Alteration of a Human Intestinal Microbiota under Extreme Life Environment in the Antarctica

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The human intestinal microbiota (HIM) settles from birth and continues to change phenotype by some factors (e.g., host’s diet) throughout life. However, the effect of extreme life environment on human HIM composition is not well known. To understand HIM fluctuation under extreme life environment in humans, fecal samples were collected from six Japanese men on a long Antarctic expedition. They explored Antarctica for 3 months and collected their fecal samples at once-monthly intervals. Using terminal restriction fragment length polymorphism (T-RFLP) and real time polymerase chain reaction (PCR) analysis, the composition of HIM in six subjects was investigated. Three subjects presented restoration of HIM after the expedition compared versus before and during the expedition. Two thirds samples collected during the expedition belonged to the same cluster in dendrogram. However, all through the expedition, T-RFLP patterns showed interindividual variability. Especially, Bifidobacterium spp. showed a tendency to decrease during and restore after the expedition. A reduction of Bifidobacterium spp. was observed in five subjects the first 1 month of the expedition. Bacteroides thetaiotaomicron, which is thought to proliferate during emotional stress, significantly decreased in one subject, indicating that other factors in addition to emotional stress may affect the composition of HIM in this study. These findings could be helpful to understand the effect of extreme life environment on HIM.

Key words Antarctic expedition; human intestinal microbiota; Bifidobacterium; terminal restriction fragment length polymorphism (T-RFLP)

A human intestinal microbiota (HIM) is essential to the health of the host and plays important roles in nutrient absorption, development of the mucosal immune system, resistance to colonization by pathogens and metabolism of xenobiotics.1–4) Humans have harbored a vast and complex society of bacteria from birth and interacted with the bacterial complex.5) The HIM includes native species that permanently colonize the tract and temporary species that transit through the tract.1) Moreover, the establishment and maintenance of the HIM is continuously influenced by several factors such as diets, drugs (e.g., antibiotics), and health status.5–10) Even emotional stress, fatigue, and extreme conditions were reported as factors such as body temperature, stress, and diets.

However, it is difficult to precisely control some possible factors such as a body temperature, stress, and diets for evaluation the effect on the fecal microbiota in human because of ethic problem and safety. As a result, some animal studies have been conducted instead of clinical studies with human.11–14) Six members whose mission is to investigate and collect meteorites explored the Sør Rondane Mountains in Antarctica for three months. They are exposed to extremely low temperature and strong ultraviolet radiation during the expedition and maybe suffer stress by carrying out their missions, possibly followed by alteration of HIM. In this study, we tried to analyze the fecal microbiota of the members of Antarctic expedition by terminal restriction fragment length polymorphism (T-RFLP) and real time polymerase chain reaction (PCR) for evaluating alteration of the microbiota induced by an expedition.

MATERIALS AND METHODS

Fecal Specimens Six healthy explorer (all Japanese men) between 37 and 55 years of age (average = 43 years old) participated in the study. The first fecal samples were collected before the departure to the Antarctica from Japan. The second, third and forth samples were collected once a month after beginning exploration. The fifth samples were collected after returning back to Japan. The study was approved by the Ethical Committee of Japan Aerospace Exploration Agency (JAXA, Tsukuba, Japan). Written informed consent was taken by the all participants in this study.

DNA Isolation from Fecal Samples About 40 to 100 mg of fresh fecal samples were suspended in 500 mL of TE (10 mM Tris–HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA)) and centrifuged at 20800 × g for 5 min. The supernatant was thrown away. This washing step was repeated 3 times. After 600 µL of TE, 600 µL of Tris-saturated phenol, 100 µL of 10% sodium dodecyl sulfate, and 0.3 g of glass beads (diameter, 0.15–0.21 mm) were added, the samples were treated at 7000rpm for 20s on a MagNA Lyser (Roche, Penzberg, Germany). And then, the samples were incubated at 70°C for 10 min. This crushing step was repeated 2 times. Six hundred microliter of upper layer was transferred in to a 1.5 mL tube, followed by addition of 600 µL of glacial isopropanol and 60 µL of 3 M sodium acetate. After shaking gently, the solutions were centrifuged at 20800 × g for 5 min and then

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the supernatant was thrown away by decantation. The DNA pellet was washed with glacial 70% ethanol and dried on a centrifuge evaporator (Eyela, Tokyo, Japan) for 15 min. Then, the pellet was dissolved in 200 µL of TE. Next, the DNA preparation was purified by using the high pure PCR template preparation kit (Roche) and quantified with a Nanodrop 2000c (Thermo Scientific, Wilmington, DE, U.S.A.).

