# Phylogenetic analysis of atmospheric halotolerant bacterial communities at high altitude in an Asian dust (KOSA) arrival region, Suzu City

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Abstract: The microbial communities transported by Asian desert dust (KOSA) events have attracted much attention as bioaerosols because the transported microorganisms are thought to influence the downwind ecosystems in Korea and Japan. However, the atmospheric microbial community has not been investigated at high attitude in the KOSA arrival area. In this study, to estimate the viability and diversity of atmospheric halotolerant bacteria, which are expected to resist to various environmental stresses as well as high salinities, bioaerosol samples were collected at 10 and 600 m above the ground within the KOSA arrival area, Suzu City, Japan, during KOSA events. During the sampling period, the particle numbers at 600 m were higher than those at less than 20 m, suggesting that large particles of aerosol fall from the high attitude of 600 m to the ground surface. The microorganisms in bioaerosol samples grew in media containing up to 15

% NaCl concentrations demonstrating the viability of the halotorerant bacteria in bioaerosol samples. The PCR-DGGE analysis using 16S rDNA revealed that the bacterial species in NaCl-amended cultures were similar to the bacteria detected from the genomic DNA directly extracted from the bioaerosol samples. The 16S rDNA sequences of bacterial communities in bioaerosol samples were classified into 4 phylotypes belonging to the B. cereus or B. subtilis group. The bioaerosol samples collected at 600 m included 2 phylotypes belonging to B. subtilis, and one phylotype among all 4 phylotypes was identical between the samples at 10 and 600 m. In the atmosphere at 600 m, the halotolerant bacterial community would remain viable, and the species composition would include a few species of the genus Bacillus. During this investigation period, these atmospheric bacteria may have been vertically transported to the ground surface, where the long-range KOSA particle transport from China is frequently observed.

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Title:

# Phylogenetic analysis of atmospheric halotolerant bacterial communities at high attitude in an Asian dust (KOSA) arrival region, Suzu City

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**Abstract**

The microbial communities transported by Asian desert dust (KOSA) events have attracted much attention as bioaerosols because the transported microorganisms are thought to influence the downwind ecosystems in Korea and Japan. However, the atmospheric microbial community has not been investigated at high attitude in the KOSA arrival area. In this study, to estimate the viability and diversity of atmospheric halotolerant bacteria, which are expected to resist to various environmental stresses as well as high salinities, bioaerosol samples were collected at 10 and 600 m above the ground within the KOSA arrival area, Suzu City, Japan, during KOSA events. During the sampling period, the particle numbers at 600 m were higher than those at less than 20 m, suggesting that large particles of aerosol fall from the high attitude of 600 m to the ground surface. The microorganisms in bioaerosol samples grew in media containing up to 15 % NaCl concentrations demonstrating the viability of the halotorerant bacteria in bioaerosol samples. The PCR-DGGE analysis using 16S rDNA revealed that the bacterial species in NaCl-amended cultures were similar to the bacteria detected from the genomic DNA directly extracted from the bioaerosol samples. The 16S rDNA sequences of bacterial communities in bioaerosol samples were classified into 4 phylotypes belonging to the *B. cereus* or *B. subtilis* group. The bioaerosol samples collected at 600 m included 2 phylotypes belonging to *B. subtilis*, and one phylotype among all 4 phylotypes was identical between the samples at 10 and 600 m. In the atmosphere at 600 m, the halotolerant bacterial community would remain viable, and the species composition would include a few species of the genus

*Bacillus*. During this investigation period, these atmospheric bacteria may have been vertically transported to the ground surface, where the long-range KOSA particle transport from China is frequently observed.

