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Four-year bacterial monitoring in the International Space Station—Japanese Experiment Module "Kibo" with culture-independent approach

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Studies on the relationships between humans and microbes in space habitation environments are critical for success in long-duration space missions, to reduce potential hazards to the crew and the spacecraft infrastructure. We performed microbial monitoring in the Japanese Experiment Module "Kibo", a part of the International Space Station, for 4 years after its completion, and analyzed samples with modern molecular microbiological techniques. Sampling was performed in September 2009, February 2011, and October 2012. The surface of the incubator, inside the door of the incubator, an air intake, air diffuser, and handrail were selected as sampling sites. Sampling was performed using the optimized swabbing method. Abundance and phylogenetic affiliation of bacteria on the interior surfaces of Kibo were determined by quantitative PCR and pyrosequencing, respectively. Bacteria in the phyla *Proteobacteria* (γ-subclass) and *Firmicutes* were frequently detected on the interior surfaces in Kibo. Families *Staphylococcaceae* and *Enterobacteriaceae* were dominant. Most bacteria detected belonged to the human microbiota; thus, we suggest that bacterial cells are transferred to the surfaces in Kibo from the astronauts. Environmental bacteria such as *Legionella* spp. were also detected. From the data on bacterial abundance and phylogenetic affiliation, Kibo has been microbiologically well maintained; however, the microbial community structure in Kibo may change with prolonged stay of astronauts. Continuous monitoring is required to obtain information on changes in the microbial community structure in Kibo.

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INTRODUCTION

Research on microbial dynamics in crewed habitats in space is indispensable to achieve safe and healthy long-duration space habitation. According to the previous research, microgravity and spaceflight affect bacterial growth, virulence, biofilm formation, and so on (reviewed in Yamaguchi *et al.*[1]). For instance, Wilson *et al.*[2] reported that spaceflight-grown *Salmonella enterica* serovar Typhimurium increased their virulence when compared with control bacteria on Earth. Kim *et al.*[3] reported that *Pseudomonas aeruginosa* showed different structure and biomass of biofilms between normal and spaceflight conditions. Furthermore, immune dysfunction occurs associated with spaceflight.[4] Therefore, the relationship between humans and microbes may change in space habitation. For successful manned space missions, it is necessary to investigate the relationship between humans and microbes, and how microbes influence the systems and materials in such space environments. That is, we have to understand how and where microbes proliferate in a confined environment in space.

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 (research title: "Microbe": http://iss.jaxa.jp/en/kiboexp/news/101101_microbe-2_start.html). The objective of this research is to monitor microbes and analyze their dynamics in Kibo from environmental microbiological viewpoints. Previously, many studies on microbial contamination in space habitats have been based on culture-based approaches, despite the fact that the majority of microbes in natural environments are hard to culture under conventional culture methods.[5,6] In environmental microbiology, some culture-independent techniques are available, and these techniques have been introduced in recent research on microbial contamination in space habitats. Ichijo *et al.*[7] used total direct counting, quantitative PCR and PCR denaturant gradient gel electrophoresis for determination of the abundance and phylogenetic affiliation of bacteria on the interior surfaces in Kibo. Venkateswaran *et al.*[8] used quantitative PCR and pyrosequencing followed by propidium monoazide treatment to understand the distribution and diversity of viable microbes in debris collected from the ISS. These reports provided the useful information that the ISS environmental microbiota comprised human-related microbes.

For long space missions, understanding changes in microbial dynamics in a confined environment is essential, as described above. In this research, we collected samples from interior surfaces in Kibo every ca. 500 days after Kibo began operation. Bacterial abundance and phylogenetic affiliation were determined by fluorescent staining, 16S ribosomal RNA (rRNA) gene-targeted quantitative PCR and pyrosequencing. This is the first report on continuous monitoring of bacterial abundance and phylogenetic affiliation in a space habitat determined by culture-independent molecular microbiological methods.

