RF-072 Study on the Sampling and Identification of KOSA bioaerosols for a Research of Borderless Health Damage (Abstract of the Final Report)

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[Abstract]
Asian dust, known in Japan as “KOSA”, contributes significantly to regional and global climate and environment changes. In order to clarify the behavior of bioaerosols diffusing in the atmosphere with KOSA and the health damage of KOSA, we attempted to sample directly the KOSA bioaerosols. The sampling was carried out in the mixed layer over Dunhuang (China) as the KOSA source region, Kanazawa (Japan), Suzu (Japan), Amakusa (Japan) as the KOSA receptor region, and Choungju (Korea) as the middle point of KOSA long-range transport, using the bioaerosol sampler and a tethered balloon. The analyses of KOSA bioaerosol were identification using the separate culture, a denaturing gradient gel electrophoresis (DGGE), 4’,6-diamino-2-phenyl- indole (DAPI) strain, direct genome (metagenome) method. From DAPI strain analysis of the mineral particle, it found that DNAs rose up over the KOSA source region attaching mineral particles. The results of many biological analyses indicated that a lot of species of bioaerosol exist in KOSA particle. Though some KOSA bioaerosol was a kind of food spoiling or pathogenic bacterium, our findings indicate that KOSA bioaerosol diffusion may influence human health and natural environments.

1. Introduction

Asian dust, known in Japan as “KOSA” (Literally, “yellow sand”), contributes significantly to regional and global climate and environment changes because particles can scatter and absorb solar radiation, act as chemical reaction site, and act as cloud condensation nuclei or ice nuclei in the atmosphere [1-6].

On the other hand, many species of bacteria and fungi can survive transoceanic transport through the atmosphere [7-8]. Since KOSA transport becomes a large social problem, such as “Health Damage of KOSA”, many researchers published the epidemiological research for a bronchitis *et al*

9-11).

In this study, we carried out the direct sampling of KOSA bioaerosols using the bioaerosol sampler and a tethered balloon over the KOSA source region, KOSA receptor region, the middle region of long-range transport, and biological analyses, *i.e.* separate culture, identification, DGGE, DAPI, direct genome (metagenome). The possibility of health damage by KOSA bioaerosol examined.

2. Research Objective

In order to investigate the existence of KOSA bioaerosol and possibility of its health damage, we attempted to sample directly in the mixed layer over Dunhuang (China) as the KOSA source region, Kanazawa (Japan), Suzu (Japan), Amakusa (Japan) as the KOSA region, and Choungju (Korea) as the middle point of KOSA long-range transport, using the bioaerosol sampler and a tethered balloon. Many biological analyses of sampling KOSA bioaerosol were carried out and we examined the possibility of health damage.

3. Research Method

3.1 Sampling at China and Japan (Sub-theme 1)

Bioaerosol sampling was performed on the campus of the Dunhuang City Meteorological Department (40.0˚N, 94.5˚E) as KOSA source region on August in 2006 and 2007 and October in 2008. Dunhuang City is located on the eastern border of the Takla Makan Desert, which is the source area of KOSA traveling toward Japan and Korea. As the KOSA region, Bioaerosol sampling was performed on the campus of the Kanazawa City (36.6˚N, 136.6˚E) on May in 2007 and the Suzu City (37.5˚N, 137.4˚E) on May in 2008 and Amakusa City (32.5˚N, 130.2˚E). Suzu City is located on the north coast of Noto peninsula in Japan, which is the arrival area of KOSA traveling from China over Japan Sea. Amakusa City is located on the west of Japan, which is the arrival area of KOSA traveling from China over East China Sea. Figure 1 shows the location of Dunhuang, Kanazawa, Suzu, Amakusa, and Chongju (as shown in Sub-theme 5).

Bioaerosols were collected at about 800 m (using a tethered balloon) and at 10 m above the ground on the roof of buildings. For the collection at each altitude, we used an air pump with a 0.45 μm or 0.2 μm membrane filter and sampled 0.7 m^3 of air in the course of 1 hour during the day. The 1 hour of sampling time was a maximum time until the battery of air pump ran out in the atmosphere at 800 m. Before the sampling, the filters were autoclaved with a filter holder. Environmental factors, such as temperatures and particle numbers in the atmosphere, were determined using a thermometer and a particle counter, respectively.