**Keywords** KOSA, Asian dust, bioaerosol, halotolerant bacteria, NaCl

## Introduction

Asian desert dust (KOSA) that originates in desert regions of northern China, such as the Gobi and the Taklamakan, can carry desert aerosols eastward over the East Sea to Japan and the Pacific Ocean (Duce et al., 1980; Iwasaka et al., 1983) and possibly impact ecosystems and human health in the downwind environments (Iwasaka et al., 1988, Chung et al., 2003). In addition to mineral dust, the microbial fractions that are transported by dust storms have received considerable attention in recent years (Prospero et al., 2005). These fractions are commonly termed “bioaerosols” including viruses, bacteria, fungi, and pollen as well as plant and animal debris (Jones et al., 2004; Jaenicke 2005) and are reported to negatively influence plant and animal health and cause diseases, such as asthma (due to allergens) or other illnesses (Griffin et al., 2004; Kellogg et al., 2006; Griffin, 2007). Moreover, bioaerosols at high altitudes may act as ice nuclei and cloud condensation nuclei affecting ice-cloud processes (Pratt et al., 2009). Viable bacteria have been identified in super-cooled cloud droplets, and their ability to grow in the environment has been confirmed (Sattler et al., 2001).

Bioaerosol research has developed very rapidly during the last two decades (Mancinelli et al., 1987; Grinshpun et al., 2005), and data of microbial communities in the atmosphere have primarily been accumulated in the Caribbean and Americas, which are the most affected by dust storms originating in the Sahara and Sahel regions of North Africa (Kellogg et al., 2004; Griffin et al., 2006). In addition, in recent times, the atmospheric bioaerosols in the KOSA source area have also been investigated to clarify the physiological characteristics (Iwasaka et al., 2009) and species composition

(Kakikawa et al. 2009) of microorganisms attached on KOSA particles. However, only a few studies have focused on the atmospheric microbial communities in the KOSA arrival regions, such as Korea (Yeo et al., 2002; Wu et al., 2004) and Japan, and there is little information available on the microbial diversity in the atmosphere at high attitudes in the KOSA arrival area.

In the atmosphere, some microorganisms remain viable and maintain their ability to withstand low moisture levels, extreme temperatures, oxygen limitations, and extended UV exposure (Imshenetsky et al., 1978; Alan et al., 2004). Halotolerant bacteria are known to tolerate high salinity and be resistant to stresses, such as high pH, extreme temperatures, or desiccation (Russell, 1989; Rothschild et al., 2001). In the KOSA source area, Dunhang City, viable halotolerant bacteria have been detected using NaCl-amendment culture techniques from bioaerosol samples collected at 600 m (Maki et al., 2008). Halotolerant bacterial communities were targeted because they have possibility to typify the atmospheric microbial transport across hundreds to thousands of kilometers and at low to extreme altitudes (Okamoto et al., 2004; Echigo et al., 2005). An experimental design facilitating the cell activities of a halotolerant bacterial community in bioaerosol samples is expected to be useful for analyzing the microorganisms transported by dust events.

In this study, bioaerosol samples were collected at heights of 10 and 600 m in Suzu City during a KOSA event. The viabilities of halotolerant bacterial communities in the bioaerosol samples were evaluated with the NaCl-amendment assay using culture media at different NaCl concentrations. The compositions of the bacterial species in the microbial cultures with NaCl amendment and in the genomic DNA of environmental

bioaerosol samples were analyzed using denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified bacterial 16S rRNA genes (16S rDNA).

## Experiment

### Sampling

Bioaerosol sampling was performed on the campus of the Suzu City (37.5˚N, 137.4˚E) on May 8 in 2008 (Fig. 1). Suzu City is located on the north coast of Noto Peninsula in Japan, which is the arrival area of KOSA traveling from China. Aerosol Index data were derived on the basis of the Ozone Monitoring Instrument (OMI) (http://jwocky.gsfc.nasa.gov/) and backward trajectories calculated from the NOAA Hybrid Single Particle Lagrangian Integrated Trajectory (HYPLIT) model, (http://www.arl.noaa.gov/HYSPLIT. php). The bioaerosol sample collected at 600 m above the ground was obtained using a tethered balloon (Kobayashi et al., 2007; Maki et al., 2008). The bioaerosol sample collected at 10 m above ground was obtained from the roof of a building. For collection at each altitude, we used an air pump with a 0.45 μm membrane filter and sampled 0.7 $m^3$ of air for 1 hour during the daytime. One hour of sampling time was the maximum time until the battery for the air pump failed at 600 m in the atmosphere. Before the sampling, the filters were autoclaved with a filter holder (In-Line Filter Holder, 47 mm; Millipore, Tokyo, Japan). Environmental factors, such as the temperature and particle numbers in the atmosphere, were determined using a thermometer (EX-501: EMPEX Instruments, Inc., Tokyo, Japan) and a particle counter (KR-12A: RION CO., Ltd., Tokyo, Japan), respectively.