**PCR Amplification and T-RFLP Analysis** The amplification of the fecal 16S ribosomal DNA (rDNA), restriction enzyme digestion, size-fractionation of terminal restriction fragments (T-RFs) and the T-RFLP analysis were carried out described previously.\(^21,22\) PCR was performed using the total fecal DNA and the primers of 5' FAM-labeled 516f (5'-TGC CAG CAG CCG CGG TA-3'; *Escherichia coli* positions 516 to 532) and 1510r (5'-GGTTACCTTGGTACGACTT-3'; *E. coli* positions 1510 to 1492). The resulting 16S rDNA amplicons were treated with 2 U of *Bsl*I (New England Biolabs, Ipswich, MA, U.S.A.) for 1 h and the digestives were fractionated using an automated sequence analyzer, ABI PRISM 3130xl DNA Sequencer (Applied Biosystems, Carlsbad, CA, U.S.A.) and GeneMapper (Applied Biosystems).

**Assignment of T-RFs Detected** The major T-RFs were identified by computer simulation, which was performed using a T-RFLP analysis program, (MiCA3 by Shyu et al.\(^23\); http://mica.ibest.uidaho.edu/), a phylogenetic assignment database for T-RFLP analysis of human colonic microbiota (PAD-HCM)\(^24\) and Microbiota Profiler (InfoCom Co., Tokyo, Japan). Cluster analysis was performed using Microbiota Profiler (InfoCom Co.) based on nineteen operational taxonomic units (OTUs) distributed from the T-RFs by the method of Nagashima \(^{22}\). The distances were calculated to determine any similarity among the samples and were represented graphically by constructing a dendrogram.

**Real-Time PCR Assays** Relative levels of *Bifidobacterium* and *Bacteroides* (*B. thetaiotaomicron*) to total fecal bacteria were analyzed using real-time PCR with SYBR Premix Ex Taq II (TaKaRa Bio, Shiga, Japan) and Thermal Cycler Dice Real Time System (TaKaRa Bio). The fold value in target bacteria, normalized to 16S rRNA gene of total bacteria and relative to the levels of samples collected before the expedition, was calculated for each sample using 2^{ΔΔCt} method.\(^{25}\) The primer sets used are shown in Table 1. Amplifications were

| Target                | Primer       | Sequence (5’ to 3’)                  | Reference |
|-----------------------|--------------|--------------------------------------|-----------|
| *Bifidobacterium* spp.| g-Bifid-F    | CTCCTGGAAACCGGGTG                         | 35)       |
|                       | g-Bifid-R    | GGTGTCTTCCCGATCTACA                      |           |
| *B. thetaiotaomicron* | *B. theta*F  | AACAGTTGAAGCTCGGGG                      | 36, 37)   |
|                       | *B. theta*R  | AGGCCTCAACCCGATCAA                      |           |
| Total bacteria        | Forward      | TCCTACGGGAGGCACGAGT                      | 38)       |
|                       | Reverse      | GGACTACGAGGGATATCTACTCTTT                |           |

**Fig. 1.** Dendrogram of the Fecal Bacterial Structure in Members of Antarctic Expedition

The T-RFs generated from fecal DNAs of 6 individuals were distributed to twenty-nine OTUs based on the method of Nagashima \(^{22}\) And then, the T-RFLP patterns were analyzed by Euclidean distance between correlation and coefficients and the UPGMA algorithm. A–F, subjects; 1, before expedition; 2, during expedition (after 1 month); 3, during expedition (after 2 months); 4, during expedition (after 3 months); 5, after expedition.
performed using the following temperature profiles: one cycle at 95°C for 30 s, 45 cycles of denaturation at 95°C for 5 s, annealing at 55°C (Bifidobacterium spp. and total bacteria) and 60°C (B. thetaiotaomicron) for 30 s, and extension at 72°C for 60 s. Fecal DNA samples were diluted to 10 ng/µL and the real time PCR reaction mixture was composed of 20-fold diluted template DNA.

**Statistical Analysis** The dendrogram was established by Euclidean distance between correlation and coefficients and the unweighted pair-group methods with arithmetic means (UPGMA) algorithm. The T-RFs were quantified as the percentage of individual T-RF peak area per total T-RF peak areas, and this was expressed as the percentage of the area under the peak curve (% AUC). The nineteen OTUs, aforementioned, were used in principal component analysis to compare and group T-RFLP patterns. The principle component analysis was performed using PASW Statistics 18 (IBM, Armonk, NY, U.S.A).