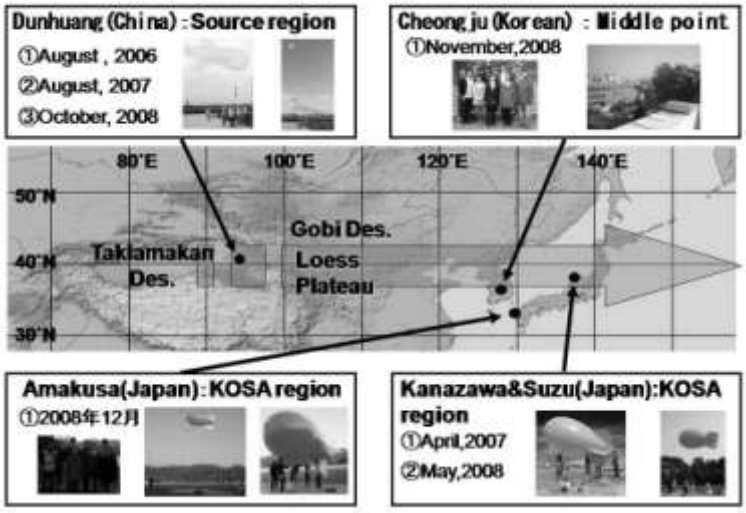

Fig.1 Locations of the sampling points.

3.2 DAPI, separate culture, DGGE analysis (Sub-theme 2)

A 1-mL aliquot of the filter wash solution was fixed with a glutaraldehyde solution at a final concentration of 1 %. The samples were stained with DAPI (4',6-diamino-2-phenyl-indole) at a final concentration of 0.5 μg mL^{-1} for 15 min and observed using an epifluorence microscope under UV excitation. The Nutrient agar was used the separate culture of fungi. To investigate the viabilities of halotolerant bacteria, 1 mL of the wash solution was inoculated to 19 mL of TS media including NaCl at final concentrations of 0 %, 3 %, 10 %, and 20 % (w/v). One mL of the filter wash solution of bioaerosol samples collected at 10 and 800 m and 5 mL of the microbial cultures with NaCl amendment were used for the extraction of genomic DNAs using SDS, proteinase K, and lysozyme as described previously[12]. Furthermore, the genomic DNAs were purified by phenol-chloroform extraction, chloroform extraction, and ethanol precipitation. A 16S rDNA region (ca. 550 bp) of the extracted genomic DNAs were amplified by polymerase chain reaction (PCR) using oligonucleotide primers for PCR-DGGE analysis, which were F341-GC; 5'- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3' and R907; 5'-CCG TCA ATT CCT TT[A/G] AGT TT-3'. The determined sequences were compared with a DDBJ (DNA Data Bank of Japan) database using the BLAST and FASTA SEARCH programs. A phylogenetic tree including all sequences was constructed according to the neighbor-joining algorithmic method using TreeViewPPC.

3.3 Biochemical information using direct genome (metagenome) analysis (Sub-theme 3)

DNA was extracted from dusts on the collected filter using cell wall lytic enzyme, lysozyme and proteinase K (Sigma-Aldrich). 16S and 18S rDNA were amplified by PCR and rDNA clone library was constructed the following procedure, as described previously[13]; Amplified 16S and 18S rDNA (1.5 kb and 1.7 kb, respectively) were cloned by ligation to the *Hinc* II site of plasmid pUC119 and introduced into *Esherichia coli* JM109 by electropolation (BIO-RAD Lab.). Inserted rDNA clones were screened by colony PCR. Then, a total of 685 clones with a rDNA insert was divided into 29 representative variants by RFLP (Restriction Fragment Length Polymorphism)

analysis using restriction enzymes *Rsa* I and *Alu* I (TAKARA BIO INC.). The DNA sequences of cloned rDNA were determined by genetic analyzer (Applied Biosystems) and the related species of dustborne microorganisms were searched by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) to DNA databases (GenBank/EMBL/DDBJ).

3.4 Health damage (Sub-theme 4)

We use the data on the total number of the KOSA observed days in Japan from Japan Meteorological Agency, and the data of the numbers of food poisoning patients due to *Bacillus cereus* from Ministry of Health, Labor and Welfare, Japan from 1999 to 2004. *Bacillus cereus* causes two distinct types of food poisoning; the diarrheal syndrome and the emetic syndrome. The emetic syndrome is caused by the ingestion of the heat-stable emetic toxin (cereulide, a cyclic peptide of 1.2 kDa). To characterization of cereulide-producing strains, we amplified cereulide synthase (CRS) gene using PCR method by Agata *et al.* [14].