During the sampling period, the mean temperatures of the atmosphere at 600 m and 10 m were 15.7 ± 0.9 °C and 14.7 ± 0.9 °C, respectively, and the mean RH values were 58.3 ± 9.6 % and 80.6 ± 13.3 %, respectively. Within 1 hour of sampling, the bacterial particles on the filter were washed off by shaking with 10 ml of sterilized water containing 0.9 % (w/v) of NaCl. The washing solution was used for the determination of the bacterial species composition by PCR-DGGE analysis. Moreover, the eluted washing solution was utilized as a cultivation spike in media containing different NaCl concentrations to investigate the viabilities of halotolerant bacteria.

**Physiological experiments**

To investigate the viabilities of halotolerant bacteria, 1 ml of the washing solution was inoculated to 19 ml of TS media including NaCl at final concentrations of 0 %, 3 %, 10 %, and 15 % (w/v). The tryptic-soy-broth (TS) medium was composed of 17 g trypticasepeptone, 5 g phytonepeptone, 2.5 g $K_2PO_4$, and 2.5 g glucose in 1 liter of pure water. A TS medium has often been used for detecting and isolating bacteria from bioaerosol samples (Maki et al., 2008). After the microorganisms in the bioaerosol samples were cultivated in the media at 20 ºC in the dark, the microbial growth was estimated using 550 nm absorbance every two days. After 15 days of incubation, 5 ml of the microbial cultures was used for determining species diversity by PCR-DGGE 16S rDNA analysis.

**PCR-DGGE analysis of bacterial 16S rDNA**

One ml of the filter washing solution of bioaerosol samples collected at 10 and 600 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 (Maki et al., 2008). 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 was amplified by 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' (Muyzer et al., 1993). For each PCR reaction, 10 ng of the extracted DNAs was added to a PCR mastermix (20 µl) containing 2 µmol/l of dNTPs (TaKaRa, Ohtsu, Japan), 2 nmol/l each of the primers, and 1U of Taq DNA polymerase (TaKaRa, Ohtsu, Japan). Thermal cycling was performed using a Program Temp Control System PC-700 (ASTEC, Fukuoka, Japan) with a thermal cycling program. Amplification was verified by agarose gel electrophoresis (1.5 % w/v agarose gel).

The DGGE analysis was performed with 6 % acrylamide gels containing a linear gradient of denaturant from 40 % to 60 % (100 % denaturant contained 7 mol/l of urea and 40 % (v/v) formamide). Electrophoresis was carried out at 60 °C and 90 V for 16 h in a 1 x TAE buffer with an electrophoresis system (AE-6290; ATTA, Tokyo, Japan). After electrophoresis, the gels were stained with SYBR Gold and scanned in a Printgraph (AE-6933FXCF: ATTA, Tokyo, Japan). Several bands on the gels were excised for sequencing. The excised gel pieces were transferred to PCR tubes, and the PCR amplicons (ca.550bp) were purified by phenol-chloroform extraction and

chloroform extraction followed by ethanol precipitation. The nucleotide sequences were determined using a Dye Deoxy$^{TM}$ Terminator Cycle Sequencing Kit (ABI, CA, USA) and a DNA autosequencing system (Model 373A, ABI, CA, USA) according to the manufacturer's recommended protocol. Primer F-341 without a GC clamp was used as the sequencing primer. 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 (Saitou et al., 1987).

**Nucleotide sequence accession numbers**

The DDBJ accession numbers for the partial 16S rRNA gene sequences are AB511910 to AB511913.