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RESULTS

Bacterial abundance

Bacterial abundance on the interior surfaces in Kibo is shown in Table 1. We determined bacterial abundance with different approaches, fluorescent microscopy and quantitative PCR targeting the bacterial 16S rRNA gene, to confirm the reliability of the results. Quantitative PCR outputs the copy number of the 16S rRNA gene in a sample. To convert the copy number to a cell number, we need to know the copy number of the target gene in a single cell. However, bacterial cells carry 1–15 copies of the 16S rRNA gene in their genomes.[9] We therefore calculated the average copy number of 16S rRNA genes in the bacterial community in Kibo as 5 copies/cell, based on the community structure determined by pyrosequencing as described below and the ribosomal RNA operon copy number database *rrn*DB.[9] As shown in Table 1, ca. 10^3 cells/cm^2 of bacteria were detected on the interior surfaces in Kibo in Microbe-I (Table 2). In Microbe-II and Microbe-III (Table 2), 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.

Bacterial community structure

Bacterial community structure on the interior surfaces in Kibo was analyzed by 16S rRNA gene-targeted pyrosequencing. Among 99,967 raw reads, 71,190 were high-quality reads after processing with the Quantitative Insights into Microbial Ecology (QIIME) pipeline. These reads were distributed in each sample (13 samples) with an average of 5,472 reads. At first, we estimated sufficient read number for bacterial community analysis using chao1 index (Supplementary Table S1) and a rarefaction curve (Supplementary Figure S1). As shown in Supplementary Table S1, we found that observed operational taxonomic units (OTUs) revealed 73–100% of bacterial family-level OTUs (equivalent to OTU at 90% similarity) that were present in our sample. We therefore confirmed the number of high-quality reads obtained was sufficient to reveal the bacterial community structure in the Kibo sample at the family level.

Figure 1a 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 frequently detected. The families *Enterobacteriaceae* and *Staphylococcaceae* were dominant in each sample (Figure 1b).

DISCUSSION

Studies on the relationship between humans and microbes in space habitation environments are critical for success in long-duration space missions. To reduce potential hazards to the crew and the spacecraft infrastructure, indoor quality control is important, as defined by the roadmap or scenario of each agency. In this study, we performed microbial monitoring in the ISS—"Kibo" for 4 years after its completion, and analyzed samples with culture-independent techniques.

The majority of bacteria in environments are hard to culture under conventional culture condition. Therefore, bacterial number is usually underestimated with culture methods. To obtain bacterial numbers more accurately, bacterial abundance was determined with culture-independent techniques. We used both total direct counting and quantitative PCR to obtain more reliable results. Total direct counting potentially gives us the absolute cell numbers. In case the number of contaminants (e.g., non-biological fluorescent particles or detritus) is relatively large in the sample, the accuracy of total direct counting tends to decrease. In this study, we set up a criterion when we enumerate bacterial cells by total direct counting; tiny particles whose size are smaller than bacteria generally distributed in environments are omitted. Under this criterion, cell number might be underestimated. The results show that the bacterial number was below 10^4 cells/cm^2. 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.

Bacterial community structure was determined by amplicon sequencing with a high-throughput sequencer. In this study, we used a two-step PCR protocol to generate amplicons. Amplicon sequencing with two-step PCR has several advantages.[10] For example, this method increases reproducibility and recovers higher genetic diversity. Selection of the amplified region can

Table 1. Bacterial abundance on interior surfaces in Kibo determined by fluorescent microscopy and quantitative PCR