**RESULTS**

**T-RFLP Analysis and Clustering of Fecal Microbiota in Members of Antarctic Expedition** Shown in Fig. 1, almost samples collected during expedition were included in cluster V. 61.1% of all samples collected during the expedition belong to cluster V. 84.6% of sample included in cluster V were the samples collected during the expedition. Shown in clusters I and II, three subjects, A, C and E, showed restoration to their original states. Subject C particularly showed typical restoration that samples collected before and after the expedition were included in same cluster and samples collected during the expedition belong to same cluster. Subject D, however, did not show the restoration because sample collected after the expedition was included in the same cluster with samples during the expedition. Subject B showed dramatic change among clusters for the entire period. In dendrogram, subject F showed little change during and after expedition. It means that the pattern of microbiota was not restored by returning to common life. Principal components analysis also showed same patterns with cluster analysis (Fig. 2).

**Characteristics of OTU to Each Clusters and Individuals** The proportion of OTU 124 that was presumed to correspond to bifidobacteria decreased in the subjects A, C and E and the proportion was restored after the expedition (Fig. 3). Subject B also showed decrease in the proportion of OUT 124, but dramatic increase was observed in the sample of B4. The mean proportion of OTU 955 was 7.7% before the expedition, but after a month, the proportion was increased to 24.1% significantly (t-test, p<0.001). OTU 955 was presumed to correspond to Clostridium subcluster XIVa including Ruminococcus obeum, Clostridium bolteae, Blautia wexlerae, and Clostridium asparagiforme (Table 2). The proportion of OTU469 that was presumed to correspond to Bacteroides was reduced to ground level during the expedition and restored to original level in subject A. The proportion of OTU650 that was presumed to correspond to Clostridium cluster XVIII showed a tendency to increase during and after the expedition. The proportion of OTU749 that was presumed to correspond to Clostridium IV decreased during the expedition in subject A and D. The proportion of OTU940 that was presumed to correspond to Clostridium subcluster XIVa and Enterobacteriales dramatically decreased during and after the expedition in subject F.

**Real Time PCR Assays** With specific primers, relative change in proportion of bifidobacteria and B. thetaiotaomicron was evaluated with real time PCR analysis (Fig. 4). Almost same pattern with T-RFLP analysis were observed. Especially, in subjects A, C and E, the relative proportion decreased during the expedition and was restored after the expedition in bifidobacteria. In B. thetaiotaomicron, subject A showed a decrease during the expedition in the relative proportion and restoration after returning to Japan.

**DISCUSSION**

In this study, we analyzed fecal microbiota of Antarctic
expeditioners by T-RFLP to make it clear whether long-term exposure to the extreme environment affects HIM. T-RFLP is a rapid and reproducible tool that allows an easy assessment of the diversity of complex bacterial communities culture-independently. For T-RFLP analysis, 516f-BstI combination which is considered to be useful for fully assigning bacterial species to the T-RFs and suitable for analysis of some species of Bifidobacterium was selected.

Most of the samples collected during the expedition were included in cluster V. There are some possible factors affected the composition of fecal bacteria during the expedition. Dietary similarity is one of the possible factors.

Next, cold stress may be another factor of the conversion because of awfully low temperature of Antarctic area. Acute cold stress may induce systemic biochemical and physiological changes which related with intestinal bacteria. The exposure to cold stress altered the urinary excretion level of some metabolites which are believed to be produced by the gut microbial community. Human physiological responses to cold exposure are systemic and extensive. The pathway how cold stress affects the composition of fecal bacteria has not fully elucidated. However, some factors can be raised. Lowering colonic temperature can affect the composition. In animal experiments the cold-stressed mice had lower colonic temperature compared with control groups. It can alter the environment for growth of certain bacteria and then influence the composition. And, the above-mentioned changes of metabolic patterns by cold stress affect the composition of human intestinal bacteria. Furthermore, a change of food intake which could affect the gastrointestinal microbiota by altering and/or
| OTU  | Predicted bacteria from T-RFLP                                                                 |
|------|-----------------------------------------------------------------------------------------------|
| 106  | Prevotella ruminicola                                                                        |
| 110  | Selenomonas ruminantium                                                                      |
| 110  | Selenomonas sputigena                                                                        |
| 124  | Bifidobacterium adolescentis                                                                  |
| 168  | ND                                                                                           |
| 332  | Streptococcus bovis                                                                          |
| 338  | Clostridium bartlettii                                                                       |
| 366  | Prevotella bryantii                                                                          |
| 469  | Bacteroides coprophilus                                                                      |
| 494  | Eubacterium cylindroides                                                                     |
| 505  | Clostridium baratii                                                                          |
| 520  | Enterococcus faecalis                                                                        |
| 650  | Holdemania filiformis                                                                        |
| 657  | Lactobacillus salivarisis                                                                     |
| 749  | Clostridium saccharogum                                                                        |
| 754  | Clostridium perfringens                                                                       |
| 770  | ND                                                                                           |
| 853  | ND                                                                                           |
| 919  | Lachnospira pectinoschizaa                                                                     |
| 940  | Clostridium litorale                                                                         |
| 955  | Ruminococcus obeum                                                                           |
| 968  | Clostridium felsineum                                                                        |
| 990  | ND                                                                                           |
favoring the multiplication of certain anaerobic species is one of the results of cold stress. Cold pain significantly delayed gastric emptying and biphasic alteration in both gastric secretion and pancreatic trypsin output, followed by losing appetite. Such a change in food intake could alter intestinal environment (e.g., pH, oxidation-reduction potential etc.) and metabolic patterns, finally induce dramatic perturbation of HIM. All through the expedition, T-RFLP patterns showed interindividual variability. The case of a treatment of ciprofloxacin, broad-spectrum antibiotic, and controlled diet can be the good examples. When ciprofloxacin was treated, fecal bacterial communities of three subjects were influenced abundantly, however, the abundance and pattern varied among individual. Likewise, because initial communities of intestinal bacteria of each Antarctic expeditioner are variable, their responses to the above-mentioned factors may vary.