## Results

**Environmental factors**

The OMI aerosol index data averaged for 3 days (6-8 May 2008) indicated higher values (>1.00) from KOSA source regions, such as the Gobi and Taklamakan Deserts and the Loess Plateau, to KOSA arrival regions, such as Korea and Japan (Fig. 1). A backward trajectory analysis for the sampling period was carried out using the NOAA HYPLIT model. The backward trajectories revealed that the higher aerosol index values originated from the desert or loess regions in China, in agreement with the OMI image, suggesting that the prevailing westerly winds in the main troposphere brought

dust particles over KOSA arrival regions (data not shown). On the other hand, according to the Japan Meteorological Agency, easterly surface winds of ~1 m/s were recorded at less than 20 m above the ground in Suzu City during the sampling period.

The atmosphere at 600 m above the ground in Suzu City during the sampling period included more aerosol particles with diameters exceeding 0.5 μm than were observed at less than 20 m above the ground, while smaller aerosol particles with diameters of 0.3 to 0.5 μm were fewer in number in the atmosphere at 600 m than in the atmosphere at less than 20 m (Fig. 2). The size distributions of the particle numbers were different in the atmosphere at 600 m and in that at less than 20 m above the ground, indicating that atmospheric constituents at 600 m originated from areas other than Suzu City. At heights of 600 m and less than 20 m, particles between 0.3 - 0.5 μm showed high concentrations of more than 78,000 particles/l and made up about 90 % of the total number of particles greater than 0.3 μm. In addition, the particles between 0.5 - 2.0 μm showed concentrations that were one order smaller than those between 0.3 - 0.5 μm and ranged from 1,400 particles/l to 7,100 particles/l. Relatively large particles greater than 2.0 μm were also detected at heights of 600 m and less than 20 m, and the total concentrations ranged from 560 particles/l to 790 particles/l.

**Physiological culture**

When the bioaerosol samples were cultivated in a TS medium including different NaCl concentrations, the microbial growth of both samples in the TS medium containing 0 % and 3 % NaCl rapidly increased within 2 days of incubation and fluctuated between 23 and 190 absorbances (approximately $10^8$ - $10^9$ cells/ml) during

the experimental period (Fig. 2). In the TS medium containing 10 % NaCl, the microorganisms of both samples (10 and 600 m) gradually grew to over 80 absorbances during 15 days of incubation. The cultures in the TS medium containing 15 % NaCl started to show weak microbial growth from the 7th day and maintained low values of absorbance of approximately 10 (approximately $10^7$ cells/ml) during the experimental period. These results indicated that the microorganisms that are tolerant to NaCl concentrations of 3%, 10% and 15% maintained their viabilities in the aerosol samples collected from 10 m and 600 m.

**DGGE analysis of the bacterial communities**

The bacterial species compositions in the bioaerosol samples were compared by PCR-DGGE analysis targeting bacterial 16S rRNA genes (Fig. 3). The PCR products amplified from the genomic DNA directly extracted from the bioaerosol samples indicated different DGGE banding patterns in the bioaerosol samples collected from 10 and 600 m, and each banding pattern was composed of a single DGGE band (SDd-1 and SDd-7, respectively) on the gel. These results indicate that different bacterial species dominated in the samples collected at both altitudes. The bacterial communities of the microbial cultures cultivated in the media containing 0 %, 3 %, 10 %, and 15 % NaCl were also investigated using PCR-DGGE analysis (Fig. 3). The DGGE bands of microbial cultures showed two dominant bands, and the banding patterns were also different in the bioaerosol samples collected at 10 and 600 m, suggesting that the bacterial community which grew in a saline media had different species compositions at 10 and 600 m. The microbial cultures of the bioaerosol samples collected at 600 m at

all NaCl concentrations tested commonly had the lower of the 2 bands (SDd-1, SAd-2, SAd-3, SAd-5, and SAd-6), and this band was also detected from the bioaerosol samples collected at 10 m at the 3 % NaCl concentrations tested. In contrast, the microbial cultures of the bioaerosol samples collected at 10 m at all NaCl concentrations tested showed the upper of the 2 bands (SDd-7, SAd-8, SAd-9, SAd-11, and SAd-13), and the same located band also appeared in the bioaerosol samples collected at 600 m at a 10 % NaCl concentration. The remaining band (SAd-12) was specific to the microbial cultures of the samples collected at 10 m in the medium containing 10 % NaCl. These results indicate that the dominant species composition of the bacterial community in the atmosphere at 600 m is different from that in the atmosphere at 10 m, while some species in the bacterial communities were common at both altitudes.

