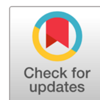

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Atmospheric Bioaerosol, *Bacillus* sp., at an Altitude of 3,500 m over the Noto Peninsula: Direct Sampling via Aircraft

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ABSTRACT

This work focuses on the analysis of bioaerosols in the atmosphere at higher altitudes over Noto Peninsula, Japan. We carried out direct sampling via aircraft, separated cultures, and identified present isolates. Atmospheric bioaerosols at higher altitudes were collected using a Cessna 404 aircraft for an hour at an altitude of 3,500 m over the Noto Peninsula. The aircraft-based direct sampling system was devised to improve upon the system of balloon-based sampling. In order to examine pre-existing microorganism contamination on the surface of the aircraft body, bioaerosol sampling was carried out just before takeoff using the same method as atmospheric sampling. Identification was carried out by a homology search for 16S or 18S rDNA isolate sequences in DNA databases (GenBank). Isolate sampling just before takeoff revealed *Stretpomyces* sp., *Micrococcus* sp., and *Cladosporium* sp. One additional strain, *Bacillus* sp., was isolated from the sample after bioaerosol collection at high altitude. As the microorganism contamination on the aircraft body before takeoff differed from that while in the air, the presence of additional, higher atmosphere-based microorganisms was confirmed. It was found that *Bacillus* sp. was floating at an altitude of 3,500 m over Noto Peninsula.

Key words: Atmospheric bioaerosols, Direct sampling, Aircraft, *Bacillus* sp., Noto Peninsula

1. INTRODUCTION

Bioaerosols, a group of organic aerosols ranging from 10 nm to 100 μm, are airborne particles or large molecules that carry living organisms or are released from living organisms (*e.g.*, bacteria and fungi) (Aria and Amyot, 2004). Many fields of study have focused on atmospheric bioaerosols. In geophysics and meteorology, there is a substantial body of work in progress on the importance of atmospheric bioaerosols as ice nuclei and cloud condensation nuclei (Bowers *et al.*, 2009; Aria and Amyot, 2004). In medicine, Ichinose *et al.* (2008) reported that Asian sand dust containing microbiological materials causes adverse respiratory health effects (Ichinose *et al.*, 2005). In biology, microorganisms (Atmosphere bioaerosols) can be transported by KOSA (yellow dust events) not only in North East Asia but also in Arctic areas (Yukimura *et al.*, 2009; Hua *et al.*, 2007). Based on this evidence, the long-range transportation of atmospheric bioaerosols seems to have a great influence on ecosystem of source and deposit regions.

In clarifying the behavior of atmospheric bioaerosols, especially their long-range transport, it is necessary to directly collect them at very high altitude. Griffin (2008, 2004) collected atmospheric bioaerosols at an altitude of 20,000 m using an aircraft flying through African dust. In East Asia, we collected atmospheric bioaerosols using a tethered balloon below an altitude of 1,000 m over Noto Peninsula, Japan, and the east side of the Tarim Basin (Taklamakan desert), China, and discovered many kinds of atmospheric bioaerosols (Chen *et al.*, 2010; Yamada *et al.*, 2010; Iwasaka *et al.*, 2010, 2009; Kakikawa *et al.*, 2010, 2008; Maki *et al.*, 2010, 2008; Kobayashi *et al.*, 2010,

2007). However, in East Asia, very few papers have reported on the collection and analysis of atmospheric bioaerosols at high altitudes.

Using a Cessna 404 aircraft, we carried out the direct sampling of atmospheric bioaerosols at an altitude of 3,500 m. In order to examine microorganism contamination at other altitudes, bioaerosols were collected just before takeoff. The identification of viable bacteria and/or fungi in the form of bioaerosols in these samples was reported.