3.5 Sampling at Korea and proteomics (Sub-theme 5)

Bioaerosol sampling was performed on the campus of the Cheongju in Korea (36°N, 127°E) in November, 2008 as the middle point of long-range transport. The bioaerosol sample was collected about 10 m above the ground on the roof of a building of Chunbuk National Unversity.

The bacterial particles on the filter were washed off by shaking with 10 mL of sterilized water. The wash solution was used for the comparative metaprotome analysis between different KOSA bioaerosol samples by 2-DE and protein fraction 2-DE system. The original compost soil was used to develop the extraction and purification method applied as previously described by Benndorf *et al.* [15].

4. Results

4.1 Sampling at China and Japan (Sub-theme 1)

We carried out sampling directly in the mixed layer over Dunhuang (China) as the KOSA source region, Kanazawa (Japan), Suzu (Japan), Amakusa (Japan) as the KOSA region. For increase of sample volume, the new type bioaerosol sampler was developed (Fig.2). The air pump was changed from diaphragm type (14 L min^{-1}) to the rotary type (23 L min^{-1}). For the decrease of pressure loss, the filter holder was developed. The tethered balloon was developed for the increase of sample volume (Fig.3). The new type tethered balloon was more volume of helium, i.e. from 12 m^3 to 18 m^3.

(a) 

(b) 

Fig.2 Bioaerosol samplers developed this study, a; the old type sampler, b; the new type sampler.

(a)  **(b)** 

Fig.3 Tethered balloon developed this study, a; the old type, b; the new type.

4.2 DAPI, separate culture, DGGE analysis (Sub-theme 2)

We investigated DAPI, separate culture of fungi and halobacteria, DGGE analysis of biochemistry. Epifluorescence microscopy after DAPI staining revealed that the mineral particles with white-blue selffluorescence and a diameter of more than 5 μm (Fig.4). Figure 5 shows the photograph of fungi using separate culture for fungi in the sampling over Dunhuang in 2007. From the DNA analysis of 18S rDNA, the black mold as shown in Fig. 5 (a) was *Cladosporium* sp. and the other mold as shown in Fig.5 (b) was *Aspergillus* sp.

The bacterial species compositions in the bioaerosol samples at Dunhuang in 2007 were compared by PCR-DGGE analysis targeting bacterial 16S rRNA genes (Fig. 6). The PCR products amplified from the genomic DNA directly extracted from each bioaerosol sample indicated two DGGE bands (DDd-30, DDd-31, DDd-32, and DDd-33) on the gel. Moreover, the DGGE banding patterns were similar in the bioaerosol samples collected at 10 and 800 m, indicating that similar bacterial species dominated in the samples collected at both altitudes. We excised and sequenced 19 bands from the DGGE gel (Fig. 6). Eight phylotypes were obtained after comparing the sequences with each other and with the bacterial 16S rDNA databases. All sequences belonged to the Gram-positive bacterial group and clustered with the members of the genus *Bacillus* and the genus *Staphylococcus* (Fig. 7). Moreover, 3 phylotypes among all 8 phylotypes were obtained from bioaerosol samples collected at both 10 and 800 m, indicating the presence of similar bacterial species in the atmospheres at both altitudes. The sequences of DAd-11, DAd-17 and DAd-22 obtained from the microbial cultures of 0 %, 3 %, and 10 % NaCl and the sequences of DDd-30 and DDd-32 from the DNA directly extracted from bioaerosol samples belonged to a single phylotype related to *Bacillus pumilus* with a high similarity of 99.6 %.

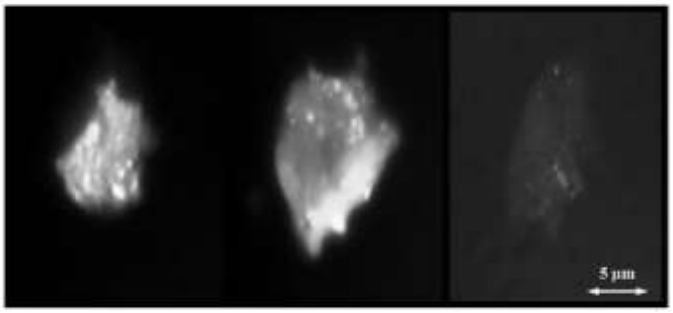

Fig.4 Epifluorescence micrograph of KOSA particle in the bioaerosol sample collected at 800 m above the ground over Dunhuang.

