Distribution of antibiotic resistance genes in glacier environments

Takahiro Segawa,1,2 Nozomu Takeuchi,4 Andres Rivera,5 Akinori Yamada,6 Yoshitaka Yoshimura,7 Gonzalo Barcaza,8 Kunio Shinburi,9 Hideaki Motoyama,3 Shiro Kohshima10 and Kazunari Ushida11*

1 Transdisciplinary Research Integration Center, 4-3-13 Toranomon, Minato-ku, Tokyo 105-0001, Japan.
2 Transdisciplinary Research Integration Center, and
3 Meteorology and Glaciology Group, National Institute of Polar Research, 10-3 Midori-cho, Tachikawa, Tokyo 190-8518, Japan.
4 Department of Earth Sciences, Graduate School of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan.
5 Centro de Estudios Científicos, Valdivia, Chile.
6 School and Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 2-12-1 Oookayama, Meguro, Tokyo 152-8550, Japan.
7 College of Agriculture, Tamagawa University, 6-1-1 Tamagawagakuen, Machida, Tokyo 194-8610, Japan.
8 Dirección General de Aguas, Morandé 59, Piso 8, Santiago, Chile.
9 Technical division, Institute of Low Temperature Science, Hokkaido University, Kita-19, Nishi-8, Sapporo 060-0819, Japan.
10 Wildlife Research Center of Kyoto University, 2-24 Tanaka-Sekiden-cho, Sakyu-ku, Kyoto 606-8203, Japan.
11 Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo, Sakyu-ku, Kyoto 606-8522, Japan.

Summary

Antibiotic resistance genes are biologically transmitted from microorganism to microorganism in particular micro-environments where dense microbial communities are often exposed to an intensive use of antibiotics, such as intestinal microflora, and the soil microflora of agricultural fields. However, recent studies have detected antibiotic-resistant bacteria and/or antibiotic resistance genes in the natural environment geographically isolated from such areas.

Here we sought to examine the prevalence of antibiotic resistance genes in 54 snow and ice samples collected from the Arctic, Antarctic, Central Asia, North and South America and Africa, to evaluate the level of these genes in environments supposedly not affected by anthropogenic factors. We observed a widespread distribution of antibiotic resistance genes in samples from various glaciers in Central Asia, North and South America, Greenland and Africa. In contrast, Antarctic glaciers were virtually free from these genes. Antibiotic resistance genes, of both clinical (i.e. aac(3), bla<sub>IMP</sub>) and agricultural (i.e. <i>strA</i> and <i>tetW</i>) origin, were detected. Our results show regional geographical distribution of antibiotic resistance genes, with the most plausible modes of transmission through airborne bacteria and migrating birds.

Introduction

Evaluation of the human impact on natural habitats has focused so far on developing methods to monitor the concentration of greenhouse gases, nitrogen compounds and other man-made chemical species. Although antibiotic resistance genes are thought to be mostly originated under natural environments (Martínez, 2008; Martinez, 2009; Allen et al., 2010; D’Costa et al., 2011), the rise in antibiotic use for medical and agricultural purposes is the major cause of propagation of antibiotic resistance genes, and can be considered a major anthropogenic environmental threat affecting the natural environment (Gon-Uriza et al., 2000; Chee-Sanford et al., 2001; Cabello, 2006; Hawkey, 2008). Under the right conditions, microorganisms carrying antibiotic resistance genes thrive in antibiotic-rich micro-environments such as the intestinal microflora of humans and livestock and the soil microflora of agricultural fields, and can be distributed into the surrounding environment by water and atmospheric circulation (Lighthart and Shaffer, 1995; Levy and Marshall, 2004; Gibbs et al., 2006). As such, regions adjacent to hospitals and agricultural fields often show a higher prevalence of antibiotic-resistant microorganisms than other similar environments (Andersen and Sandaa, 1994; Heuer et al., 2002; Furushita et al., 2003; Koike et al., 2007; Jakobsen et al., 2008; Malik et al., 2008). However, recent studies have detected antibiotic-resistant bacteria and/or antibiotic resistance genes in environments...
geographically isolated from these sources (Allen et al., 2008; Dib et al., 2009; Thaller et al., 2010; Ushida et al., 2010). In this context, we sought to examine the prevalence of antibiotic resistance genes in the glacier environment, an area usually distant from human industrialized activities, as a potent indicator of human impact on the global environment.

