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Review:

Bacterial Monitoring in the International Space Station – "Kibo"

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Since 2009, we have been continuously performing bacterial monitoring in Kibo, the Japanese Experiment Module of the International Space Station (ISS), in cooperation with the Japan Aerospace Exploration Agency (JAXA). The objective of this research is to monitor microbes and analyze their dynamics in Kibo from environmental microbiological viewpoints. In this review, we summarize our research related to bacterial monitoring in Kibo.

Keywords: bacterial monitoring, culture independent approach, international space station

1. Introduction

Microbes are present even in space habitats, and humans and microbes have a close relationship. Research on the relationship between humans and microbes in space habitation environments is critical for success in long-duration missions to reduce potential hazards for the crew and the infrastructure. The human-microbial relationship may change in the space habitation; for example, some bacterial virulence increases during space flight [1], and immune dysfunction associated with space flight occurs [2]. Therefore, we have to understand microbial dynamics in confined environments in space.

Since 2009, we have been continuously performing bacterial monitoring in Kibo, the Japanese Experiment Module of the ISS, in cooperation with JAXA (experiment title: "Microbe"). The objective of this experiment is monitoring of microbes and their dynamics in Kibo from environmental microbiological viewpoints. We have completed experimental phases Microbe-I, II, and III (**Table 1**), and Microbe-IV has just started. In this review, we summarize our research related to bacterial monitoring in Kibo.

2. On-Board Sampling

2.1. Sampling

Sampling points in Kibo were selected as follows: (1) frequently touched surface: handrail, laptop palm rest; (2) air of Kibo: diffuser and intake of the air circulation system; (3) less frequently touched surface: outer and inner surfaces of the incubator [3]. Samples were collected four times (**Table 1**). Astronauts stationed on the ISS performed all sampling. Samples were stored in the Minus Eighty-degree Laboratory Freezer for ISS (MELFI; freezer with storage at -95°C) after sampling and were transported to our laboratory at -80°C.

2.2. Sampling Devices

The sampling protocol should be simple because sampling is performed by astronauts who are not specialists in microbiology. Therefore, the procedures for sampling were optimized before use in Kibo. In the Microbe research, we have used two kinds of sampling devices, swabs and adhesive sheets.

2.2.1. Swab

The swab method is widely used in pharmaceutical and food industries for bacterial sampling. Initially, we determined a swab procedure with higher operability and lower variation among individual samples than current methods [4]. We evaluated six swabbing protocols (A–E and the conventional method used in Japan; **Fig. 1**) as the following procedure; (i) spreading known amounts of bacterial cells on the painted stainless steel plate; (ii) swabbing the plate to recover bacterial cells; (iii) enumeration of recovered bacterial cells; (iv) calculation of the recovery rate by comparing recovered bacterial cells with the initial bacterial number. Swabbing protocol "C" yielded a high bacterial recovery rate ($69\% \pm 11\%$, $n = 10$; **Fig. 1**) and low individual differences ($p>0.01$, Student's t-test). Therefore, we adopted this technique, in which the operative swabs one-way horizontally and then vertically on the surface at 1 cm intervals without changing the contact face of the swab (**Fig. 1C**).

2.2.2. Adhesive Sheet

For sampling microbial cells, we also developed an adhesive sheet [5]. This sheet has high operability and needs no water for sampling. Because of the retirement of the Space Shuttle, the availability of return vehicles from the ISS is limited. Because the adhesive sheet is thin, it requires less storage space and is easier to transport and store than swabs. We improved this sheet for microbial monitoring in the space habitat (**Fig. 2**) [6]. The bacterial recovery rate using adhesive sheets was estimated as 78% $\pm 12\%$ ($n = 5$). The rate determined with adhesive sheets

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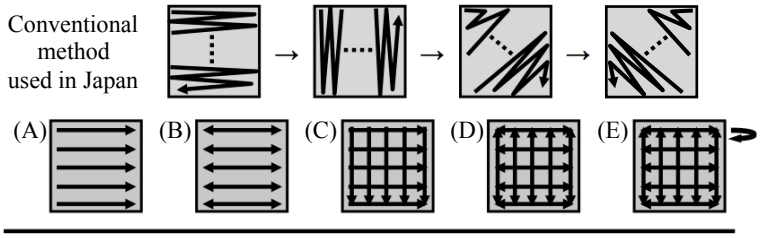
Table 1. Launch, sampling and return date.