When evaluating the change of fecal microbiota induced by the expedition, controlled factors (e.g., diet) make clear the response to the certain factor. However, it was not investigated in this study. Controlled life patterns (e.g., diet) during the expedition are likely to be a stress for the members. Of course, not all stressors are equally influential on all individuals, and the members cope with these stressors in the different way. Moreover, since they already had a mission which they undertook during the expedition, it is hard to restrict the environments for other researches.

A certain of psychological stress induced by extreme environments (e.g., cold exposure and ultraviolet), physical work, and circadian rhythm could be expected during the expedition. It has been reported that exposure to such stressors significantly change microbial populations in intestine. Holdeman et al. showed that emotional stress affected human intestinal bacteria, especially the population of *B. thetaiotaomicron* and *Blautia producta*. In this study, the change of *Blautia producta* was not evaluated because OTU505 was presumed to contain the species did not show remarkable change and the culture-independent method for detection of the species was not established yet. For evaluating the change of *B. thetaiotaomicron*, we analyzed *B. thetaiotaomicron* by real time PCR with specific primers because the proportion of OTU469 was presumed to correspond to Bacteroides did not detected during the expedition and restored by returning to the common life in subject A. Actually, *B. thetaiotaomicron* was not significantly changed in the experiment with graduate students having an upcoming exam. It assumed that in

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**Fig. 4. Changes in Relative Proportions of *Bifidobacterium* spp. and *Bacteroides thetaiotaomicron* by Antarctic Expedition**

(A) Changes of *Bifidobacterium* spp. in subjects A, B, C, E, and F. (B) Change of *Bifidobacterium* spp. in subjects D. (C) Changes of *Bacteroides thetaiotaomicron* in subject A and E. Each set of experiments was repeated at least three times. ND, not detected or below detection limit (19 copies per reaction). Values are expressed as mean±S.D.
addition to emotional stress, other factor may affect the composition of human intestinal bacteria. Liźko et al.\textsuperscript{14} reported the decrease in counts of bifidobacteria and Lactobacilli due to nervous-emotional stress and Logan et al.\textsuperscript{13} showed that patient with chronic fatigue syndrome had marked alterations in microbiota, including lowered levels of bifidobacteria. The reports are identical to the present result. Same patterns of decreased bifidobacteria during the expedition were detected in not only subject A, but subjects B, C and E. However, dramatic and slight increase of OTU124 after three months of the expedition observed in subjects B and F, respectively. For confirming the alterations of *Bifidobacterium* spp., real-time PCR was conducted with the same fecal DNA samples. Then, almost same patterns with T-RFLP were observed in relative values to total bacteria, indicating that the proportion of bifidobacteria decreased during the expedition in subjects A, B, C and E. Because absolute quantities of bifidobacteria were so small, in subject D, inflated changes were observed (Fig. 4B).

Also, in subject E, the population of OTU657 corresponding to lactic acid bacteria (LAC) was decreased to ground level during the expedition and slightly recovered after expedition. However, subjects B and F showed increased population of LAC during the expedition. Different responses to stress and adaption to the extreme surroundings can be reasons of various patterns.

In this study, the approaches with culture-independent method, T-RFLP, was used for analyzing the alteration of HIM induced by long time exposure to extreme environment during Antarctic expedition. And, a change at the species level was concretely analyzed with real time PCR. However, an entire picture of the change was not revealed yet.

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