2. EXPERIMENTAL

2.1 Use of an Aircraft-Based Sampling Device

Using a Cessna 404 aircraft (Nakanihon Air Service Co., Ltd.), bioaerosol collection was carried out as shown in Fig. 1(a). There is a 20 mm diameter hole in the roof of the craft (Fig. 1(b)). Before sampling, the hole was sealed. Upon beginning the sampling, the hole was opened, and the sterilized inlet was inserted as shown in Fig. 1(b). The inlet connected to a sterilized tube. The inlet and tube used for sampling were a conductive polytetrafluoroethylene (PTFE) tube (ASONE Co.) and a conductive silicon tube (SIBATA Sciencific Technology Ltd.), respectively. Since both products are highly conductive and can be sterilized with steam, they are suitable for collecting a sampling of bioaerosols. In calculating streamlines of air near the aircraft body, it was found that there was little influence when the inlet length was less than 10 mm. The sampling examined using 15 mm length of the inlet for no influence of air near the aircraft body. The tube was connected to a bioaerosol sampler.

This bioaerosol sampler was the same as that used on a tethered balloon in previous studies (Chen *et al.*, 2010; Yamada *et al.*, 2010; Iwasaka *et al.*, 2010, 2009; Kakikawa *et al.*, 2010, 2008; Maki *et al.*, 2010, 2008; Kobayashi *et al.*, 2010, 2007). Atmospheric bioaerosols were collected on a 0.45 μm pore-size membrane filter. The filter was set into a filter holder (In-Line Filter Holder, 47 mm; Millipore Co., Ltd.) in the sampler under sterile conditions after autoclaving. The sampling volume of the air was estimated at 1.0 m^3 over a 60-minute sampling and flow rate of 17.0 L/min by an air pump. To avoid contamination during non-sampling, the inlet and outlet of the filter holder were closed by shutter valves.

2.2 Sampling

The sampling of atmospheric bioaerosols was carried out using an aircraft over Noto Peninsula. Though Noto Peninsula juts out into the center of the Sea of Japan (Fig. 2), the collection of atmospheric bioaerosol samples while crossing the peninsula is not affected

Fig. 1. Cessna 404 aircraft used for the direct sampling of atmospheric bioaerosol (a) and the outside of the inlet (b). The inlet was sterilized just before sampling.

