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Detection of the *sul2–strA–strB* gene cluster in an ice core from Dome Fuji Station, East Antarctica

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ABSTRACT

Objectives: Bacteria harbouring antimicrobial resistance genes (ARGs) have been isolated from various locations, including ancient microbiomes, indicating that these genes pre-date the discovery of antibiotics. To gain further information regarding ARGs in the pre-antibiotic era, ice samples derived from Dome Fuji Station, Eastern Antarctica, were examined.
Methods: DNA was extracted from firn or ice core samples ($n = 3$; 1200–1400 ybp, 1700–2100 ybp and 2200–2800 ybp, respectively) under sterile conditions. Whole-genome amplification and PCR analyses were utilised to detect ARGs.
Results: A 2764-bp gene cluster containing the type II dihydropteroate synthase gene *sul2* and the aminoglycoside phosphotransferase genes *strA* and *strB* was detected in the 1200–1400-year-old Antarctic ice core (DF-63.5). The *sul2–strA–strB* gene cluster is frequently associated with plasmid RSF1010 and transposon Tn*5393*; however, these elements were not detected in sample DF-63.5. The gene cluster exhibited a high level of sequence identity to sequences harboured in present-day bacteria, although there were sequence polymorphisms in the *strA* gene. Furthermore, expression of this gene cluster in *Escherichia coli* resulted in reduced susceptibility to dihydrostreptomycin and sulfamethoxazole.
Conclusion: The results of this study provide further evidence that certain ARGs existed in the pre-antibiotic era. Because the *sul2* gene confers resistance to the synthetic compound sulfamethoxazole, these findings suggest that ARGs against synthetic antimicrobials emerged in bacteria during the pre-antibiotic era.

1. Introduction

In recent years, the emergence and global dissemination of antimicrobial-resistant bacteria (ARB) has occurred. Indeed, ARB have been isolated not only from clinical settings but also from natural environments such as the faeces of wild animals, soil, rivers, sea water, Antarctic snow and glacier ice [1–3]. Notably, however, ARB and antimicrobial resistance genes (ARGs) have also been detected in secluded conditions such as permafrost, deep-sea sediments and deep cave sediments [4–6]. Because these samples were isolated from pristine environments lacking modern anthropogenic influences, these findings suggest that ARB and ARGs arose in the 'pre-antibiotic era', which comprises the period prior to the 20th century development of antimicrobial agents [5]. It has been postulated that the emergence of ARGs in the modern world results from the application of clinical and agricultural antimicrobials to environmental bacteria that harbour intrinsic ARGs in their chromosome. Conversely, the presence of ARGs in ancient microbiota is considered a natural phenomenon [1,4].

When studying ancient microbiomes, it is crucial to ensure that the sample has been isolated from a location lacking anthropogenic influences, which can act as a source of selective pressure on the microbiota or by way of contamination by modern microbiomes. Ice core or firn samples, which are

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obtained by vertical drilling into ice sheets or glaciers, are a good source of ancient environmental samples [7]. Since ice sheets or glaciers are formed by the accumulation of snow, they contain dust and other particles that have been deposited and preserved over time [8]. Indeed, some ice cores have provided records of the environmental conditions from up to one hundred thousand years before present (ybp), as reported by the Greenland Ice Sheet Project, the European Project for Ice Coring in Antarctica, and examination of ice cores from Dome Fuji Station in East Antarctica [9–11]. Although permafrost soil is a potentially good source of ancient microbiomes, it has been reported that a number of non-spore-forming bacteria can still survive [12], suggesting that the microbial communities and DNA in permafrost soils are not completely stable. In contrast, bacterial activity in ice sheets appears to be minimal due to the very low temperature and the lack of liquid water [13]. Furthermore, previous studies of micro-organisms in ice core samples indicated that the cold temperatures and isolated locations of ice sheets are favourable conditions for the preservation of ancient microbiomes [14–16]. However, the antimicrobial susceptibility of those isolates was not studied. Although a few culture-independent studies reported the presence of ARGs in ice core samples from Greenland and mountain glaciers, information regarding ARGs in Antarctic ice core samples is limited [3].