	Microbe-I	Microbe-II		Microbe-III
Launch (Vehicle)	29 Aug. 2009 (Discovery)	15 May 2010 (Atlantis)	22 Jan. 2011 (HTV2)	21 Jul. 2012 (HTV3)
Sampling	5 Sep. 2009	29 Oct. 2010	27 Feb. 2011	16 Oct. 2012
Astronaut	Frank de Winne	Shanonn Walker	Scott Kelly	Akihiko Hoshide
Return (Vehicle)	13 Sep. 2009 (Discovery)	14 May 2011 (Endeavor)	9 Mar. 2011 (Discovery)	28 Oct. 2012 (Dragon)



	Initial bacterial number[a] (cells/area)	Collected bacterial number (cells)	Recovery (%)
A	1.0×10^7	$(5.9\pm1.0)\times10^6$	59±10
B	1.0×10^7	$(5.0\pm1.0)\times10^6$	50±9.9
C	1.0×10^7	$(6.9\pm1.1)\times10^6$	69±11
D	1.0×10^7	$(7.2\pm1.5)\times10^6$	72±15
E	1.0×10^7	$(7.1\pm1.4)\times10^6$	71±14
Conventional	1.0×10^7	$(7.1\pm1.3)\times10^6$	71±13

(n=10)

a. *L. lactis* on painted stainless steel plate

Fig. 1. Swabbing techniques examined in this study and bacterial recovery rate. *Lactococcus lactis* subsp. *Lactis* GTC00232 was used as a test strain [4].
(A) Moving the swab one-way with five horizontal strokes without rotating the swab. (B) Moving the swab back and forth with five horizontal strokes without rotating the swab. (C) Moving the swab one-way with five horizontal and five vertical strokes without rotating the swab. (D) Moving the swab back and forth with five horizontal and five vertical strokes without rotating the swab. (E) Moving the swab back and forth with five horizontal and five vertical strokes with rotating the swab at every stroke.

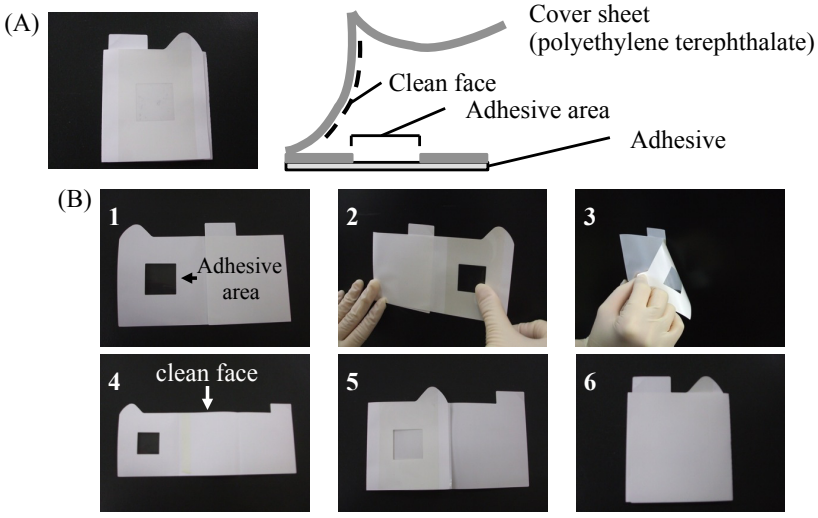


Fig. 2. Adhesive sheet for microbial monitoring in space habitat [5]. Sheets were sterilized by gamma radiation and packed individually in small bags. (A) Photograph and schematic illustration of the adhesive sheet. (B) Procedure for microbial sampling: 1, open the triangular tab; 2, attach the adhesive area to the sampling site and press three times; 3, peel off the adhesive sheet from the sampling site; 4, open the rectangular tab; 5, close the triangular tab; 6, close the rectangular tab.

Table 2. Bacterial abundance on interior surfaces in Kibo determined by fluorescent microscopy and quantitative PCR [7].

