluster in DF-63.5**	**LC010221**
pVI678	*Escherichia coli*	EF090911
ICEVfind1	*Vibrio fluvialis*	KM213605
pSRC15	*Salmonella* Typhimurium	GQ379901
RSF1010	*Escherichia coli*	M28829
pAb5S9	*Aeromonas bestium*	EF495198
pYT3	*Salmonella* Typhimurium	AB591424
pASL01a	*Escherichia coli*	JQ480155
pKA1	*Vibrio cholerae*	AY958065

Fig. 8. Comparison of the *sul2-strA-strB* nucleotide sequence from DF-63.5 (2,764 bp) and reference sequences from various hosts. All the gene sequences were derived from the NCBI database. The phylogenetic tree was constructed by neighbor-joining analysis (Jukes-Cantor method) with 1,000 bootstrap replications using the MEGA software version 6.06. Numbers at branch nodes are bootstrap values. The scale bar indicates the Jukes-Cantor distance.

3.3.4. Antimicrobial susceptibility test

The results of susceptibility test are presented in Table 7. Compared with the untransformed *E. coli* strain DH5α, the strain transformed with the empty pTA2 vector (strain DH5α/pTA2) showed reduced susceptibility to ABPC because pTA2 contains the *bla* gene as a resistance marker. *E. coli* transformed with *sul2-strA-strB* (strain

DH5α/pTA2[*sul2-strA-strB*]) showed a 7-fold reduction in susceptibility to DSM and 1-fold or more reduction to SMX, but similar susceptibility to KM and GM in comparison with untransformed strain.

Table 7. Antimicrobial susceptibility of *E. coli* strains of competent cell and transformant.

Strain	MIC (mg/L)				
	ABPC	DSM	KM	GM	SMX
DH5α	1	1	1	0.5	512
DH5α/pTA2	> 512	1	1	0.5	512
DH5α/pTA2[*sul2-strA-strB*]	> 512	64	1	0.5	> 512

3.4. Discussion

We detected and successfully amplified the *sul2-strA-strB* gene cluster from the Antarctic ice core sample DF-63.5 of approximately 1,200 - 1,400 ybp. The *sul2* gene encodes type II dihydropteroate synthase that confers sulfonamide resistance, while *strA* and *strB* encode aminoglycoside phosphotransferases that determine streptomycin resistance [91, 96]. First, we confirmed that the *sul2-strA-strB* gene cluster originated from DF-63.5 because the RSF1010 family, belonging to the IncQ group of non-conjugative plasmids with a broad host range and encoding *sul2-strA-strB*, has been widely utilized in molecular biology as cloning or shuttle vector [50, 52]. As shown in Table 6, the polymorphisms in the nucleotide sequence of *strA* from DF-63.5 were different from those in the RSF1010 and pLS88 vector plasmid. The other RSF1010-derivative vector plasmids such as pAY201 (GenBank accession number AB526842), pYN401 (AB531985), pRL1383a (AF403426), and pCVD046 (KM017897) do not harbor the complete *sul2-strA-strB* sequence because their antimicrobial resistance marker genes have been exchanged for β-lactamase or chloramphenicol resistance genes. In addition, we did not detect the *sul2-strA-strB* gene cluster in other samples including the control ice although the same decontamination, melting, whole genome amplification, and PCR procedures were used as for all samples. Therefore, we concluded that the *sul2-strA-strB* gene cluster was contained in the ice core DF-63.5.

As shown in Fig. 7, the *sul2-strA-strB* gene cluster is widely distributed among gram-negative bacteria such as *Enterobacteriaceae, Aeromonas* spp., and *Vibrio* spp. As these bacteria have been isolated from various sources including human clinical and

veterinary sources, plants, and aquatic habitats [103, 105], it is obvious that this ARG cluster is globally distributed among bacteria in various habitats. From this point of view, it is plausible that bacteria harboring the *sul2-strA-strB* gene cluster were transferred to Antarctica by wind (see discussion in Chapter 2) and entrapped in the Antarctic ice sheets. The bacterial host of the ARG cluster in DF-63.5 is unknown, but considering the wide distribution of this cluster among gram-negative bacteria, environmental gram-negatives could be suspected. Interestingly, previous studies of ARB and ARGs in Siberian permafrost samples isolated *Pseudomonas* sp., *Acinetobacter* sp., and *Psychrobacter psychrophilus* which harbored Tn*5393*-derivative *strA* and *strB* genes [71, 87]. As gram-negative bacteria were detected in the 1,670 and 2,860 ybp Antarctic ice cores of Dome Fuji Station as described in Chapter 2, it is possible that gram-negative bacteria were the host of the *sul2-strA-strB* gene cluster in DF-63.5.