We excised and sequenced 13 bands from the DGGE gel (Fig. 4). Four phylotypes were obtained after comparing the sequences with each other and with the bacterial 16S rDNA databases (Table 1). All sequences belonged to the genus of the *Bacillus* bacterial group and clustered with the members of the *B. cereus* group and the *B. subtilis* group (Fig. 4). A single phylotype among all 4 phylotypes included the sequences of SDd-1, SAd-2, SAd-3, SAd-5, and SAd-6 from the bioaerosol sample collected at 600 m and SAd-10 from the sample collected at 10 m, and their sequences were identical to that of *Bacillus* sp. Z17 with a high similarity of 100 % and belonged to the *B. subtilis* group. This result means that some species of bacterial communities were common in the atmospheres at both altitudes. Another phylotype of 4 sequences of SDd-7, SAd-8, SAd-9, SAd-11, and SAd-13 was specific to the bioaerosol samples

collected at 10 m and related to the bacterial members of the *B. cereus* group, such as *B. cereus*, *B. thuringiensis*, and *B. mycoides*, with a high similarity of 100 %. Moreover, SDd-7 was obtained from the DNA directly extracted from bioaerosol samples suggesting that this phylotype would dominate in the atmosphere at 10 m. Another sequence of SAd-4, which was obtained from the medium containing 10 % NaCl of the bioaerosol sample collected at 600 m, indicated a low similarity of below 99.4 % to other known bacteria in the DDBJ sequence database and was relatively related to the members of the *B. subtilis* group. The remaining phylotype of SAd-12 obtained from microbial cultures with 10 % NaCl of the samples collected at 10 m was closely related to *B. subtilis* with a high similarity of 100 % and formed a cluster with the members of *B. subtilis* group, including *B. amyloliquefaciens*, in the phylogenetic tree. These results indicated that the bacterial community of the bioaerosol sample collected at 10 m belonged to the *Bacillus cereus* group and the *B. subtilis* group, while only members of the *B. subtilis* group were dominant in the samples collected at 600 m.

**Discussion**

Several results from field investigations (Griffin, 2007; Kellogg et al., 2006) have suggested that microorganisms in the atmosphere were transported around the world and influence ecological systems. The aerosol index data and the backward trajectory supported the demonstration that some amounts of aerosol particles collected in Suzu City were transported from KOSA source regions, such as the Gobi and Taklamakan Deserts and the Loess Plateau, by prevailing westerly winds in the main troposphere

(Fig. 1). The relatively larger size of aerosol particles at heights of 600 m indicated greater numbers there than at the heights of less than 20 m (Fig. 2), and the Japan Meteorological Agency reported easterly surface winds of ~1 m/s at less than 20 m above ground during the sampling period. Presumably, the aerosol particles in the atmosphere at 600 m originated from other areas in the direction of the East Sea, while those in the atmosphere at below 20 m mainly was transported from the east of Suzu City. The vertical distribution of microorganisms in this KOSA arrival region (Suzu City, Japan) during this sampling period was of interest for identifying the species composition of the bacterial population transported from the Eastern Sea and China to Japan.