	Microbe-I		Microbe-II		Microbe-III	
	TDC[a]	qPCR[b]	TDC	qPCR	TDC	qPCR
Outer surface of incubator	2×10^3	4×10^3	2×10^2	$< 1\times 10^2$	2×10^2	$< 1\times 10^2$
Air diffuser	9×10^2	2×10^3	$< 2\times 10^2$	3×10^2	$< 2\times 10^2$	$< 1\times 10^2$
Handrail	7×10^2	5×10^2	$< 2\times 10^2$	1×10^2	2×10^2	$< 1\times 10^2$
Air return grill	NT[c]	NT	$< 2\times 10^2$	1×10^2	$< 2\times 10^2$	$< 1\times 10^2$
Internal surface of incubator	NT	NT	$< 2\times 10^2$	1×10^2	$< 2\times 10^2$	$< 1\times 10^2$

(Unit: cells/cm^2)

[a] TDC, total direct counting with fluorescent microscopy.
[b] qPCR, quantitative PCR.
[c] NT, not tested.

was equivalent to that determined with swabs ($p>0.05$, Student's t-test). We also confirmed that the number of bacterial cells collected on the adhesive sheet did not significantly change with freezing for up to 12 months. We therefore concluded that the sheet was a suitable alternative device for sampling in the space habitat.

3. Results Obtained from "Microbe-I" – "Microbe-III"

The majority of microbes in natural environments are hard-to-culture in conventional culture conditions. To understand the real "microbial world" in Kibo, culture-independent methods are required. Therefore, in this study, we used the state-of-the-art molecular microbiological methods to determine bacterial abundance and phylogenetic affiliation. Total direct counting with a fluorescence microscope and 16S rRNA gene targeted quantitative PCR were used to determine bacterial abundance. For phylogenetic analysis, amplicon sequencing with a high throughput sequencer (pyrosequencing) was used. Results were reported in a research article [7]. We summarize the results as follows:

Table 2 shows bacterial abundance on the interior surfaces in Kibo. Approximately 10^3 cells/cm^2 of bacteria were detected on the interior surfaces in Kibo in Microbe-I. In Microbe-II and Microbe-III, bacterial abundance was *ca.* 10^2 cells/cm^2, or less than the quantification limit. Overall, bacterial abundance did not exceed 10^4 cells/cm^2 in any measurement. Our previous study showed that bacterial abundance on the surfaces of equipment in our laboratory on Earth, such as handrails, elevator buttons, faucets and telephone receivers, was *ca.* 10^5 cells/cm^2. Bacterial abundance in Kibo was more than 10-fold lower than that in the ground-based laboratory. Therefore, we conclude that the bacterial abundance in Kibo was well controlled during the 1,596-day operation covered by our study. However, Kibo is a module for performing experiments and not for living activities. It is therefore important to compare results obtained in Kibo with those in modules for living activities to evaluate the whole microbial world in the ISS.

Figure 3A shows the bacterial community structure at the phylum level on the interior surfaces in Kibo. Bacteria of the phyla *Proteobacteria* (beta and gamma subclasses), *Firmicutes* and *Actinobacteria* were detected frequently. The families *Enterobacteriaceae* and *Staphylococcaceae* were dominant in each sample (**Fig. 3B**). The microbial community structure on the equipment in Kibo varied during the first 4-years of operation (**Fig. 3B**). Because the bacterial community in the space habitat was not established, it is considered that the community was easily changed by external factors such as astronauts' activities and experiments in Kibo.

4. "Microbe-IV" as the Next Phase of Bacterial Monitoring

In February 2015, "Microbe-IV," the next phase of "Microbe," commenced. Four sampling are scheduled in fiscal year 2014–2016. The first and second samplings were performed on 27 February and 28 August 2015. Four new sampling points were added to the six points used in "Microbe I–III": a foot hold (high frequency of human contact); the MELFI1 door (high frequency of human contact; located on the floor); the Intermodule Ventilator Fan (air of Kibo; located between Kibo and the module next to Kibo); and the wall of Kibo (low frequency of human contact) [3]. Continuous monitoring provides information on changes in the microbial community structure in Kibo and the stability of the microbial ecosystem during prolonged stay in confined environments in space.

5. Application on Earth

These modern microbiological methods for microbial monitoring and community analysis used in Kibo give us more precise information than conventional methods. These new methods will be applicable to various fields, especially microbiological quality control for pharmaceutical and food processing industries. These methods are introduced in the Japanese Pharmacopoeia 17$^{\text{th}}$ edition [8]. Microbiological monitoring in Kibo will lead to a new era of microbiological control in space and on Earth.