Although the original host of the *sul2*, *strA*, and *strB* genes could not be identified because of the wide distribution of these genes [91, 96], the evolutionary history of the genetic structure of *sul2-strA-strB* has been studied. Briefly, it has been postulated that: 1) linkage and distribution of *strA-strB* occurred originally as one genetic element within transposon Tn*5393*, 2) Tn*5393* was inserted into the *CR2-sul2* gene cluster (GenBank accession number AB277723) on an ancestor plasmid of RSF1010, forming the *CR2-sul2-strA-strB* gene cluster, 3) transposase genes removed part of *CR2* from the gene cluster [105, 118]. This hypothesis is supported by the fact that Tn*5393* encoding *strA-strB* gene cluster without *sul2* was detected in bacteria that had been derived from permafrost samples of 15,000 to 40,000 years old, suggesting the high age and persistence of Tn*5393* [71, 87]. As DF-63.5 was older than contemporary

samples but younger than these permafrost samples, our finding may indicate that genetic transposition of Tn*5393* into RSF1010 occurred within this several tens of thousands of years.

Our results showed that *sul2-strA-strB* were distributed among bacteria as a genetic cluster in the absence of selective pressure. Why *sul2-strA-strB* gene cluster was conformed and distributed in bacteria of the pre-antibiotic era is still unclear, but *strA-strB* genes may improve survival in natural environments that are home to antibiotic-producing bacteria such as *Streptomyces* sp. [4, 91]. Another possibility is that *strA-strB* was accidentally translocated to RSF1010, which has a very broad host range. Therefore, the spread of *sul2-strA-strB* may be a result of the RSF1010 dissemination regardless of its antimicrobial resistance advantage. Alternatively, *sul2-strA-strB* may have additional functions coupled with evolutionary advantages other than antimicrobial resistance. This hypothesis is supported by a report that an *sul2-strA-strB*-encoding plasmid conferred enhanced fitness to its host without antimicrobial pressure [36]. Further study using a variety of samples is needed to clarify the reason of the presence of ARG clusters in pristine environments.

Previous studies on ARGs in permafrost or Antarctic surface snow detected several ARGs such as *bla* for β-lactamase resistance, *tet*(M) for tetracycline resistance, *vanX* for vancomycin resistance, *erm* and *mef* for macrolide resistance, and *strA* and *strB* for aminoglycoside resistance [26, 71, 94]. In agreement with these reports, our results showed the existence and distribution of ARG among environmental bacteria in pre-antibiotic era although we could not detect ARG in other sea ice or ice core samples. To the best of our knowledge, this is the first report on the detection of the *sul2* gene in Antarctic ice core. Given the fact that sulfonamide is a synthetic antimicrobial agent,

our finding strongly supports the hypothesis that most of ARGs that encode resistance against synthetic antibiotics such as sulfonamide, trimethoprim, and fluoroquinolone, originate from environmental bacteria [4]. The nucleotide sequence of this ARG cluster was homologous to contemporary *sul2-strA-strB,* and this ARG cluster could successfully be expressed in *E. coli* DH5α. These results suggest that the *sul2-strA-strB* cluster in the Antarctic ice core DF-63.5, the sample of pre-antibiotic era, may be very similar to that of in clinical or environmental bacteria in the present, post-antibiotic era.

3.5. Summary of Chapter 3

Bacteria harboring antimicrobial resistance genes (ARGs) has been isolated from various habitats not only clinical setting but also natural environments including extremely isolated area. To detect the ARGs in pristine environment, we examined the sea ice and the Antarctic ice cores from Dome Fuji Station in East Antarctica. DNA in ice samples were extracted in a sterilized condition, and the ARGs were detected by standard PCR method. We detected the type II dihydropteroate synthase gene *sul2* and aminoglycoside phosphotransferase genes *strA* and *strB* conforming 2,764 bp of *sul2-strA-strB* gene cluster in the Antarctic ice core sample DF-63.5 of approximately 1,200 - 1,400 years old. The nucleotide sequence of this gene cluster were showed high identity to that of in plasmids, transposons, or integrative conjugation elements in other present-day gram-negative bacteria. Although this gene cluster was reported to frequently relate with plasmid RSF1010 or transposon Tn*5393*, we could not detect these genetic elements. The gene cluster could be expressed in *Escherichia coli* strain DH5α by transformation, exhibiting reduced susceptibility to dihydrostreptomycin and sulfamethoxazole. These results showed that some ARGs existed and conformed the gene cluster in pre-antibiotic era. Since *sul2* is a resistance gene which confers resistance against sulfamethoxazole, it is suggested that ARGs against systemic antimicrobials are ancient and may have played some roles in pristine environments.