Further studies of ARB and ARGs in the pre-antibiotic era are essential to achieve a better understanding of the origin and evolutionary history of ARGs [5]. In the current study, Antarctic ice core samples were assayed for ARGs to obtain additional information regarding ancient ARGs that were present in the pre-antibiotic era. Using genetic analyses, the *sul2–strA–strB* gene cluster was detected in one sample and this sequence was compared with that harboured by modern bacteria. In addition, the effects of expression of these ARGs in a laboratory strain of *Escherichia coli* were examined.

2. Materials and methods

2.1. Samples

Three ice samples at depths of 63.57 m (sample name DF-63.5), 85.34 m (DF-85.3) and 107.86 m (DF-107.8) at Dome Fuji Station in East Antarctica (39°42′E, 77°19′S) were examined during the 39th Japanese Antarctic Research Expedition between 1998 and 1999. Analysis of volcanic chronology analysis data indicated that the ages of the ice cores were as follows: DF-63.5, 1200–1400 ybp; DF-85.3, 1700–2100 ybp; and DF-107.8, 2200–2800 ybp [7]. The density of ice samples in this study was measured previously: 0.719 g/cm^3 at 63.57 ± 1 m deep; 0.787 g/cm^3 at 85.34 ± 1 m deep; 0.828 g/cm^3 at 102.53 ± 1 m deep; and 0.873 g/cm^3 at 111.27 ± 1 m deep [17]. The accumulated snow with a density between 0.550 g/cm^3 to 0.830 g/cm^3 is defined as 'firn', whilst 'ice core' has a density of >0.830 g/cm^3 [18]. Thus, DF-63.5 and DF-85.3 were defined as firn samples. Although the accurate density of DF-107.8 (at a depth of 107.86 m) was not available, it can be regarded as an 'ice core' given the density of 102.53 ± 1 m sample (0.828 g/cm^3). As a control, distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was frozen and treated in a similar manner. All samples were maintained below −30 °C until melting.

2.2. Ice sample preparation and DNA extraction

Ice samples were decontaminated as described in our previous study [19]. Briefly, to remove contaminants from the surface of the ice, blocks of ice core (ca. 10.0 cm × 10.0 cm × 10.0 cm) were soaked three times each for 1 min in ice-cooled 99.5% ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and were then carefully rinsed three times with deionised sterile distilled water. Approximately 10 mm was removed from each surface by these procedures. As a previous study of ice samples reported that surface contaminants could be cleaned by removing ca. 10 mm from the surface of the firn or ice core samples [20–22], we confirmed decontamination of the sample and utilised only the core part of samples for further analysis. After cleaning, the ice cores were placed in sterilised glass beakers and were allowed to thaw at room temperature. The thawed samples were filtered using an Isopore™ Track-Etched Membrane Filter with 3.0-μm pore size (Merck Millipore, Billerica, MA) to remove coarse particles that may contain enzyme inhibitors. Bacterial cells were then collected using a 0.22-μm pore size Millipore Express PLUS Membrane Filter (Merck Millipore). The 0.22-μm filter was washed in 2 mL of phosphate-buffered saline containing 0.05% Tween-80 (Wako Pure Chemical Industries) with shaking for 1 min. The bacterial suspension was then centrifuged at 22 000 × *g* for 5 min, the supernatant was discarded and the resulting pellet was subjected to DNA extraction using an InstaGene™ Matrix Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions for extraction of DNA from general bacteria. To allow for the extraction of DNA from bacteria with thick, rigid cell walls, such as *Mycobacteria*, the pellets were first resuspended in 25 μL of bacteriolysis buffer from the EXTRAGEN MB Mycobacterial DNA Extraction Kit (Tosoh Corporation, Tokyo, Japan). All procedures were conducted on a clean bench, and all instruments, such as glass beakers and forceps, were sterilised prior to use by exposure to ultraviolet light for ≥12 h. The control ice (frozen distilled water) was also processed in the same manner.