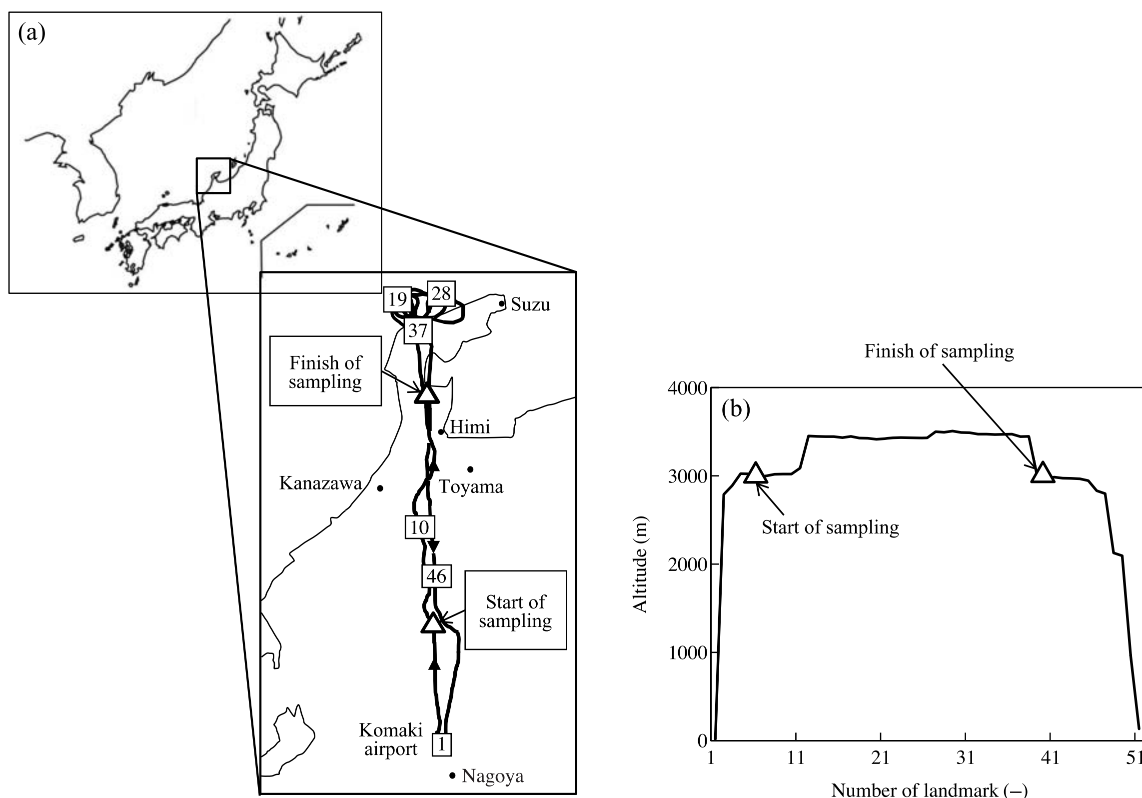

Fig. 2. Flight route (a) and altitude chart (b) for the direct sampling of atmospheric bioaerosol via aircraft.

by microorganisms originating in the soil of the Japanese island. The sampling was carried out in the afternoon (15:00-16:00) on November 21, 2009. The weather on the sampling date was cloudy.

Fig. 2 shows the flight route (a) and altitude chart (b). The number in Fig. 2(a) and horizontal axis in Fig. 2(b) indicate landmarks during our flight. The landmarks indicate Global Positioning System (GPS) locations at intervals of 2 minutes. The triangles indicate the beginning and conclusion of the sampling. The aircraft took off from Komaki Airport, and sampling was carried out for 1 hour in a circular flight over Noto Peninsula. The altitude for sampling was approximately 3,500 m.

In order to examine pre-existing microorganism contamination on the surface of the aircraft body, bioaerosol sampling was carried out just before takeoff using the same method as atmospheric sampling. To address the issue of microorganisms attaching to the surface of the aircraft, the aircraft surface was disinfected with ethanol before and after sampling (76.9-81.4 vol%, Wako Pure Chemical Industries, Ltd.).

2.3 Trajectory Analysis

For the estimation of origin of atmospheric bioaerosols, backward trajectories of air masses were calculated using the U.S. National Oceanic and Atmospheric Administration (NOAA) Air Resources Laboratory Hybrid Single Particle Lagrangian Integrated Trajectory (HYSPLIT) model (http://ready.arl.noaa.gov/HYSPLIT.php, Draxler and Hess, 1998). In this study, the sampling was carried out by wide field circling of the aircraft over Noto Peninsula. Thus, the backward trajectories were calculated from the trajectory ensemble as a trend.

2.4 Separated Culture and Identification

The atmospheric bioaerosols were cultivated immediately after collection in a clean booth. The filter sample was placed on a plate containing Nutrient Agar (Diffco BD Co., Ltd.) for general bacterium and Sabouraud dextrose agar (Wako Pure Chemical Industries, Ltd.) for the eumycetes, *i.e.*, fungi. DNA was extracted from isolates on the plate using a cell-wall lytic enzyme, lysozyme, and proteinase K (Sigma-Aldrich). 16S rDNA for prokaryotes was amplified by polymerase chain reaction (PCR) as described by Weisburg *et al.* (1991). 18S rDNA for eukaryotes was amplified by PCR using primer F1 (5′-TGGTTGATCCTGCCAGAGG-3′) and R1 (5′-GGCTACCTTGTTACG ACTT-3′).

The PCR reaction mixture (vol. 20 μL) included the following: 4 μL of 5 × Buffer, 1.6 μL of 10 × dNTP (2.5 mM each, dATP, dCTP, GTP, dTTP), 0.2 μL of each primer (20 mM), 12.8 μL of sterile deionized H_2O, 1 U of PrimeSTAR DNA polymerase (TAKARA BIO INC. Co., Ltd.), and 1 μL of DNA (~30 ng). A thermal cycler (Dice, TAKARA BIO INC. Co., Ltd.) was used under the following conditions for amplification: initial 2 min denaturation at 98°C; 35 cycle-10 s denaturation at 98°C; 10 s annealing at 54°C; 1.5 min extension at 72°C; and a final 3 min extension at 72°C.

The DNA sequencing of cloned rDNA was determined using a genetic analyzer (Applied Biosystems Co., Ltd.), and the related species of isolates were searched by Basic Local Alignment Search Tool (BLAST) analysis (http://www.ncbi.nlm.nih.gov/BLAST) against DNA databases (GenBank/EMBL/DDBJ).