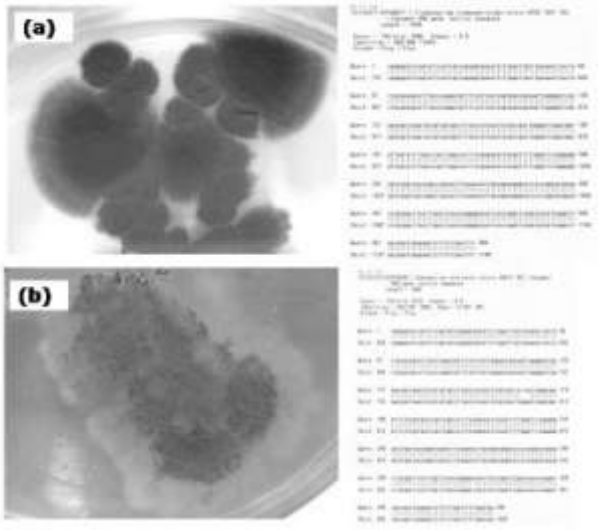

Fig.5 Photograph of molds in the bioaerosol sample collected at 800 m above the ground over Dunhuang.

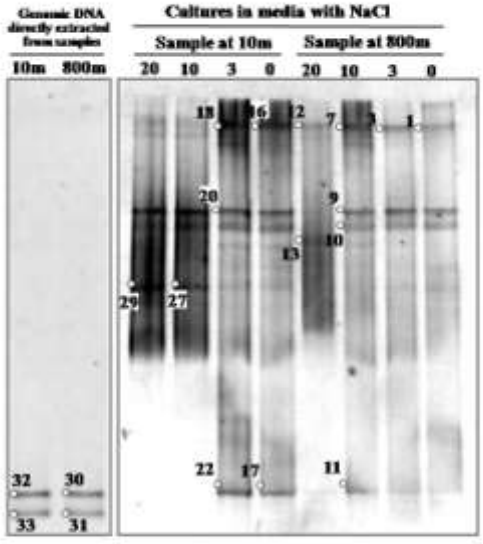

Fig.6 DGGE profile (band patterns) of amplified 16S rDNA from genomic DNA directory extracted from bioaerosol samples collected at 10 and 800 m above the ground over Dunhuang.

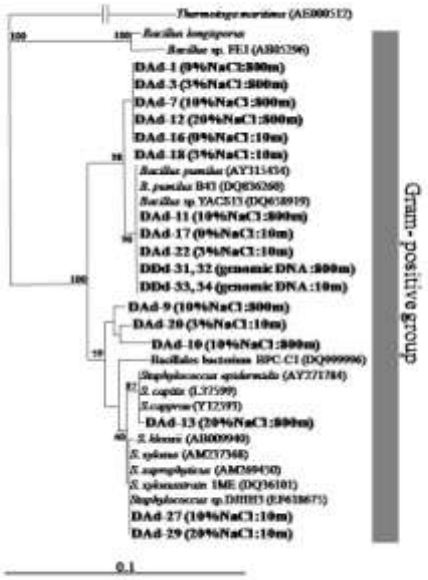

Fig.7 Phylogebetic tree including the partial sequences of 16 S rDNA amplicons excised from the DGGE gell shown in Fig.6.