2.3. Detection and cloning of antimicrobial resistance genes

We anticipated that the concentrations of ARGs, if present, in the melted samples of the ice core and blank ice would be as low as or below the detection limit of PCR. Therefore, whole-genome amplification was conducted using an illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Using primers designed in previous studies, ARGs for β-lactam resistance, aminoglycoside resistance, macrolide resistance, chloramphenicol resistance, plasmid-mediated fluoroquinolone resistance and vancomycin resistance were amplified (Supplementary Table S1). Furthermore, an additional primer set was designed for amplification of the *strB* gene (primer set *strB*-2 in Supplementary Table S1). Genes known to confer resistance to heavy metals (*merA*, *merB*, *tcrB* and *cadA*) were also screened for using previously published primers (Supplementary Table S1). All amplification reactions were performed in duplicate using TaKaRa ExTaq Polymerase (TaKaRa Bio Inc., Shiga, Japan) and KOD FX Neo Polymerase (Toyobo Co., Ltd., Life Science Department, Osaka, Japan) according to the manufacturer's instructions. PCR products were purified using a FastGene® Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo, Japan) and were analysed by direct sequencing at Hokkaido System Science Co. Ltd. (Hokkaido, Japan).

2.4. Sequence polymorphisms in strA

A previous study identified nucleotide sequence polymorphisms in the *strA* gene at positions 1, 69, 80, 204, 467, 470 and 593 in bacteria of various origins [23]. Therefore, the *strA* sequence found in sample DF-63.5 was compared with the other *strA* sequences deposited at the National Center for Biotechnology Information (NCBI) nucleotide database using BioEdit Sequence Alignment Editor software v.7.0.5.3.

2.5. Genetic elements surrounding the sul2–strA–strB gene cluster

Because the sulfonamide resistance gene *sul2* has frequently been detected in the upstream region of the *strA–strB* gene cluster [24,25], primers for amplification of *sul2* (primer set *sul2* in Supplementary Table S1) were designed. In addition, to obtain the complete nucleotide sequence of this ARG cluster, primers to amplify the intermediate regions between the *sul2* and *strA* genes (primer set *sul2–strA*) and the *strA* and *strB* genes (primer set *strA–strB*) were designed, as well as the region downstream of the *strB* gene (primer set *strB*-U) (Supplementary Table S1). Furthermore, because *strA* and *strB* are frequently located on plasmid RSF1010 and transposon Tn*5393* [26], portions of these genetic elements were amplified. The primers used to PCR amplify the *mobB–repB* region of RSF1010 and the *tnpA* gene and the *tnpR–strA* region of Tn*5393* are shown in Supplementary Table S1. To examine the phylogenetic lineage of the *sul2–strA–strB* gene cluster identified in this study, a phylogenetic tree was constructed by the maximum-likelihood method with 1000 bootstrap replicates using MEGA software v.6.06. The phylogenetic relationship between the *sul2* gene and other sulfonamide resistance genes was also compared using the same software.

2.6. Cloning of the sul2–strA–strB gene cluster

The whole sequence of the *sul2–strA–strB* gene cluster was amplified by PCR using the forward primer of *sul2* and the reverse primer of *strB* (Supplementary Table S1). The PCR product was cloned into the pTA2 vector using a TArget Clone-Plus Kit (Toyobo Co., Ltd.) and was then transformed into competent *E. coli* DH5α cells using a Competent High DH5α Competent Cell Kit (Toyobo Co., Ltd.) according to the manufacturer's protocol. The pTA2 vector encodes the β-lactamase gene $bla_{TEM\text{-}116}$ for the selection of positive transformants. Transformants were selected on Luria–Bertani agar with 50 mg/L ampicillin, 0.2 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for induction and 40 μg/mL of X-Gal for blue–white selection.

2.7. Antimicrobial susceptibility testing

To examine whether the ARGs identified in the present study could confer heterogeneous resistance to *E. coli*, the antimicrobial susceptibility of *E. coli* strain DH5α was compared with that of *E. coli* DH5α transformed with the pTA2[*sul2-strA-strB*] vector (*E. coli* DH5α/pTA2[*sul2-strA-strB*]). Minimum inhibitory concentrations (MIC) for each strain were determined to ampicillin, dihydrostreptomycin, kanamycin, gentamicin and sulfamethoxazole. MIC tests were performed by the agar dilution method or broth dilution method (for sulfamethoxazole) according to the protocol recommended by the Clinical and Laboratory Standards Institute (CLSI) [27]. Mueller–Hinton agar (Oxoid Ltd., Basingstoke, UK) was used for the susceptibility tests according to CLSI recommendations [27]. All antimicrobial agents were purchased from Sigma-Aldrich (St Louis, MO). *Staphylococcus aureus* ATCC 29123 and *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA) were used as quality control strains for the MIC tests [27].