	Microbe-I		*Microbe-II*		*Microbe-III*	
	TDC (cells/cm^2)	*qPCR (cells/cm^2)*	*TDC (cells/cm^2)*	*qPCR (cells/cm^2)*	*TDC (cells/cm^2)*	*qPCR (cells/cm^2)*
Outer surface of incubator	2×10^3	4×10^3	2×10^2	$<1\times10^2$	2×10^2	$<1\times10^2$
Air diffuser	9×10^2	2×10^3	$<2\times10^2$	3×10^2	$<2\times10^2$	$<1\times10^2$
Handrail	7×10^2	5×10^2	$<2\times10^2$	1×10^2	2×10^2	$<1\times10^2$
Air return grill	NT	NT	$<2\times10^2$	1×10^2	$<2\times10^2$	$<1\times10^2$
Internal surface of incubator	NT	NT	$<2\times10^2$	1×10^2	$<2\times10^2$	$<1\times10^2$

Abbreviations: NT, not tested; qPCR, quantitative PCR; TDC, total direct counting with fluorescent microscopy.

Table 2. Sampling and shipping to our laboratory

	Microbe-I	*Microbe-II*	*Microbe-III*
Sampling date (cumulative duration of Kibo operation)[a]	5 September 2009 (459 days)	27 February 2011 (999 days)	16 October 2012 (1,596 days)
Return to the Earth (mission)	12 September 2009 (STS-128)	9 March 2011 (STS-133)	28 October 2012 (SpaceX CRS-1)
Sample arrival (days after sampling)	25 September 2009 (20 days)	20 March 2011 (22 days)	6 November 2012 (21 days)

[a]Kibo operation was started on 4 June 2008.