3. RESULTS AND DISCUSSION

3.1 Trajectory Analysis

In order to discuss the history of the sampled air mass, HYSPLIT backward-trajectories were tracked for a duration of 48 hours (Fig. 3). Since the sampling was conducted by circular flight, the center point of the flight was selected as the starting point of the trajectory analysis, and the trajectories were calculated with an ensemble option. This resulted in 27 members for all possible offsets in X, Y, and Z. As a trend, the trajectories suggested that the sampled air mass came to Noto Peninsula from Siberia through eastern Mongolia, northeastern China, the northern section of the Korean Peninsula, and the Sea of Japan. Altitudes of the trajectories decreased from about 5,000 and 7,000 m. Though KOSA seems to be transported through the Gobi desert region, as calculated by the trajectory analysis, the Japan Metrological Agency reported that the KOSA phenomenon was not present on the sampling date. Additionally, concentrated dust clouds were not observed from Total Ozone Mapping Spectrometer (TOMS, http://toms.gsfc.nasa.gov/index.html) data. Therefore, it was suggested that the sample in this study would not be related to KOSA bioaerosol.

3.2 Bioaerosols Just Before Takeoff

In order to examine pre-existing microorganism contamination on the surface of the aircraft body, bioaerosol sampling was carried out just before takeoff using the same method as atmospheric sampling. First, the same sampling method as used in atmospheric conditions was carried out for 1 hour before takeoff. From the separated culture, two isolates grew from a Nutrient Agar medium and Sabouraud dextrose agar medium. They were named in order from A01 strain to A04 strain. Second, after the above sampling, the aircraft was cleaned and sterilized using an ethanol solution, particularly around the inlet. Using the same method, sampling was carried out just before takeoff. After separate culturing from a Nutrient Agar medium and Sabouraud dextrose agar medium, one and two isolates grew, respectively. They were named in order from B01 strain to B03 strain.

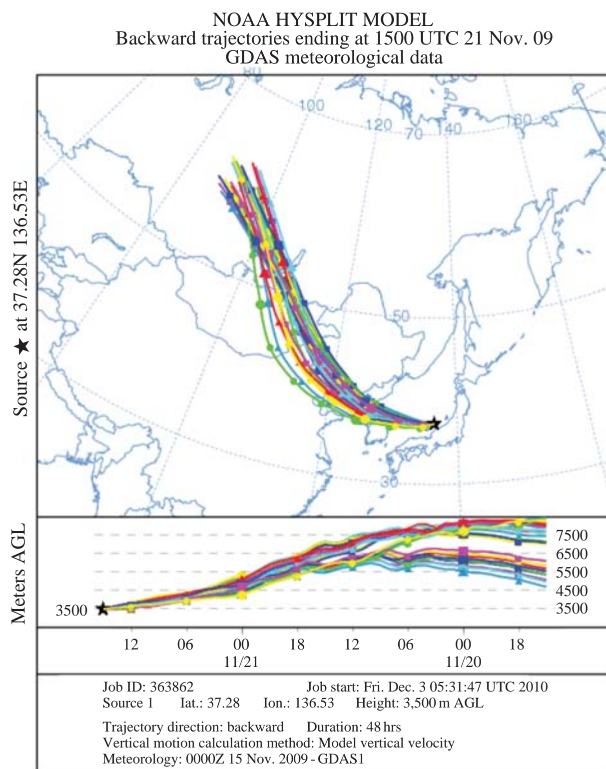

Fig. 3. HYSPLIT back-trajectories of air masses arriving at the Noto Peninsula. Plots show 24-hour air mass back-trajectories on November 21, 2009.

Fig. 4 shows a photograph of colonies of A01 strain (a) to A04 strain (d) before ethanol cleaning and B01 strain (e) to B03 strain (g) after ethanol cleaning. The A01 and B01 strain colonies, as shown in Fig. 4(a) and (e), were almost the same form and color. The colonies of these strains were pale yellow with a wrinkled surface. They seem to be a kind of actinomycetes. The colony of A02 strain, as shown in Fig. 4(b), was yellow, smooth, and convex with a regular edge. A03, A04, B02, and B03 strains, as shown in Fig. 4(c), (d), (f), and (g), respectively, were moderately expanding, velvety to powdery, and pale brown. They were observed to be of a kind of mold.