2.8. Nucleotide accession number

The ARG sequence obtained in this study was deposited in the NCBI database under GenBank accession no. **LC010221**.

3. Results

3.1. Amplification of the strA and strB genes from an Antarctic ice sample

The aminoglycoside phosphotransferase genes *strA* [synonym *aph(3″)-Ib*] and *strB* [*aph(6)-Id*] were detected in ice core sample DF-63.5, which dated to 1200–1400 ybp. Amplification of *strA* and *strB* was confirmed using different primer pairs (*strA*-2 and *strB*-2; Supplementary Table S1). In addition, the *strA–strB* gene cluster was successfully amplified from DF-63.5 using the *strA* forward and the *strB* reverse primers, as confirmed by direct sequencing. No significant amplicons of other ARGs or heavy metal resistance genes were obtained from sample DF-63.5. The other ice core samples did not indicate ARGs or heavy metal resistance genes.

3.2. Analysis of sequence polymorphisms in the strA gene detected in Antarctic ice

To detect putative nucleotide polymorphisms in the *strA* gene amplified from sample DF-63.5, the sequence of this gene was compared with those of previously characterised *strA* genes, including five RSF1010 variants, three Tn*5393* variants and one integrative conjugation element (ICE) (Table 1). The *strA* sequence obtained from DF-63.5 was identical to those of the RSF1010 variants pASL01a, pYT3 and pAB5S9 as well as to that of

Table 1
Comparison of polymorphisms present in various *strA* loci.

Genetic name	Host organism	Source of isolation	Polymorphism and position in *strA* sequence[a]							GenBank accession no.
			1	69	80	204	467	470	593	
sul2–strA–strB gene cluster from DF-63.5	Uncultured bacterium	Ice core	T	T	T	C	A	T	A	**LC010221**
RSF1010 variants										
RSF1010	*Escherichia coli* and other Gram-negative bacteria	Various	T	T	T	C	**T**	**A**	A	**M28829**
pASL01a	*E. coli*	Clinical	T	T	T	C	A	T	A	**JQ480155**
pYT3	*Salmonella* Typhimurium	Cattle	T	T	T	C	A	T	A	**AB591424**
pAb5S9	*Aeromonas bestiarum*	River	T	T	T	C	A	T	A	**EF495198**
pLS88	*Haemophilus ducreyi* and cloning vector	Vector	T	**C**	T	**G**	A	T	A	**L23118**
Tn*5393* variants										
pEa34	*Erwinia amylovora*	Plant	T	T	T	**G**	A	T	A	**M96392**
pRAS2	*Aeromonas salmonicida* subsp. *salmonicida*	Fish	T	T	T	**G**	A	T	A	**AF262622**
pI-35	*Pseudomonas* sp.	Permafrost	T	T	T	**G**	A	T	A	Not available[b]
Integrative and conjugative elements (ICE)										
ICEVchind4	*Vibrio cholerae*	Clinical	T	T	T	C	A	T	A	**GQ463141**

[a] Nucleotide polymorphisms that were different from that of the gene cluster in DF-63.5 are shown in bold face.
[b] The pI-35 *strA* sequence was obtained from a reference [33].