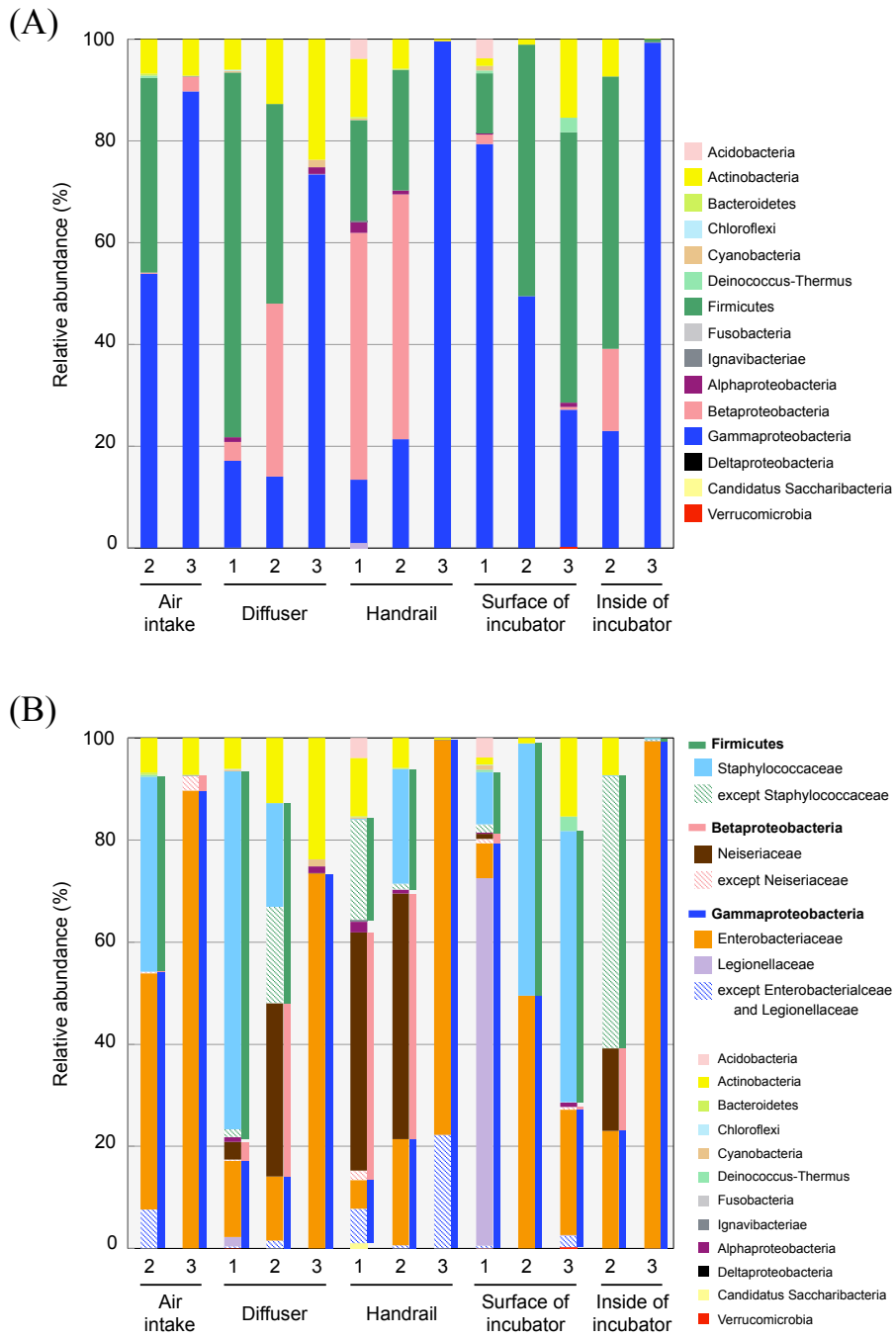


Fig. 3. Bacterial community structure on the interior surfaces in Kibo [7]. (A) At the phylum level. (B) Expanding beta- and gamma-*Proteobacteria* and *Firmicutes* to the family level.

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