DNA was extracted from the isolates; the 16S rDNA

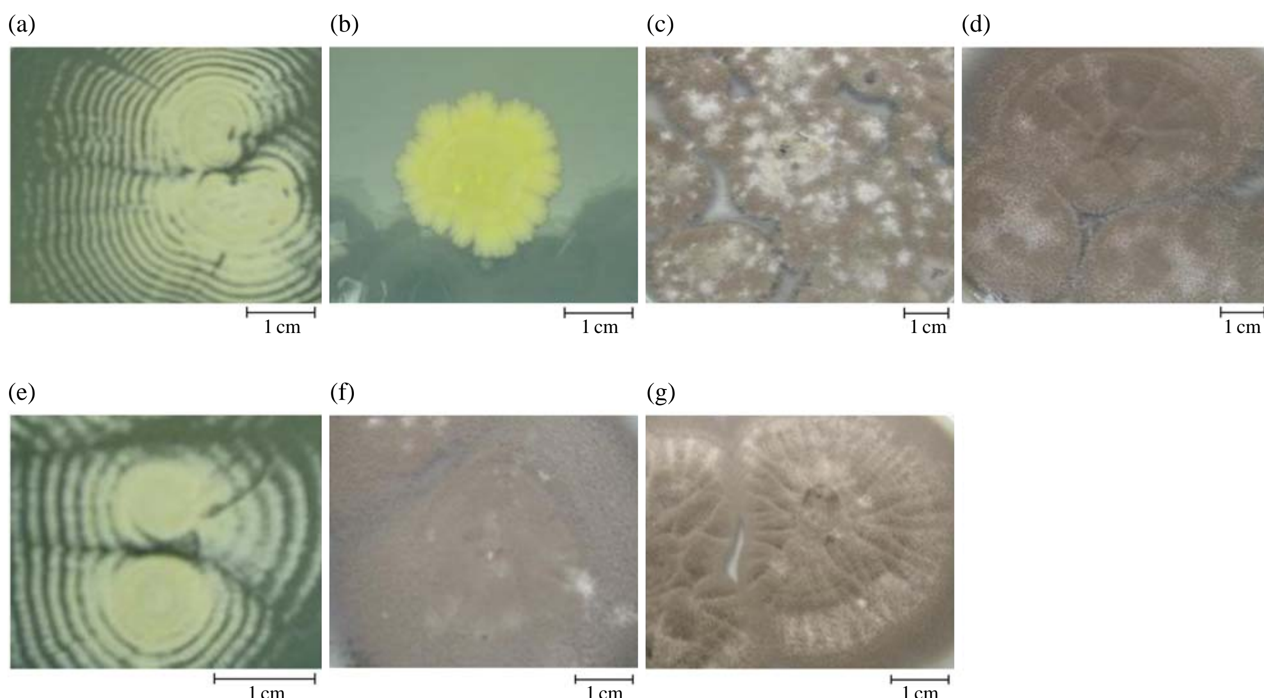

Fig. 4. Photographs of colonies of A01 (a), A02 (b), A03 (c), A04 (d), B01 (e), B02 (f), and B03 (g) strains on plates isolated from samplings just before takeoff. The A01, A02, A03, and A04 strains were isolated from sampling before ethanol cleaning. B01, B02, and B03 strains were isolated from sampling after ethanol cleaning.

Table 1. The results of homology research of isolates collecting just before takeoff.