ICEVchind4. Conversely, the DF-63.5 *strA* gene contained two polymorphisms that were not present in the original RSF1010 sequence: one at position 467 (T in RSF1010 to A in DF-63.5) and another at position 470 (A in RSF1010 to T in DF-63.5). Meanwhile, comparison of the DF-63.5 *strA* gene with those of the Tn*5393* variants pEa34, pRAS2 and pI-35 identified a transversion in the DF-63.5 *strA* gene at position 204 (G in Tn*5393* variants to C in DF-63.5). Lastly, comparison with the *strA* gene of pLS88, a plasmid that is widely used as a cloning vector [28], detected two polymorphisms: one at position 69 (C in pLS88 to T in DF-63.5) and another at position 204 (G in pLS88 to C in DF-63.5). Other RSF1010-derived vector plasmids, including pAY201 (GenBank accession no. **AB526842**), pYN401 (**AB531985**), pRLl383a (**AF403426**) and pCVD046 (**KM017897**), do not harbour the complete *sul2–strA–strB* sequence because their antimicrobial resistance marker genes have been exchanged for β-lactamase or chloramphenicol acetyltransferase genes.

3.3. Detection of the sul2 gene in the DF-63.5 sample

In addition to the *strA* and *strB* genes, the complete sequence of the *sul2* gene (816 bp) from sample DF-63.5 was successfully amplified. Subsequent BLAST and phylogenetic analyses demonstrated that this gene exhibited high levels of sequence identity to other *sul2* gene sequences deposited in the NCBI database; 100% (816/816 bp) identical to *sul2* in pAb5S9 and pYT3; 99.6% (813/816 bp) to *sul2* in pASL01a; and 99.5% (812/816 bp) to *sul2* in RSF1010 and pLS88 (Fig. 1). In addition, this gene exhibited similarity to the dihydropteroate synthase (DHPS) gene encoded on the chromosome of *Parvibaculum lavamentivorans* [66.1% identity; pairwise score = 188; Expect (E) Value = 6e-45]. Meanwhile, phylogenetic analyses indicated that the *P. lavamentivorans* DHPS sequence was more similar to *sul2* gene sequences than to *sul1* and *sul3* gene sequences or to the sequence of the chromosomally-encoded *E. coli* DHPS gene *folP* (Fig. 1).

3.4. Characterisation of genetic elements surrounding the sul2–strA–strB gene cluster

The *sul2–strA* gene cluster as well as the region downstream of *strB* were successfully amplified using the *sul2* forward and *strA* reverse primers and the downstream *strB* primers, respectively. Sequencing of the PCR products demonstrated that these ARGs were located within a 2764-bp *sul2–strA–strB* gene cluster. Meanwhile, BLAST and phylogenetic analyses revealed high levels of sequence similarity between this gene cluster and various sequences in GenBank (Fig. 2). Notably, the sequence of the DF-63.5 *sul2–strA–strB* gene cluster exhibited 100% (2764/2764 bp) identity to that of ICEVchind4. The sequence also showed high levels of identity to the RSF1010 (2757/2764 bp; 99.7%), pASL01a (2596/2764 bp; 93.9%), pYT3 (2711/2764 bp; 98.1%) and pAb5S9 (2733/2764 bp; 98.9%) sequences. However, despite repeated attempts, genetic elements present in RSF1010 and Tn*5393* were not detected in the DF-63.5 sample. Thus, we concluded that the genetic elements relating RSF1010 and Tn*5393* were not present in ice core sample DF-63.5.

3.5. The sul2–strA–strB gene cluster confers antimicrobial resistance to Escherichia coli DH5α

Results of the antimicrobial susceptibility testing are presented in Table 2. Compared with the untransformed *E. coli* DH5α strain, the strain transformed with the empty pTA2 vector (strain DH5α/pTA2) exhibited reduced susceptibility to ampicillin but did not exhibit any changes in susceptibility to the other antimicrobial agents tested. Conversely, the *E. coli* transformant with the pTA2 [*sul2-strA-strB*] vector (strain DH5α/pTA2[*sul2-strA-strB*]) exhibited reductions in susceptibility to dihydrostreptomycin (64 mg/L) and sulfamethoxazole (>4096 mg/L). Meanwhile, the susceptibility of this strain to kanamycin and gentamicin was similar to that of the untransformed DH5α strain.