CONCLUSION

First, we developed a safe and reliable decontamination method for ice samples using sea ice of the Southern Ocean and the Sea of Okhotsk, as described in Chapter 1. 16S rDNA-based analysis revealed the predominance of class *Alphaproteobacteria* and *Gammaproteobacteria* in both samples. At the genus level, however, there were clear differences in the bacterial community between the 2 sea ice samples. The predominance of bacteria from phylum *Bacteroidetes* was a distinctive feature of the Okhotsk sea ice. These results can be utilized to reveal the characteristics of bacterial communities in sea ice and provide information in the field of marine and psychrophilic microbiology.

Next, We identified the microbial diversity in Antarctic ice core samples by metagenomic analysis, as described in Chapter 2. The results showed that only a small number of microorganisms were contained in the Antarctic ice core samples, except for phylum *Deinococcus-Thermus* in the 2,860 ybp ice. The abundance of *Deinococcus-Thermus* was remarkable as compared with other microbial studies of Greenland ice core or glacier ice. Our study was the first metagenomic analysis in microbiota of Antarctic ice and can give insights into the ancient bacterial flora.

Finally, we detected an ARG cluster in one of Antarctic ice core samples, as described in Chapter 3. The nucleotide sequence of the ARG cluster obtained from the ice core was homologous to that of present-day bacteria. The ARG cluster from the ice sample could be expressed *E. coli* strain DH5α. These results suggest the existence of ARGs in pre-antibiotic era, supporting the theory that the ARGs are ancient. Our findings were important because the ARGs in our sample were located on the same gene

cluster without selective pressure, indicating that some ARGs may have been clustered before human usage of antimicrobial agents had started. In addition, our results suggest that the dissemination of the *sul2* gene that confers resistance to sulfonamide, a synthetic antimicrobial agent, occurred in pre-antibiotic era.

In conclusion, we successfully identified an ARG cluster from an Antarctic ice core. This thesis provides significant evidence of the presence of ARGs in a pristine environment. We expect that our data can be utilized for further researches with regard to the origin, history, and dissemination of ARB and ARGs in the environment.

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RESEARCH ACHIEVEMENTS

Publication relating this thesis

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Conference presentations

Okubo T., Sato T., Nakamura S., Iida T., Usui M., Noda J., Hagiwara K., Tamura Y. Poster presentation "Analysis of bacterial diversity in Antarctic ice cores". June 14th, 2012. The 1st symposium of research achievements in Hokkaido University Research Center for Zoonosis Control at Hokkaido University Graduate School of Veterinary Medicine.

Okubo T., Sato T., Nakamura S., Iida T., Usui M., Noda J., Hagiwara K., Tamura Y. Oral presentation No. S2-9 "Analysis of bacterial diversity in Antarctic ice cores". August 28th, 2012. The 79th Annual Meeting of Japanese Society of Bacteriology Hokkaido Branch at Tokachi Plaza, Obihiro.

Okubo T., Sato T., Nakamura S., Iida T., Usui M., Noda J., Hagiwara K., Tamura Y. Oral presentation No. DB-33 "Analysis of bacterial diversity in Antarctic ice cores". September 15th, 2012. The 154th Annual Meeting of Japanese Society of Veterinary Medicine at Iwate University.

ABSTRACT IN JAPANESE （和文要旨）

抗菌薬が人医療および獣医療において広く用いられるのに伴い、薬剤耐性菌の出現と増加が公衆衛生学上の問題として世界的に懸念されている。また、薬剤耐性菌がもつ耐性遺伝子は、その多くが非病原性の環境細菌に由来するとみられていることから、現在蔓延している耐性遺伝子は、環境細菌から病原性細菌へと伝達されてから拡散したものと考えられている。これに加えて、合成抗菌薬に対する耐性遺伝子を染色体上にもつ細菌が報告されている点や、永久凍土層や洞窟などの隔離条件下からも耐性菌と耐性遺伝子が検出されている点から、耐性菌と耐性遺伝子は人為的な抗菌薬の使用による選択圧の有無に関わらず、自然環境中に存在しているものと予見されている。