Isolate clones (Accession No.)	Separate curture medium	Closest microorganism in GenBank	% DNA identity (matched bases)	Comments
(1) Before ethanol clean up				
A01 *Streptomyces* sp. (AB603644)	Nutrient agar	*Streptomyces* sp. SOK4/28-05 (EU098024)	100% (299/299)	country="Czech Republic" (Information from GenBank submisson data)
A02 *Micrococcus* sp. (AB603645)	Nutrient agar	*Micrococcus* sp. PA-E028 (FJ233852)	100% (312/312)	isolation_source="Kizhanelli root" (Information from GenBank submisson data)
A03 *Cladosporium* sp. (AB603646)	Sabouraud dextrose agar	*Cladosporium* sp. 6027 (FJ235525)	98% (387/391)	isolation_source="leaf" (Information from GenBank submisson data)
A04 *Cladosporium* sp. (AB603647)	Sabouraud dextrose agar	*Cladosporium* sp. 6027 (FJ235525)	99% (402/405)	isolation_source="leaf" (Information from GenBank submisson data)
(2) After ethanol clean up				
B01 *Streptomyces* sp. (AB603648)	Nutrient agar	*Streptomyces* sp. SOK4/28-05 (EU098024)	100% (334/334)	country="Czech Republic" (Information from GenBank submisson data)
B02 *Cladosporium* sp. (AB603649)	Sabouraud dextrose agar	*Cladosporium* sp. 6027 (FJ235525)	99% (388/391)	isolation_source="leaf" (Information from GenBank submisson data)
B03 *Cladosporium* sp. (AB603650)	Sabouraud dextrose agar	*Cladosporium cladosporioides* (EU723484)	100% (369/369)	isolation_source="deep-sea basin" (Information from GenBank submisson data)

for prokaryotes and 18S rDNA for eukaryotes were amplified by PCR, sequenced, and analyzed for homologies using a BLAST program. Table 1 shows the homology analysis of isolates collected before takeoff. The partial DNA sequence data of the A01 (Accession no. AB603644) and B01 strains (Accession no. AB6 03648), 299 and 334 base pairs, were closely related to *Streptomyces* sp. SOK4/28-05 (Accession no. EU098 024, 100%). Observation of colony forms, as shown in Fig. 4(a) and (b), confirmed the homology analysis results (Tresner and Backus, 1963). The A01 and B01 strains appeared to be *Streptomyces* sp. The DNA of the A02 strain (Accession no. AB603645), 312 base pairs in length, was closely related to *Micrococcus* sp.

Table 2. Isolates of atomospheric bioaerosol at an altitude of 3,500 m over Noto peninsula.

Isolate clones (Accession No.)	Separate curture medium	Closest microorganism in GenBank	% DNA identity (matched bases)	Comments
BASZHN0901 *Bacillus* sp. (AB603643)	Nutrient agar	*Bacillus subtilis* (FR729926)	99% (371/372)	isolation_source="soil" (Information from GenBank submisson data)

PA-E028 (Accession no. FJ233852, 100%). The observation results of colony forms of the A02 strain identified it as *Micrococcus* sp. (Fig. 4(b), Baird-Parker, 1974). The DNA of A03 (Accession no. AB603646), A04 (Accession no. AB603647), and B02 strains (Accession no. AB603649), 391, 405, and 391 base pairs in length, respectively, were related to *Cladosporium* sp. 6027 (Accession no. FJ235525, 98, 99, and 99%, respectively). The B03 strain (Accession no. AB603650), 369 base pairs in length, was closely related to *Cladosprorium cladosporioides* (Accession no. EU723484, 100%). From the observation of colonies of the B03 strain, as shown in Fig. 4(g), it was not clear that the B03 strain could be identified as *Cladosporium* "*cladosporioides*". Thus, A03, A04, B02, and B03 strains suggested to be *Cladosporium* sp.

From the results of colony form observation and homology analysis in 16S or 18S rDNA before takeoff, *Streptomyces* sp., *Micrococcus* sp., and *Cladosporium* sp. were present before ethanol cleaning, and *Streptomyces* sp. and *Cladosporium* sp. were present after. It was found that *Micrococcus* sp., present on the body of the aircraft, was dead after the ethanol cleaning.