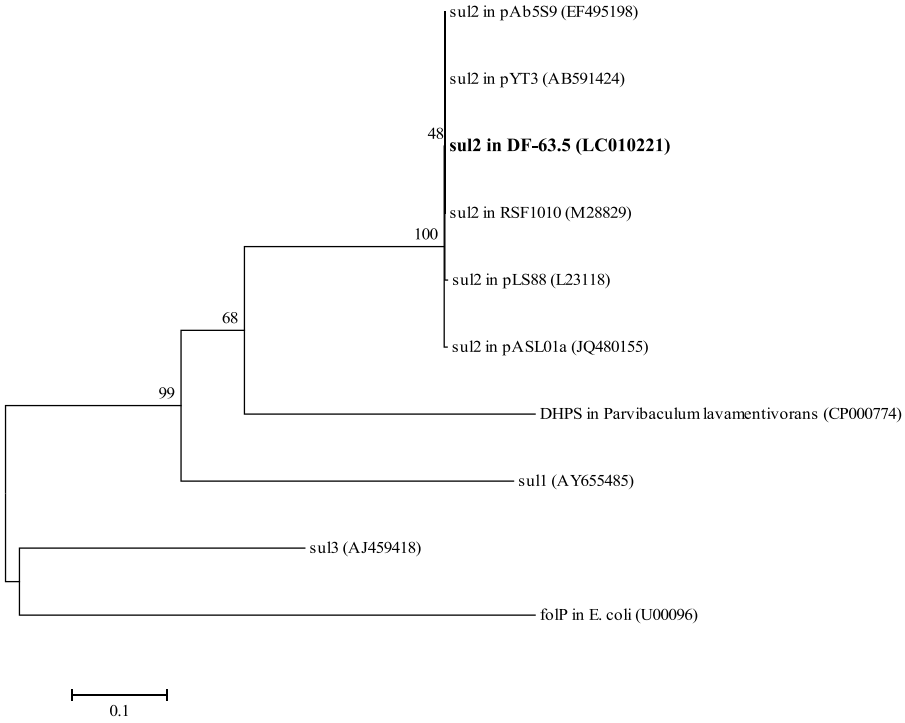
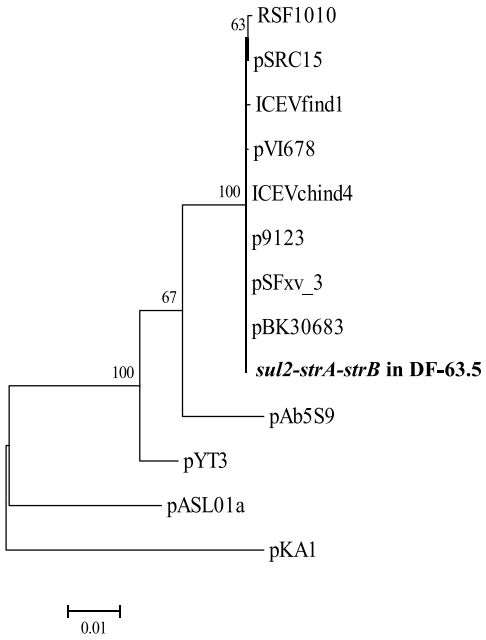


Fig. 1. Comparison of the *sul2* sequence (816 bp) from sample DF-63.5 with sequences of the *sul2*, *sul1* and *sul3* genes and the dihydropteroate synthase (DHPS) gene of *Parvibaculum lavamentivorans*. The gene sequences were obtained from the NCBI database using their respective accession numbers. The phylogenetic tree was constructed by the maximum-likelihood method with 1000 bootstrap replicates. The numbers at branch nodes denote bootstrap values. The scale bar indicates the maximum-likelihood distance.



Host	Accession No.
Escherichia coli	M28829
Salmonella Typhimurium	GQ379901
Vibrio fluvialis	KM213605
Escherichia coli	EF090911
Vibrio cholerae	GQ463141
Escherichia coli	AY360321
Shigella flexneri	CP001386
Klebsiella pneumoniae	KF954760
Uncultured bacterium	**LC010221**
Aeromonas bestium	EF495198
Salmonella Typhimurium	AB591424
Escherichia coli	JQ480155
Vibrio cholerae	AY958065

Fig. 2. Comparison of the nucleotide sequence of the *sul2–strA–strB* gene cluster (2764 bp) amplified from ice core sample DF-63.5 with the reference sequences from various hosts. All gene sequences were obtained from the NCBI database. The phylogenetic tree was constructed by the maximum-likelihood method with 1000 bootstrap replicates. The numbers at branch nodes denote bootstrap values. The scale bar indicates the maximum-likelihood distance.