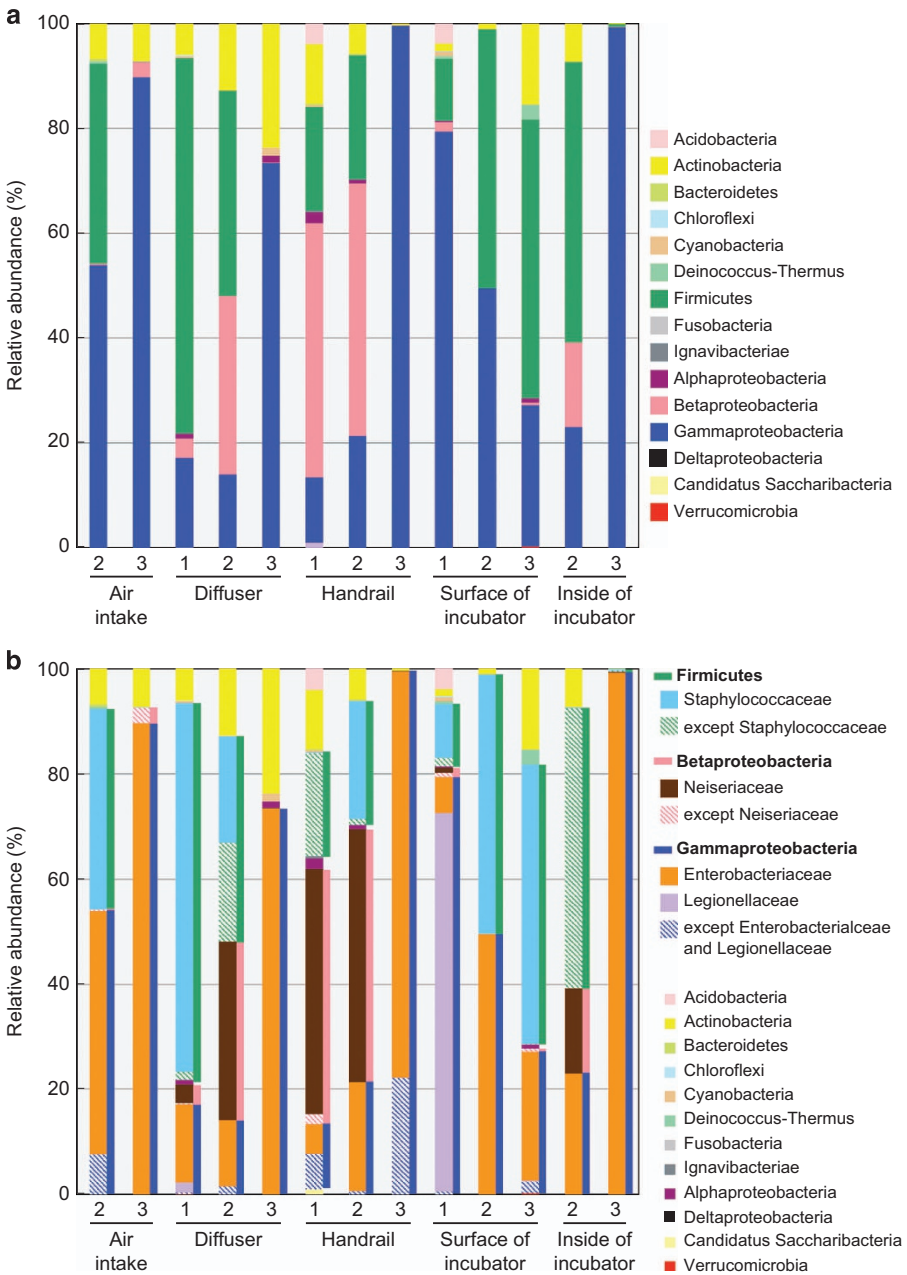

Figure 1. Bacterial community structure on the interior surfaces in Kibo. (**a**) At the phylum level. (**b**) Expanding beta- and gamma-*Proteobacteria* and *Firmicutes* to the family level.

influence the profile of the bacterial community generated by pyrosequencing.[11] Various regions such as V1–V2,[12,13] V3–V5,[14,15] V4–V5,[16,17] and V6–V8 (refs 18,19) have been analyzed for bacterial community structure by pyrosequencing. In this study, before the experiment, we evaluated several primer sets (targeting V1–V3, V3–V5, V7–V8, and V6–V8) by analyzing bacterial diversity on the incubator surface of Microbe-I by denaturing gradient gel electrophoresis. We selected a primer set of 968f and 1401r, which can amplify the V6–V8 region of the 16S rRNA gene based on greater diversity (data not shown).

The results of bacterial community analysis show that *Actinobacteria*, *Firmicutes*, and *Proteobacteria* were frequently detected on the surfaces of equipment in Kibo. In particular, *Staphylococcaceae* belonging to the phylum *Firmicutes*, *Enterobacteriaceae* belonging to the *Proteobacteria* (gamma-subclass), and *Neisseriaceae* belonging to the *Proteobacteria* (beta-subclass) were dominant taxonomic groups on the equipment surfaces (Figure 1b). *Actinobacteria* are commonly found in both terrestrial and aquatic ecosystems.[20] This phylum is known as the dominant species of human gut microbiota.[21,22] *Enterobacteriaceae*, *Staphylococcaceae*, and *Neisseriaceae* are also human related. *Enterobacteriaceae*, a large group of the gamma-*Proteobacteria*, are commonly found in the human gut microbiota.[23] Fierer *et al.*[12] reported that *Actinobacteria*, *Firmicutes*, and *Proteobacteria* contain the majority of the sequences retrieved from human palm surfaces. According to this report,[12] *Staphylococcaceae*, one of the most abundant taxonomic groups collected from Kibo, are relatively highly abundant on human palm surfaces; *Neisseriaceae* made up ca. 3% of the sequences from human palm surfaces; *Enterobacteriales* were also commonly found there. As described