3. 3 Direct Sampling of Atmospheric Bioaerosol via Aircraft

Direct sampling using an aircraft was carried out at an altitude of 3,500 m over Noto Peninsula (Fig. 2). After separate culturing overnight, one strain grew from the Nutrient Agar medium. Fig. 5 shows the colony of isolated atmospheric bioaerosol on a plate. This isolate was named BASZHN 0901. The colony of this isolate was observed as thick, opaque, and cream-colored. Table 2 shows the results of the homology analysis of the 16S rDNA sequence of the BASZHN 0901 strain. The partial DNA sequence data of the BASZHN 0901 strain (Accession no. AB603643), 372 base pairs in length, showed close relation to *Bacillus subtilis* (Accession no. FR729926, 99%). The DNA identity was a high value (99%), but it was difficult to be identified as *Bacillus subtilis*. The BASZHN 0901 strain was identified as *Bacillus* sp. The observation of a colony of BASZHN 0901 (Fig. 5) indicated this result (Gibson and Gordon, 1974). As there was no *Bacillus* sp. among the isolated microorganisms from just before takeoff samples, the contamination, *i.e.* in other altitude or attaching the aircraft body, was not occurred.

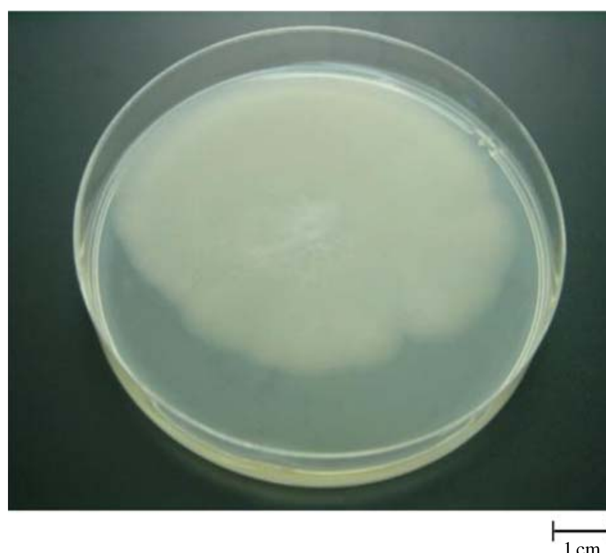

Fig. 5. Photograph of a colony of BASZHN 0901 strain as an atmospheric bioaerosol at an altitude of 3,500 m over Noto Peninsula.

Griffin (2004) reported that *Bacillus* is present at an altitude of 20,000 m in Earth's atmosphere. He noted in the paper mentioned above that *Bacillus* is a spore-former and that its spores are egg-like vesicles that can protect microorganisms from physical stress such as ultraviolet light-induced DNA damage, desiccation, and extreme temperatures (Saffary *et al.*, 2002; Setlow, 2001). In East Asia, *Bacillus* sp. was found to be a spore-former. The possible origin of *Bacillus* sp. BASZHN 0901 at altitude of 3,500 m over Noto Peninsula is very interesting. From the trajectory analysis (Fig. 3), the air containing *Bacillus* sp. BASZHN 0901 came from Siberia, eastern Mongolia, northeast China, the northern section of the Korean peninsula, or the Sea of Japan. Analyses of soil and/or seawater have shown that *Bacillus* exists in Siberia (Rikhvanov *et al.*, 1999), northeast China (Wang *et al.*, 2010; Carrasco *et al.*, 2007), and the Sea of Japan (Yoon *et al.*, 2004). In order to clarify the origin of high-altitude atmospheric bioaerosols such as *Bacillus* sp. BASZHN 0901, it is necessary to obtain more detailed data on the DNA of various strains of *Bacillus* in East Asia. We hope that future study will focus on this effort.

4. CONCLUSIONS

A system of direct bioaerosol sampling via aircraft was developed and carried out at an altitude of 3,500 m over Noto Peninsula on November 21, 2009. From the trajectory analysis, the sampled air mass appeared to come from the Siberia through the Sea of Japan to Noto Peninsula. Just before takeoff, *Streptomyces* sp., *Micrococcus* sp., and *Cladosporium* sp. were isolated through sampling. *Micrococcus* sp. appeared deceased from ethanol cleaning. Bio-analyzing high-altitude samples for cultivable microorganisms revealed the formation of a colony identified as *Bacillus* sp. In this aircraft-based sampling system, there was no contamination by other microorganisms because high-altitude atmospheric bioaerosol sampling results did not agree with those just before takeoff. It was found that there was a living bacterium, *Bacillus* sp., present as an atmospheric bioaerosol at an altitude of 3,500 m over Noto Peninsula.

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