Table 2
Antimicrobial susceptibility of *Escherichia coli* DH5α strains used in this study.

E. coli strain	MIC (mg/L)				
	AMP	DSM	KAN	GEN	SMX
DH5α	1	1	1	0.5	512
DH5α/pTA2[a]	>512	1	1	0.5	512
DH5α/pTA2[*sul2-strA-strB*][b]	>512	64	1	0.5	>4096

MIC, minimum inhibitory concentration; AMP, ampicillin; DSM, dihydrostreptomycin; KAN, kanamycin; GEN, gentamicin; SMX, sulfamethoxazole.
[a] *E. coli* DH5α transformed with the empty pTA2 vector.
[b] *E. coli* DH5α transformed with the pTA2[*sul2-strA-strB*] vector.

4. Discussion and conclusion

The *sul2–strA–strB* gene cluster was detected in the Antarctic ice sample DF-63.5, which was dated to 1200–1400 ybp. First, we confirmed that the gene cluster originated from DF-63.5 because the RSF1010 plasmid family, which encodes *sul2–strA–strB*, has been commonly utilised in molecular biology as a component of cloning or shuttle vectors [28]. As shown in Table 1, the polymorphisms in the nucleotide sequence of the *strA* gene amplified from DF-63.5 were different from those in the original RSF1010 sequence as well as from those present in other vectors such as pLS88. On other hand, the possibility of contamination of modern bacteria cannot be entirely ruled out because it is suggested that particles can penetrate into the pores of the ice sample [29] while the surface decontamination procedure was performed in the manner as described previously under clean conditions. In addition, the ice samples in this study were collected in 1998 or 1999. As they were kept in a freezer for the past 2 decades, the condition of ice samples may have changed from the original. However, the *sul2–strA–strB* gene cluster was not detected in other samples, including the control ice. In addition, genetic elements relating RSF1010 and Tn*5393*, which are frequently encoded together with *sul2–strA–strB* in modern bacteria, were not detected from DF-63.5. This supporting evidence suggest that the *sul2–strA–strB* gene cluster in DF-63.5 originated in the period of deposition.

The species of origin of the *strA* and *strB* genes has yet to be identified owing to the wide distribution of these genes among various bacterial species, including those of the Enterobacteriaceae family as well as *Aeromonas* and *Vibrio* spp. [26,30,31]. Currently, these genes are suspected to have originated in aminoglycoside-producing *Streptomyces* spp. as these organisms encode chromosomal aminoglycoside phosphotransferase genes such as *aphE* [*aph(3″)*-Ia], *aphD* [*aph(6)-Ia*], *sph* [*aph(6)-Ib*], *spcN* [*aph(9)-Ib*] and *aph(7″)*-Ia [32]; however, the sequences of these loci are markedly different from those of the *strA* and *strB* genes. The current results are consistent with a previous report that detected *strA* and *strB* from ancient samples [33], although those samples were permafrost soil. The existence of *strA* and *strB* in these ancient samples indicate that *strA* and *strB* pre-date the discovery of antimicrobials, especially aminoglycosides.

Because the *sul2* gene encodes resistance to sulfonamides, a class of synthetic antibiotics, the origin of this coding sequence is more controversial. It has been proposed that *sul2* also originated from micro-organisms, although the species in which this gene developed is unknown [31]. Interestingly, BLASTn analysis revealed 66.1% identity between *sul2* and the chromosomal DHPS gene of *P. lavamentivorans*. Bacteria of the genus *Parvibaculum*, which belongs to the Rhodobiaceae family of class Alphaproteobacteria, are known for their alkane-oxidising ability and can be isolated from natural environments such as seawater and deep-sea vents [34]. As there is only moderate sequence similarity between the *P. lavamentivorans* DHPS and the modern *sul2* gene, the DHPS gene is not considered the direct ancestor of *sul2*. However, this sequence similarity does suggest that the ancestral gene of *sul2* may exist on the chromosome of certain environmental bacteria in the absence of selective pressure with sulfonamides. Indeed, we propose that the *sul2* gene in DF-63.5 originated from environmental bacteria that lived during the period of deposition in the ice. This idea can be supported by the fact that present-day

environmental bacteria often harbour *sul2* as part of class 1 integron-associated genetic content [35]. In addition, Segawa et al. performed 16S rRNA gene analyses on samples from Antarctic ice cores that were dated to 2000–4000 ybp and revealed that various groups of bacteria, including uncultured environmental bacteria, were trapped in Antarctic ice [16].