4.3 Biochemical information using direct genome (metagenome) analysis (Sub-theme 3)

DNA was extracted from dust particles on the filters with cell wall lytic enzyme, lysozyme and proteinase K. The amount of DNA was about 0.1 μg from the filter of 800 m above the ground, and about 0.2 μg from the filter of 10 m height above the ground. It was estimated that the number of microorganisms at 800 m above the ground was ~2 x 10^6 in the 0.7 m^3 atmosphere air, because 1 μg of DNA contains 2 x 10^7 molecules of bacterial or fungi genome. In 10 m above the ground, it was estimated ~4 x 10^6 microorganisms in the 0.7 m^3 atmosphere air. It remains to be seen whether these microorganisms were suspended freely or attached to dust particles. By nearly complete 18S rDNA sequence analysis, it was indicated that closest species to *Rickenella fibula* (AY771599) and *Ceriporiopsis gilvescens* (AY219403) were included in the atmosphere 800 m above the ground in Dunhuang. These species are known as spore-forming fungi. The clone DHUP10 fungus shows a high DNA homology of 99.6% to an uncultured fungus in soil (AM114819)[16]. In DNA database, however, there was no sequence similar to two 18S rDNA species derived from the dust particles in atmosphere air of 800 m height. This result suggests the existence of undiscovered eukaryotic species. Recent studies of aquatic environments show unexpected eukaryotic diversity, by similar molecular approach [17, 18]. It is possible that many novel microorganisms markedly resistant to ultraviolet light or desiccation exist in atmosphere. 16S rDNA data indicated that dust particles from 800 m height in Dunhuang included bacteria closely related to *Brevibacillus sp.* (AJ313027, 99.8%), *Rhodococcus sp.* (DQ285075, 99.8%), *Delftia sp.* (EU880508, 100%), *Pseudomonas sp.* (AM411620, 99.8%) and *Agrobacterium tumefaciens* (EU592041, 99.9%). The clone DHUP7, DHUP34 and DHUP66 bacteria show a high DNA homology to uncultured *Staphylococcus sp.* (EU660426, 99.4%), *uncultured Pseudomonas sp.* (DQ088809, 99.7%) and uncultured bacterium (AY958912, 99.7%) isolated by culture-independent method. Three bacterial species of *Brevibacillus*, *Staphylococcus* and *Rhodococcus,* belong to Gram-positive bacteria. *Brevibacillus*

sp. is a spore-former, whereas *Rhodococcus sp.* has a feature of photosynthetic bacteria. Three species of *Delftia, Pseudomonas* and *Agrobacterium tumefaciens* belong to Gram-negative bacteria, which have lipopolysaccaride and can cause and aggravate respiratory diseases. *Agrobacterium tumefaciens* was typically found in soil and has been known as a plant pathogen. It should be noted that clone DHUP23 is closely related to *Delftia sp.* (EU880508) isolated from river estuary sediment in southern China. Clone DHUP49 is also closely related to *Pseudomonas sp.* (AM411620) isolated from deep sea. These ocean bacteria, *Delftia sp.* (EU880508) and *Pseudomonas sp.* (AM411620) might be of Asian desert origin and have been transported to the sea by desert wind.

These results on dustborne microorganisms of 800 m above the ground indicate that dusts contain at least eight bacterial and three fungal species including soil bacterium, spore-forming bacterium and plant pathogenic fungi. Thus these dustborne bacteria and fungi have possibilities of affecting downwind ecosystem.

4.4 Health damage (Sub-theme 4)

As shown in Table 1, there were no relation between KOSA observed days and food poisoning cases and patients due to *Bacillus cereus*. One possibility is that the isolated strains did not produce the cereulide. Figures 8 and 9 show the PCR products of CRS gene. These results show that almost strains were non-cereulide producing strains due to lack of CRS gene.

Table. 1 Total numbers of KOSA observed days and the food poisoning cases and patients due to *Bacillus cereus* from 1999- 2004

year	KOSA observed days	Food poisoning due to *Bacillus cereus*		
		cases (%)	patients (%)	Patients of the KOSA observed month
1999	248	11 (0.4)	59 (0.2)	5
2000	663	10 (0.4)	86 (0.2)	0
2001	753	9 (0.5)	444 (1.7)	0
2002	1109	7 (0.4)	30 (0.1)	4
2003	155	12 (0.8)	118 (0.4)	20
2004	466	25 (1.5)	397 (1.4)	3

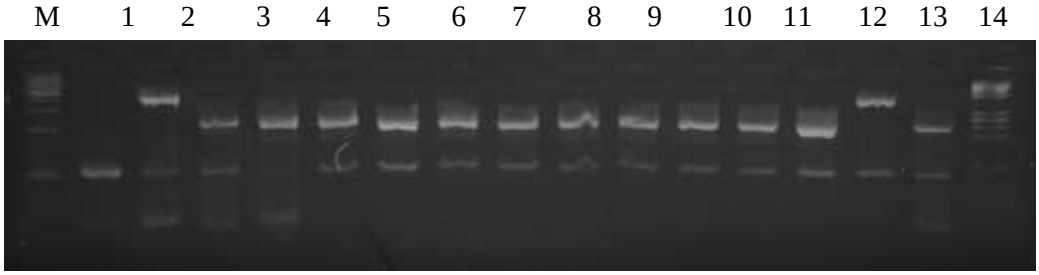

Fig. 8 PCR products amplified with CRS gene specific primer.