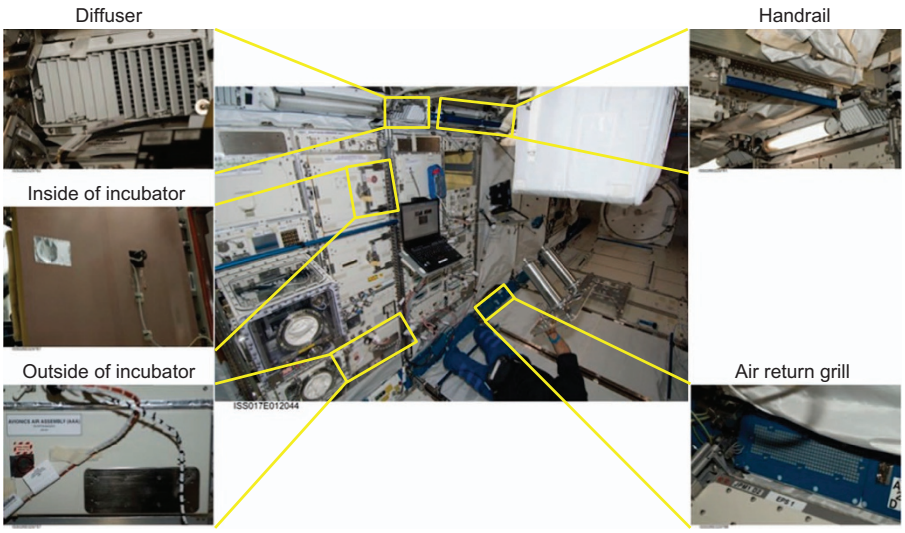

Figure 2. Photographs of sampling points in Kibo ((c) NASA/JAXA).

above, most of the bacteria detected on the Kibo equipment surfaces belong to human microbiota; thus, we suggest that bacterial cells are transferred to the surfaces in Kibo from the astronauts. Kibo was disinfected with isopropyl alcohol before launch. We measured bacterial numbers on the surfaces in Kibo before launch by fluorescent microscopy, but the bacterial number was below the quantification limit ($< 2 \times 10^2$ cells/cm^2). Our results show that immediately after beginning the operation of Kibo, few bacteria were present there. The bacterial community in Kibo formed from human-related bacteria through astronaut activities in the early stage of operation.

Microbial community structure on the equipments in Kibo varied during the first 4-year operation (Figure 1b). For example, on the surfaces of diffuser, handrail, and inside of incubator, *Neiseriaceae* occupied 3.4–48% of total bacterial communities on Microbe-I and Microbe-II; however, on Microbe-III, *Neiseriaceae* was rarely detected there ($< 1\%$). Relative abundance of *Enterobacteriaceae* seemed to increase in Microbe-III. Although human-related bacteria occupied majority of bacterial community in Kibo in the early stage of operation, their bacterial community was not stable during this period (Figure 1b). Because bacterial community in the space habitat was not established, it is considered that the community is easy to change affected by external factors such as astronauts' activities and experiments in Kibo.

Legionella were dominant in the sample collected from the outer surface of the incubator on Microbe-I (Figure 1b). *Legionella* is normally found in aquatic and terrestrial environments,[23] but not among human microbiota. Therefore, it seems unlikely that *Legionella* cells were transferred to the surface via the astronauts. In the monitoring of space habitat, *Legionella* species was only found in the Russian Space Station Mir.[24] As environmental bacteria have been detected in Kibo in this study and they are unlikely to have derived from astronaut activities, humans may not be the only source of bacteria in Kibo, and resupply materials could be the vehicles of microbes to the space habitats. We have to monitor such microbes.

As mentioned above, Kibo was microbiologically well maintained during the first 4-year operation. Microbiological cleanliness was controlled by wiping interior surfaces with disinfectant (benzalkonium chloride) once a week. However, benzalkonium chloride may increase the antimicrobial resistance of bacteria.[25,26] Prolonged stay of astronauts may increase microbial abundance and affect the microbial community in Kibo. In February 2015, "Microbe-IV", the next phase of "Microbe", was started. Four sampling are scheduled in fiscal year 2014–2016. 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). Continuous monitoring provides further 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, and leads to assure crew safety and the success of long-duration missions.