Although the host of the *sul2*, *strA* and *strB* genes has yet to be identified, the evolutionary history of the genetic structure of the *sul2–strA–strB* gene cluster has been studied. Briefly, it was postulated that (i) linkage and distribution of *strA–strB* occurred originally as one genetic element within transposon Tn*5393*; (ii) Tn*5393* was inserted into the *CR2–sul2* gene cluster (GenBank accession no. **AB277723**) on an ancestral plasmid of RSF1010, thereby forming the *CR2–sul2–strA–strB* gene cluster; and (iii) transposase genes subsequently removed part of the *CR2* sequence from the gene cluster [23,25]. This hypothesis is supported by the fact that the Tn*5393*-derived transposon Tn*5393c*, which encodes a *strA–strB* gene cluster lacking *sul2*, was detected in environmental bacteria (*Pseudomonas* spp., *Acinetobacter* spp. and *Psychrobacter psychrophilus*) from Siberian permafrost sediments from 15 000–40 000 ybp [6,33]. Although microbial communities in permafrost samples may be quite different from those in ice core samples, the existence of *strA–strB* and Tn*5393* from permafrost bacteria hints at the persistence of these genetic elements in ancient, pristine samples. The result in the current study is the first detection of *sul2–strA–strB* on a single gene cluster from ancient samples. Because the DF-63.5 sample was markedly older than present-day samples but younger than the permafrost samples, these findings may indicate that genetic transposition of Tn*5393* into RSF1010 occurred within the time period between the age of the ice core and the permafrost sample.

Antimicrobial susceptibility testing indicated that *E. coli* strain DH5α/pTA2[*sul2-strA-strB*] exhibited reduced susceptibility to ampicillin, dihydrostreptomycin and sulfamethoxazole compared with the untransformed *E. coli* DH5α strain. The reduction in susceptibility to ampicillin resulted from the β-lactamase gene $bla_{\text{TEM-116}}$ on the pTA2 vector. In contrast, as the pTA2 vector does not encode any aminoglycoside resistance genes, the reduced susceptibility to dihydrostreptomycin appeared to be caused by cloning of the *strA* and *strB* genes, which confer resistance to streptomycin but not to kanamycin or gentamicin [32]. The reduced susceptibility to sulfamethoxazole also resulted from cloning of the *sul2* gene. Overall, the decreases in susceptibility of strain DH5α/pTA2[*sul2-strA-strB*] to dihydrostreptomycin and sulfamethoxazole demonstrate that the ARGs amplified from DF-63.5 are functionally identical to the *sul2*, *strA* and *strB* genes encoded by present-day bacteria.

To the best of our knowledge, this is the first report demonstrating the presence of the *sul2* gene in a pristine sample. Isolation of a genetic cluster containing the *sul2*, *strA* and *strB* genes indicates that the formation of ARG clusters can occur in the absence of anthropogenic selective pressure. Given that sulfonamides are synthetic antibiotics, these findings support the hypothesis that most ARGs with activity against synthetic antimicrobials originated in environmental bacteria. As such, the results suggest that no current class of antibiotics, including synthetic antimicrobials, can inhibit the emergence of ARB. A limitation of this study is the age of ice core samples; although we attempted to utilise deeper ice core, we could not get such samples because of their scarceness. Further studies using other ice core samples may provide more information about ARGs in the pre-antibiotic era.

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Competing interests

None declared.

Ethical approval

No specific permissions were required for the sampling site because this area is out of the Antarctic Specially Protected Area. Samples were utilised for research purpose only. This present study did not involve endangered or protected animals and plants.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2018.11.005.

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