M、100bp ladder marker; 1、internal control; 2、CRS positive control; 3、negative control ; 4-15、samples from Dunhuang, China

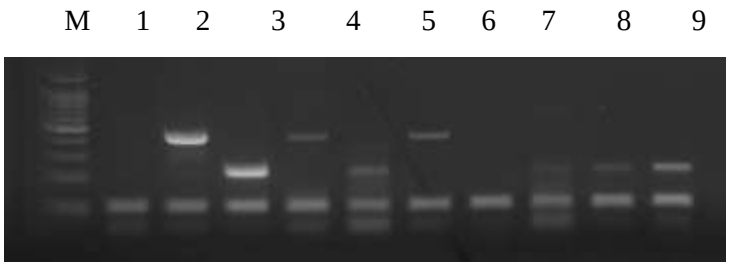

Fig. 9 PCR products amplified with CRS gene specific primer.

M、100bp ladder marker; 1、internal control; 2、CRS positive control; 3、negative control

4-10、samples from Japan

4.5 Sampling at Korea and proteomics (Sub-theme 5)

Bioaerosol sampling was performed on the campus of Cheongju in Korea (36°N, 127°E) on November 13-15 in 2008.

The identification of a protein with the respective theoretical parameters (pI, molecular mass) was accepted if the peptide mass matched with a mass tolerance within 10 ppm. The accessibility of such data has been revolutionized by the use of internet protocols, such as SWISS-2D-PAGE (http://www.expasy.org/). As shown in Figure 10, for each protein sample, two-dimensional SDS/PAGE was repeated 4–8 times, and after silver staining an average 2-DE image was constructed for comparative image analysis using the software ImageMaster (Amersham Biosciences). On every average gel image, approx. 300 spots were visualized and the 2-DE images for the DH-A (N 40˚01'4", E 94˚47'39") and DH-B (N 40˚21‘28", E 93˚48‘49") were systematically compared.

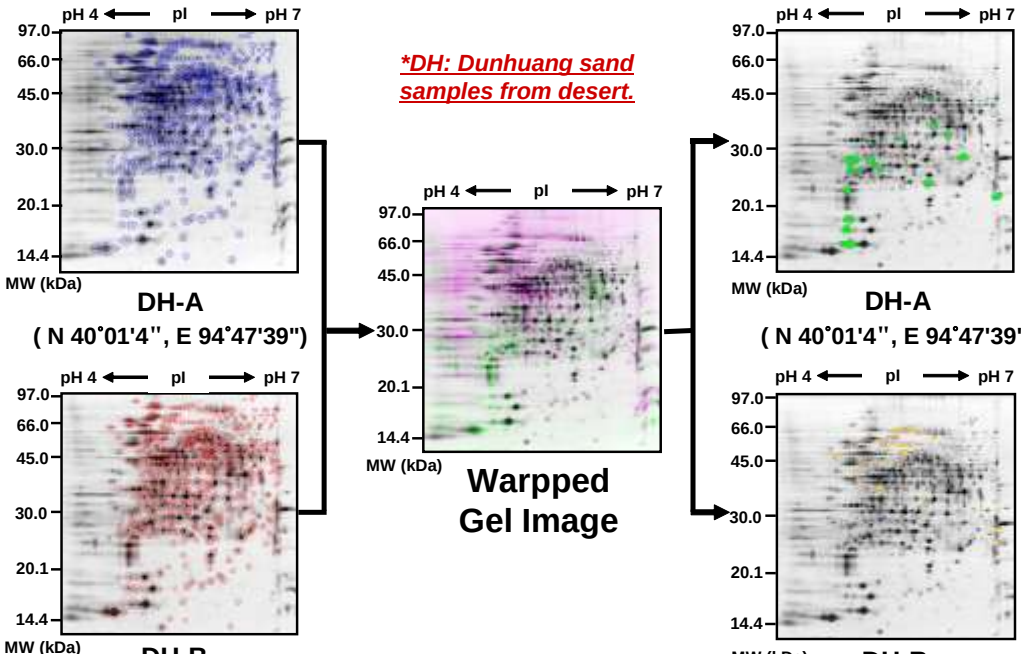

Fig. 10. Comparative metaproteome analysis between DH-A and DH-B.