本研究では、上記の仮説を補完しうる知見を収集するため、絶対的抗菌薬非存在環境として南極のアイスコアを選択し、その中に含まれる耐性遺伝子の検出を目標とした。まず第1章では、氷サンプルの安全かつ十分なクリーニング方法を立ち上げるため、南氷洋およびオホーツク海で採材した海氷をサンプルとして、氷の洗浄および氷からのDNA抽出方法を検討した。また、海氷中の細菌叢を16S rDNAに基づく分類で特定し、その構成を両サンプル間で比較した。続いて第2章では、上記のクリーニング方法に基づいて実際の南極アイスコアを処理するとともに、含まれる微生物叢をメタゲノム解析によって網羅的に特定した。第3章では、海氷および南極アイスコア中から薬剤耐性遺伝子の検出を試み、検出した遺伝子について既知の遺伝子との比較を行なった。

第1章における検討の結果、氷サンプルの洗浄方法が開発された。また、海氷に含まれる細菌叢を南氷洋とオホーツク海とで比較したところ、門レベルあるいは網レベルの広い分類ではおおむね似た傾向を示したが、科レベルや属レベルの詳細な解析で明確に菌叢が異なることがわかった。また、オホーツク海の海氷はバクテロイデス門の細菌を多く含むことが特徴であった。海氷を対象とする研究は数が少なく、また本実験で用いた採材地域でのサンプリングは過去に行なわれていないため、本研究の結果は海氷や低温環境中での細菌叢の構成に関する新たな知見を加えるものとなった。

第2章では、1,670年前および2,860年前の南極アイスコアについて、細菌および真菌の構成をメタゲノム解析で特定した。グリーンランドなどのアイスコアを対象としたメタゲノム解析の既報と比べ、南極アイスコアのメタゲノム解析では遺伝子のヒット数が大幅に少ないことが特徴であったが、これは南極（ドームふじ基地）が周囲から隔絶された環境にあることが影響したものと考えられた。一方、2,860年前の氷については、デイノコッカス-テルムス門の細菌が極端に多く検出された。本菌は極限環境微生物として知られており、ドームふじ基地における南極アイスコア中の微粒子の起源であるオーストラリアの砂漠環境にも分布しうる細菌であることから、これは細菌の付着した微粒子が当該アイスコア中に多量に含まれていた結果であると考えられた。アイスコアを対象としたメタゲノム解析は本研究が初報告であることから、今回得られた南極アイスコア固有の特性が今後の他のアイスコア研究の参考となることが期待される。

第3章においては、南極アイスコアのうち約1,200～1,400年前の氷（サンプル名DF-63.5）から、*sul2-strA-strB*の耐性遺伝子クラスターを検出することに成功した。この遺伝子群は現代でも非常に広範な種類の細菌が保有していることから、この遺伝子

は古くから環境細菌が保有しており、その細菌が南極の氷にトラップされていたことが予想された。また、DF-63.5から得られた遺伝子の塩基配列が現代の当該遺伝子と相同的であったこと、およびこの遺伝子群を組み込んだ形質転換株（大腸菌）が抗菌薬耐性を発現したことから、今回得られた耐性遺伝子群は現代の相同遺伝子と極めて近縁であることが確認された。さらに、耐性遺伝子が単体ではなくクラスターとして検出されたことから、人為的な抗菌薬使用が無い環境においても、薬剤耐性遺伝子がまとまって細菌間を移動していることが考えられた。これらに加え、*sul2*遺伝子が合成抗菌薬であるサルファ剤に対する耐性遺伝子であることから、合成抗菌薬の発明以前にもその耐性遺伝子が存在しうることが改めて示された。

以上の成績から、抗菌薬の非存在下においても薬剤耐性遺伝子が存在することが証明された。これまでに永久凍土層や南極表層の雪などからの耐性菌および耐性遺伝子の検出報告はあるが、南極アイスコアという完全に外界から隔絶された環境からの報告は今回が初めてである。抗菌薬耐性菌の出現と蔓延を予見するためには、それらの起源や環境中における動態を明らかにすることが必要であることから、本研究の結果は抗菌薬の選択圧非存在下における耐性遺伝子の存在を証明したものであり、新たな薬剤耐性菌対策の構築に有用な知見を提供した。