MATERIALS AND METHODS

Sampling in Kibo

Sampling in Kibo was performed three times by an astronaut as part of the "Microbe" research program (Table 2). The sampling locations inside the Kibo module were the internal and outer surface of the incubator named CBEF (Cell Biology Experiment Facilities; low frequency of astronaut contact), an air return grill and an air diffuser (reflection of the bacterial community of the air in Kibo), and handrail (high frequency of astronaut contact; Figure 2). The air return grill and the inside of the incubator were selected as sampling points from Microbe-II onwards. An optimized swabbing protocol[27] was used for sampling the interior surfaces. Samples were stored in the freezer (−95 °C) installed in Kibo and transferred to our laboratory via the NASA Kennedy Space Center and Japan Aerospace Exploration Agency Tsukuba Space Center at −80 °C. We previously confirmed that long-term storage with freezing does not affect the number of bacteria collected with swab method.[27]

Total direct counting of microbes

Bacteria collected with swabs were suspended in 10 ml of particle-free sterilized water and trapped on an autoclaved polycarbonate membrane filter (pore size 0.2 μm; Advantec, Tokyo, Japan) in a funnel (filtration area 13 mm^2; KGS-04, Advantec). Bacterial cells on the filter were stained with filter-sterilized 1× SYBR Green II nucleic acid staining dye (Invitrogen, Carlsbad, CA, USA). We enumerated fluorescently stained bacterial cells under an epifluorescent microscope (E-400; Nikon, Tokyo, Japan) with the Nikon filter sets B2-A (EX450–490, DM505, BA520). One-hundred microscopic fields (1 mm^2) per sample were observed.

DNA extraction

Following microscopic observation, DNA was extracted from bacterial cells on the polycarbonate filter as described previously.[6] Extracted DNA was suspended in TE buffer (10 mmol/l Tris-HC1, 1 mmol/l EDTA, pH 8.0) and

Table 3. Primers and probes used in this study

Purpose	*Primer and probe*	*Target gene and position*[a]	*Sequence (5′–3′)*	*Reference*
Quantitative PCR (DNA recovery)	pgL1908f	*luc* gene, 1,908–1,927	AGGAAGCTTTCCATGGAAGA	Nishimura *et al.*[28]
	Luc175r	*luc* gene, 2,082–2,063	CAGCGTAAGTGATGTCCACC	Nishimura *et al.*[28]
	n-LucHP1[b]	*luc* gene, 2,008–2,029	TGAAGAGATACGCCCTGGTTCC	Nishimura *et al.*[28]
	n-LucHP2[c]	*luc* gene, 2,030–2,058	GGAACAATTGCTTTTACAGATGCACATA	Nishimura *et al.*[28]
Quantitative PCR (bacterial abundance)	EUB f933	16S rRNA, 933–954	GCACAAGCGGTGGAGCATGTGG	Iwamoto *et al.*[35]
	EUB r1387	16S rRNA, 1,387–1,368	GCCCGGGAACGTATTCACCG	Iwamoto *et al.*[35]
Pyrosequencing	968f[d]	16S rRNA, 968–984	AACGCGAAGAACCTTAC	Felske *et al.*[36]
	1401r[e]	16S rRNA, 1,401–1,385	CGGTGTGTACAAGACCC	Felske *et al.*[36]

Abbreviations: FITC, fluorescein isothiocyanate; rRNA, ribosomal RNA.
[a]*luc* gene: numbering as for bases of pGeneGRIP-Luc (Genlantis, San Diego, CA, USA); 16S rRNA gene: *Escherichia coli* numbering system.
[b]3′ Terminus was labeled with FITC.
[c]5′ Terminus was labeled with LCRed640.
[d]454 FLX-titanium adapter "A" sequence (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) and barcode sequence were added at the 5′ terminus for the second PCR.
[e]454 FLX-titanium adapter "B" sequence (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′) was added at the 5′ terminus for the second PCR.

stored at −20 °C before use. To estimate the DNA recovery rate during extraction, known amounts of PCR products of the luciferase gene (*luc*) fragment were inoculated into the samples as an internal standard and quantified after DNA extraction according to Nishimura *et al.*[28] The DNA recovery rate was calculated by comparing the copy number of the inoculated *luc* gene before and after DNA extraction.