Most previous studies on bioaerosols identified bacteria and fungi using traditional culture methods, focusing on the fact that the fact that the number of culturable bacteria in aerosol samples consistently increased during African dust events (Kellogg et al., 2004; Griffin et al., 2006). However, it is recognized that 90% to 99% of bacteria in natural environments cannot be cultivated by traditional methods and that many are viable but unculturable (Olsen et al., 1987). The spectrum of cultured isolates is narrower than that of diverse bacterial lineages detected using culture-independent cloning and sequencing of 16S rDNA directly collected from aerosol samples (e.g. Maron et al., 2005). A culture-independent method, which consisted of PCR-DGGE analysis of bacterial genomic DNA directly extracted from environmental samples (Muyzer et al., 1993), demonstrated that the bacterial species compositions were influenced by the dynamics of environmental factors in atmospheric environments (Maron et al., 2005). However, studies at the DNA level can detect both live

microorganisms that cannot grow in culture media and dead microorganisms that are attached to dust particles. In this study, the experimental design carried out in the TS media containing NaCl concentrations ranging from 0 % to 15 % facilitated the cell activation and growth of halotolerant bacteria that survive in bioaerosol samples collected at heights of 10 and 600 m. The PCR-DGGE analysis revealed that the viable bacterial species with NaCl amendment were similar to the bacteria detected from the DNA directly extracted from the bioaerosol samples and that the sequences of this bacterial population were classified into 4 phylotypes belonging to the *B. cereus* group and the *B. subtilis* group (Gram-positive bacteria) (Fig. 4, Table 1). The data suggested that the atmospheric bacterial community over Suzu City is dominated by a few Gram-positive bacterial species of the genus *Bacillus*. *Bacillus* sp. is known to form endospores, which are resistant to environmental stress and enhance their survival in the atmosphere (Riesenman et al., 2000). The desiccation conditions in the atmosphere appear to require the spore-forming bacterial survival and reduce the diversity of the microbial community.

Halotolerant bacteria, including Gram-positive bacteria are capable of survival in extreme environments through resistance to several types of stress, such as desiccation, UV irradiation, extreme temperatures, oxygen limitations and high salinity (Russell et al., 1989; Rothschild et al., 2001). Furthermore, since most KOSA particles traveling to Japan mixed with salt over the East Sea and contained NaCl (Tobo et al., 2009), the microorganisms attached onto KOSA particles in the atmosphere are expected to tolerate high NaCl concentrations. The PCR-DGGE analysis revealed that some of the phylotypes of the genus *Bacillus* could grow in media with wide ranges of NaCl

concentrations. In particular, species of *Bacillus* are tolerant to wide ranges of NaCl concentrations. Echigo et al. reported that similar members of halotolerant bacteria inhabit non-saline environments in an area surrounding Tokyo, Japan and indicated that they may have been introduced by KOSA events (Echigo et al., 2005). This investigation suggested that, at high attitudes, extremely halotolerant bacteria would maintain the viability and become to the strict environmental stresses of the atmosphere. In a previous investigation at Dunhuang City in August 2007, Gram-positive bacterial species of the genus *Bacillus* were isolated from bioaerosol samples collected at a height of 800 m (Maki et al., 2008). Most of the genera cultured from the aerosol samples in various worldwide investigations are Gram-positive bacteria (Shaffer et al., 1997), including *Bacillus* spp. as the most abundant genus (e.g., Di Giorgio et al., 1996; Prospero et al., 2005; Gorbushina et al., 2007). The bacterial populations detected from the atmosphere in Suzu City may be transported from other regions, such as the East Sea and China to spread their habitats around various environments.

The phylogenetic analysis using partial 16S rDNA sequences revealed that the species compositions of major bacteria were different in the bioaerosol samples collected at 10 and 600 m (Fig. 4). The sequences of the bioaerosol samples collected at 600 m belonged to only the *B. subtilis* group, while those of the bioaerosol samples collected at 10 m was composed of the phylotypes of the *B. subtilis* group and the *B. cereus* group. However, a single phylotype that originated from dominant bacteria in the bioaerosol samples collected at 600 m (the sequences of SDd-1, SAd-2, SAd-3, SAd-5, and SAd-6) was also detected from the cultures with 3 % NaCl of bioaerosol samples collected at 10 m (the sequence of SAd-10). Aerosol particles with a

relatively larger size observed at a height of 600 m would be transported from the west of Suzu City, such as the East Sea and China (Fig. 2). Furthermore, the backward trajectories and the Japan Meteorological Agency suggested different directions of wind between 10 and 600 m (the easterly wind and the westerly wind, respectively) during the sampling period. Therefore, bioaerosol particles in the atmosphere at 600 m are thought to fall vertically to the ground surface. In a previous investigation, terrestrial bacteria belonging to the genus *Bacillus* were isolated at 20,000 m above the ground, supporting the idea of the vertical transport of microorganisms into the Earth's upper atmosphere (Griffin, 2004). Therefore, mixing processes in the Japanese KOSA arrival region may transport a part of the biomass of viable halotolerant bacterial cells associated with mineral particles from the atmosphere to the ground in Suzu City.