In this study, we used advanced polymerase chain reaction (PCR) techniques to show that antibiotic resistance genes were present in significant numbers in samples from various glaciers and snowfields. We analysed 54 surface snow or ice core samples from 51 glacier sites in Central Asia (China, Kyrgyzstan and Tajikistan); North America (Alaska); South America (Chile); the Arctic (Greenland); the Himalayas (Nepal and Bhutan); Africa (Uganda) and Antarctica to evaluate the level of antibiotic resistance genes in environments supposedly not affected by anthropogenic factors (Fig. 1, Table S1). We included snow samples from the Tateyama mountain range in Japan, which is adjacent to a highly populated and industrialized area, and ice core samples taken by the Japanese Arctic Glaciological Expedition from Austdømen on the Austfonna ice cap (Svalbard Islands), which dated from 1900 to 1991. We hypothesize that this type of extensive surveillance may reveal the global circulation of antibiotic resistance genes.

Results and discussion

In this survey, we analysed 54 surface snow and ice core samples from 19 geographically dispersed glaciers located outside of Antarctica and eight sites within Antarctica. The detection pattern of antibiotic resistance genes largely reflected the history of antibiotic use and the propagation of antibiotic resistance genes. The antibiotic resistance genes or antibiotic-resistant bacteria in a glacier environment might therefore be used as an indicator of anthropological impact on the natural environment.

We chose antibiotic resistance genes that had previously been detected in the natural aquatic environment. Primers and Taqman probes for 94 antibiotic resistance genes and for the bacterial 16S rRNA gene were prepared as described previously (Ushida et al., 2010).

We detected antibiotic resistance genes in 54 environmental samples (Table S1). Rigorous protocols were followed at the collection sites and in the laboratory to avoid sample contamination. For unknown reasons, however, the tetC gene was detected in all samples, including negative controls. The tetC gene was therefore excluded from analysis due to the possibility of reagent contamination.

Among the 93 antibiotic resistance genes analysed, 45 were detected in 51 surface snow/ice samples and three in ice core samples (Fig. 2). The most frequently encountered genes were aac(3) (24/54 sites), strA #2 (21/54), blamb #2 (20/54), and strA #1 (18/54). The Chilean and Antarctic snow samples were relatively free of resistance genes such as aac(3) and strA, while the Greenland glacier samples were free of blamb.

Elevated numbers of antibiotic resistance genes (≥ 10 genes) were detected in several samples from the 51 sampling sites (50 surface snow/ice and one ice core sampling sites), from 19 glacier and eight Antarctic sites. The Central Asian and Himalayan glaciers had the highest level of gene detection, followed by the African glaciers. Fewer genes were detected in Greenlandic and Alaskan glaciers, while the Chilean and the Antarctic samples...
were virtually free from antibiotic resistance genes. The number of detected resistance genes per glacier site was generally higher in the lower region of the glacier than in the upper region of the glacier, with the exception of Greenland-QA3, which is located in the upper region. Values for Tateyama samples obtained in March (TA-Mar., two resistance genes) were lower than those obtained during the melting season (TA-Apr., eight genes, TA-Aug., six genes). It is noteworthy that, outside of the Antarctic ice sheet samples, the Tateyama sample (TA-Mar.) showed the lowest delta Ct value (2.7), followed by the Iver glacier sample (3.6), and the relatively high Ct values for the 16S rRNA gene in these two samples suggests that they contained relatively few bacteria. The average Ct values for the 16S rRNA genes in the samples from the upper regions and from Tateyama’s snowfall season were relatively high: Antarctica (24.6 ± 1.9), the Arctic (19.3 ± 2.7), Chile (19.4 ± 0.9), Greenland (12.2), Tateyama (23.3), the Himalayas (24.2), and Alaska (18.8).

The values from the lower regions and Tateyama’s melting seasons were relatively low: Chile (13.2 ± 3.1), Greenland (13.0 ± 4.1), Tateyama (13.0 ± 1.4), Nepali and Bhutanese Himalayas (10.0 ± 1.3), China (10.8 ± 1.7), Kyrgyzstan (8.4 ± 1.1), Alaska (10.7 ± 1.4), and Africa (7.2 ± 0.8). Antarctic snow samples showed much higher Ct values for the 16S rRNA gene despite the large volume of sample analysed (about 2 l per sample).

We observed a widespread presence of antibiotic resistance genes in all regions studied. Indeed, 45 out of 54 samples showed the presence of antibiotic resistance genes. Higher detection was observed in the Central Asian and Himalayan glaciers. Interestingly, most of the Chilean glacier and Antarctic samples were free from antibiotic resistance genes. The Ct values for the 16S rRNA gene in Central Asian and Himalayan samples demonstrate the higher bacterial density compared with other sampling sites. In particular, the Antarctic and Chilean glacier samples had a low bacterial density, suggesting...
the relative isolation of these areas from the global transmission of airborne bacteria and/or harsher conditions for autochthonous growth of bacteria.