Quantitative PCR for quantification of bacteria

For quantification of bacteria, the bacterial 16S rRNA gene was quantified with the primer set EUB f933 and EUB r1387 (Table 3) using a LightCycler (Roche Diagnostics, Mannheim, Germany) according to the protocol reported by Yamaguchi *et al.*[6] The copy number of the 16S rRNA gene in a sample was calibrated based on the DNA recovery rate.

16S rRNA gene-targeted amplicon sequencing

16S rRNA gene fragments, including V6–8 regions, were amplified and analyzed by amplicon sequencing with the GS FLX System (Roche Diagnostics). In this study, we used a two-step PCR protocol, as previously described by Sutton *et al.*[29] The first PCR amplification was performed using primers 968f and 1401r (Table 3), which are specific for conserved bacterial 16S rRNA gene sequences. The PCR mixture, containing 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 25 pmol of each primer, 75 nmol of $MgCl_2$, 10 nmol of each deoxyribonucleoside triphosphate, 5 µl of 10× PCR buffer, and 0.25 µl of 2.5 mg/ml 8-methoxypsoralen (Sigma-Aldrich, St Louis, MO) in dimethyl sulfoxide, was made up to 48 µl with DNA-free water. A DNA suspension was added last in a 2-µl volume after irradiation of the PCR mixture with ultraviolet light ($\lambda = 365$ nm).[30,31] A hot-start PCR was performed at 95 °C for 9 min, and touchdown PCR was performed as follows: the annealing temperature was initially set at 63 °C and was then decreased by 1 °C every cycle until it was 53 °C. Twenty additional cycles were carried out at 53 °C. Denaturing was carried out at 94 °C for 1 min. Annealing was performed using the scheme described above for 1 min, and extension was performed at 72 °C for 3 min. The final extension step was 7 min at 72 °C. The first PCR product was purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and eluted with nuclease-free water. The second PCR used the purified products of the first PCR as templates. Amplification was performed as described above, except that primers with an adapter and 10-nt-length barcode were used, and the total number of amplification cycles was reduced to 18. The second PCR product was also purified with a Wizard SV Gel and PCR Clean-Up System and eluted with nuclease-free water. PCR product was quantified with a Qubit dsDNA HS Assay Kit (Invitrogen) and mixed in approximately equal concentrations (5×10^{10} copies/µl). The sample was sequenced with a GS FLX System at Hokkaido System Science (Hokkaido, Japan).

Data analysis

Sequencing data were processed using the QIIME pipeline.[32] Sequences were quality filtered by removing reads that (a) did not contain the primer sequence, (b) contained an uncorrectable barcode, (c) were < 250 nt in length, (d) had homopolymers > 8 nt, or (e) had a quality score of < 25, and then demultiplexed using the respective sample nucleotide barcodes.[8] These sequences were clustered into OTUs based on their similarity (97%). The taxonomic affiliation of each OTU was determined using the RDP Classifier at a confidence threshold of 80%.[33] Chao1 index and rarefaction curves based on family-level OTUs were estimated with the RDPipeline[34] (Ribosomal Database Project). The sequences obtained from amplicon sequencing were deposited in the DNA Data Bank of Japan Sequence Read Archive under the accession number DRA004228.

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CONTRIBUTIONS

TI and NY contributed equally to this work. MN conceived the study; TI performed quantitative PCR and phylogenetic analysis; NY performed fluorescent microscopy; FT and MS managed sampling programs; TI, NY, and MN wrote the paper; all authors discussed the results in the manuscript.

COMPETING INTERESTS

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the *npj Microgravity* (http://www.nature.com/npmgrav)