5. Discussion

5.1 Sampling at China and Japan (Sub-theme 1)

We succeed in the direct sampling of KOSA bioaerosols over Dunhuang (China) as the KOSA source region, Kanazawa (Japan), Suzu (Japan), Amakusa (Japan) as the KOSA region, and Choungju (Korea) as the middle point of KOSA long-range transport, using the developed bioaerosol sampler and the developed tethered balloon. For the sampling at higher altitude, the air plane seems to be necessary and important.

5.2 DAPI, separate culture, DGGE analysis (Sub-theme 2)

The bacterial particles stainded with DAPI, which were obsereved as small particles and were coccoid-like, with a diameter of less than 1 μm. The mineral particles with attached bacteria made up approximately 10 % of the total mineral particles, with the remaining mineral particles possessing few or no bacterial cells. From the results of Fig.5, it was found that *Cladosporium* sp. and *Aspergillus* sp. rise up at KOSA source region as KOSA bioaerosols. As both molds can form a spore, it is necessary for KOSA bioaerosol living and rising to form a spore. It seems that some KOSA bioaerosol influence the human health because a kind of *Asperigillus* sp. is to act as allergens.

The microbial cultures with NaCl amendment and the DNA directly extracted from the bioaerosol samples were examined using PCR-DGGE analysis, which revealed 8 phylotypes belonging to the genus *Bacillus* and the genus *Staphylococcus* (Gram-positive bacteria) (Fig. 7). The data indicates that the atmospheric bacterial community over Dunhuang City is of low diversity and is dominated by a few Gram-positive bacterial species of the genus *Bacillus* and *Staphylococcus*. In a previous investigation at Dunhuang City in August 2006, Gram-positive bacterial species of the genus *Bacillus* were isolated from bioaerosol samples collected at a height of 100 m. *Bacillus* sp. is known to form endospores, which were resistant to environment stress and enhance their survival in the atmosphere. The desiccation condition in atmosphere would select the spore-forming bacterial survival and reduce the diversity of microbial community.

5.3 Biochemical information using direct genome (metagenome) analysis (Sub-theme 3)

Yeo *et al.* [19] reported that four fungi of *Aspergillus, Basipetospora, Fusarium* and *Penicillium* were detected from suspended particulate matter (SPM) samples taken at Seosan, Korea. According to Wu *et al.* [20] and Ho *et al.* [21], fungal spores such as *Cladsporium, Ganderma, Arthrium, Cercospora, Stemphylium, Pithomyces, Periconia, Alternaria, Botrytis* and *Nigrospora,* had significantly higher number concentrations in Taiwan, during Asian dust event. From the results of our and other research group, it has been revealed that microorganisms could be transported by airborne dust, although these have differences in the genera of bacteria or fungi. The differences might have been caused by another methods, or dust sampling points.

5.4 Health damage (Sub-theme 4)

Bacillus cereus is a spore-forming aerobic bacterium and one of the major food-borne pathogens. Many of spore-forming aerobic bacterium such as *Bacillus* sp. and *Clostridium* sp. have

relation to human infection disease. Although the present results showed no relation between KOSA and *Bacillus cereus*, more analysis considered to be important for the future.

5.5 Sampling at Korea and proteomics (Sub-theme 5)

Through this comparative image analysis, we noticed that 17 intracellular proteins showed more than 30% difference in spot intensity between DH-A and DH-B. Now we are performing MALDI–TOF analysis for each protein spot and subsequent peptide mass fingerprinting on the sample-specific protein peaks using Expasy database.

6. Conclusion

In this study, we found many kinds of microorganisms in the KOSA bioaerosols rising in the sky and that some KOSA bioaerosol might have the possibily to be pathogens or act as allergens. Future study will be focused on the isolation and identification of KOSA bioaerosols, the mechanism of the long-range transportation process of KOSA bioaerosols, and the influence analysis of KOSA bioaerosols to human health, agriculture, forestry, fisheries, and ecosystem.

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