Ct values for the 16S rRNA gene and all antibiotic resistance genes did not show a strong correlation in either fresh snow or glacial samples ($r = 0.166, P < 0.05$ for glacial samples; $r = 0.103, P > 0.05$ for fresh snow) (Fig. 3A). The correlations between Ct values for the 16S rRNA gene and $aac(3)$, the most frequently encountered antibiotic resistance gene, were also very low ($r = 0.274, P > 0.05$) (Fig. 3B).

This study concentrated on resistance genes rather than resistant bacteria, and it is difficult to define the source and origin of these genes. However, the low correlation between Ct values for the 16S rRNA gene and antibiotics resistance genes (Fig. 3) suggests that, when detected on glaciers, the abundance of antibiotic resistance genes is not affected by the site of sampling. A NMDS (non-metric multidimensional scaling) plot of antibiotic resistance genes and 16S rRNA genes shows the regional geographical distribution of resistance genes (Fig. 4). The relative paucity of antibiotic resistance genes

![Fig. 3. Correlation between Ct values for antibiotic resistance genes and those for 16S rRNA genes. A. Correlation between Ct values of all detected antibiotic resistance genes and the 16S rRNA gene. B. Correlation between Ct values for $aac(3)$ and those for the 16S rRNA gene in samples where both are detected.](image)

![Fig. 4. Non-metric multidimensional scaling (NMDS) plot of antibiotic resistance genes and 16S rRNA gene from snow and ice samples. A reciprocal of the matrix of the Ct values for antibiotic resistance genes and those for 16S rRNA genes was used to perform NMDS based on the Bray-Curtis similarity measure using PAST 2.15 software (Hammer et al., 2001). The sampling regions are differentiated by colour. Samples marked ‘S’ are fresh snow samples.](image)
on Antarctic and Chilean glaciers suggests the contribution of airborne bacteria to the presence of antibiotic resistance genes. Airborne antibiotic-resistant bacteria may be limited to the Northern Hemisphere and adjacent lower latitude zone of the Southern Hemisphere. This hypothesis is supported by previous observations of global aerosol and dust transmission, in which the sources of aerosol and pollution are concentrated in the Northern Hemisphere, and their export pathway from the Northern Hemisphere does not affect the Southern Hemisphere further south than 45°S (Stohl et al., 2002).

The detection of resistance genes in Greenland may suggest some other method of transmission of resistant bacteria, because major air circulation towards Greenland originates from the North Pole and katabatic winds from the ice sheet (Hobbs, 1945; Klein and Heinemann, 2002), where no source of resistant bacteria is known. The involvement of animals in the distribution of resistant bacteria is highly plausible. Certain drug resistant bacteria were detected in the cloaca swab or feces of wild birds in this area (Sjölund et al., 2008) and a significant number of intestinal bacteria were detected in ice samples (Sheridan et al., 2003). Moreover, previous reports demonstrated that Greenland White-fronted Geese (Anser albirotras flavirostris) migrate between Britain and areas adjacent to the Greenland sampling sites (Wernham et al., 2002), and Northern Wheatear (Oenanthe oenanthe) migrate from the eastern Canadian Arctic across Greenland and Eurasia and into Alaska (Bairlein et al., 2012). However, it is difficult to make a proper quantitative comparison of the potential role of winds and wild birds in the dissemination of antibiotic-resistant bacteria with our current data.

Of the resistance genes that were the more widely detected among study sites, some are definitely of clinical origin and others of agriculture origin. For example, aminoglycoside acetyl transferases (AACs) such as aac(3) and aacC, and aminoglycoside O-adényl transferases (AADs) such as aadA, can be defined as being of clinical origin (Boehr et al., 2005). Aminoglycoside antibiotics were developed as early as 1950, and the rapid emergence of resistant bacteria induced the introduction of fluoroquinolons in 1970s. In Asian countries located to the west of the Central Asian glacier sampling sites, such as Iran, nosocomial bacteria have shown a very high resistance to gentamicin and tobramycin (Soroush et al., 2010). If other western, central and southern Asian countries show similar tendencies to that of Iran, frequent detection of these types of resistance genes in Central Asian and Himalayan glaciers can be explained. This may be true for India and China, where higher prevalence of this type of resistance has been reported (Biedenbach et al., 2009).

The gene strA has been detected with a high prevalence in many resistant bacteria (Sunde and Norström, 2005). Streptomycin is of particular importance in agriculture, and intensive horticulture and animal agriculture might have contributed to the global dissemination of this type of resistance over 50 years. Indeed, the presence of the strA gene in plant pathogens has previously been attributed to the widespread introduction of streptomycin to agricultural crops (Sundin and Bender, 1996).

The metallo-β-lactamase encoding gene (blaIMP) was detected in Asian and African glaciers (2002–2008) and Japanese surface snow samples (1998). Our failure to detect blaIMP in Greenlandic and Alaskan glacier samples (2007 and 2001 respectively) was unexpected. Detection of blalM0 indicates the distribution of clinical bacteria resistant to carbapenem antibiotics. This type of resistance was reported for the first time in 1995 (Arakawa et al., 1995). Since then, nosocomial infection by these bacteria has been reported in Europe, North America and Japan, mainly associated with the Pseudomonads and Enterobacteriaceae (Osano et al., 1994; Minami et al., 1996; Riccio et al., 2000). These types of resistant bacteria have become endemic in many countries, though more recently the prevalence in developed countries has decreased (Fritsche et al., 2005). Contrary to this decline in prevalence of blalM0-carrying bacteria in developed countries, the prevalence is still reportedly high in India (Varaiya et al., 2008). The detection pattern of blalM0 in this study may reflect this tendency of higher prevalence of blalM0-carrying bacteria in countries with less severe control of antibiotic use (Smith and Coast, 2002; Zhang et al., 2006). This point needs further elucidation. blaIMP is located on large plasmids (Arakawa et al., 1995; Ito et al., 1995) which enables the transfer of resistance to the various Gram-negative rods. In this context, the dissemination of resistant P. aeruginosa could cause the onsite emergence of blalM0 harbouring bacteria, particularly with cold tolerant pseudomonads such as P. putida or P. fluorescens. Environmental transmission of this type of resistance should be monitored in the future.

Sporadic detection of tetW, including on Chilean glaciers, is interesting, because this ribosomal protection protein has been reported in livestock such as sheep and pig, and it has been widely detected in a range of livestock (Scott et al., 2000; Patterson et al., 2007) and in environments affected by animal agriculture (Koike et al., 2007). Emergence of tetracycline resistance, ribosomal protection proteins, was first reported in Streptococcus agalactiae (Burdett, 1980) and is now frequently detected in nosocomial multidrug-resistant pathogens (Alekshun and Levy, 2007; Mak et al., 2009).

In contrast to our earlier findings that Antarctic surface snow samples are virtually free from resistance genes (Ushida et al., 2010), here we detected the mefA/E gene in one Antarctic snow sample from Patriot Hill, a site at which researchers have been actively engaged in scien-
tific expeditions since 1986 (Mellor, 1993). Although this antibiotic resistance gene is thought to have originated in surface bacteria on the Antarctic ice sheet, this was the only Antarctic sampling site to have had regular human activity in the last 25 years, and snow samples at all other Antarctic sites were collected in areas which had not been disturbed by previous or concurrent activity; therefore, we speculate that human activity may be responsible for the introduction of bacteria harbouring the \textit{mefA/E} gene. As previously shown for the Antarctic research base, the direct human impact on transmission of antibiotic-resistant bacteria cannot be completely excluded. The international ‘Alien Program’ now catalogues materials introduced into Antarctica by expeditions and research programmes (Hughes et al., 2011). We propose here that antibiotic-resistant bacteria may be classified as one such ’material’ introduced by human activity.

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References


non-native species transfer to the Antarctic region with fresh produce. *Bioll Conserv* **144**: 1682–1689.


**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Table. S1.** Snow and ice sample list.
Fig. S1. Melting device for ice core analysis. Upper panel: Top view of the melting head. Lower panel: Cross-section of the melting head. The ice core samples were melted using a device developed by our group that enabled us to obtain water only from the inner portion of the cores; the 20 mm thick outer layer was remained intact. For the Austdomen ice core samples taken in 1999 by the Japanese Arctic Glaciological Expedition (JAGE) (Motoyama et al., 2001), dating was calculated by the delta 18O described by Watanabe and colleagues (2001) and Isaksson and colleagues (2003). In this experiment, the following three cores were studied: 6.34–6.78 m in depth (dated at 1991 year), 12.02–12.55 m in depth (dated at 1980 year) and 50.78–51.28 m in depth (dated at 1900 year). The ice cores analysed in this study consisted of superimposed ice (Motoyama et al., 2001). Complete separation of the inner and outer cores is required to avoid contamination by bacteria that can adhere to the cores during drilling and storage. To determine whether any contamination had occurred during handling or due to ice core cracks, a solution of ~2 ng µl⁻¹ bacterial plasmid vector was applied to the surface of the core samples. The vector contaminant was not amplified from the inner part of ice core samples, but amplified only with the outer layer of ice core samples by 45 cycles of PCR with vector-specific primers. Therefore, we proceeded with further DNA analyses on the inner portion of ice core samples.