The sequence of the phylotype detected from 10 and 600 m was identical to those of *Bacillus* sp. Z17 and belonged to the bacterial members of the *B. subtilis* group. The DDBJ sequence database indicated that *Bacillus* sp. Z17 was isolated from the alpine grassland in China. Moreover, the phylotypes including SAd-4 and SAd-12 in the cultures of bioaerosol samples collected at 10 and 600 m, respectively, were also closely related to the members of *B. subtilis* group (Fig. 4). *B. subtilis*, which was found in both the Higashi-Hiroshima City and the atmospheric dust source region of the Gobi Desert, had a similar genetic identity, supporting the hypothesis of KOSA transport and deposition (Hua et al., 2007). Relative bacterial species, such as *B. subtilis*, have been identified in aerosol collected from the northern Caribbean during African dust events (Kellogg et al., 2004) and from the Taklamakan Desert, which is a KOSA source region (Kobayashi et al., 2007). The findings of this study support the

possibility that the relative bacterial population of *B. subtilis* can maintain its viability during atmospheric dust transport and distribute its habitats toward Suzu City by atmospheric dust transport.

The phylotype including SDd-7, SAd-8, SAd-9, SAd-11, and SAd-13 of the bioaerosol sample collected at 10 m was identical to those of the bacterial members of the *B. cereus* group (Fig. 4). Moreover, this phylotype, identified as *B. cereus*, was also obtained from band SDd-7 of the DNA directly extracted from bioaerosol samples collected at 10 m. This phylotype was not detected from the samples collected at 600 m and, accordingly, this phylotype would be specific to the dominant bacterial community in the atmosphere at 10 m in Suzu City.

In this study, the viable halotolerant bacterial community in the atmosphere over Suzu City during KOSA events was found to be composed of members of the genus *Bacillus*. The relative members of *B. subtilis* group were demonstrated to dominate in the atmosphere at 600 m in Suzu City and were detected from a single culture of bioaerosol samples collected at 10 m. The members of the *B. cereus* group appear to have their habitat in the atmosphere at 10 m, together with the members of the *B. subtilis* group. Consequently, the falling processes through the boundary layer were thought to transport viable cells associated with mineral particles from an altitude of 600 m to the ground surface in Suzu City during KOSA events. This bacterial community may be carried by the wind over vast distances and travel with the KOSA particles. The bacterial members of the genus *Bacillus* were detected in the atmosphere of both the KOSA arrival region (Suzu City) and the KOSA source region (Dunhuang City), but the bacterial 16S rDNA sequences detected in this study were not

perfectly identical to the bacterial sequences obtained from the bioaerosols of Dunhuang City in the course of our investigation in August 2007 (Maki et al., 2008). This inconsistency of bacterial species is thought to be the result of differences in the sampling periods. In the future, during the same KOSA event, bioaerosols at high attitudes in both the KOSA arrival and the KOSA source regions will be analyzed to demonstrate the long-range microbial transport from China to Japan by KOSA events and explain the ecological changes and possible affects on human and animal health in downwind environments.

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Figure Legends

Fig. 1 OMI aerosol index data averaged for 3 days (6-8 May 2008).

Fig. 2 Particle concentrations of each diameter size in the atmosphere at 600 m (white bars) and to less than 20 m (grey bars) above the ground.

Fig. 3 Microbial growth of bioaerosol samples collected at heights of 600 m (a) and 10 m (b) in media containing NaCl at concentrations of 0 % (square), 3 % (circle), 10 % (triangle) and 15 % (diamond). All experiments were performed in five test tubes.

Fig. 4 DGGE profile (band patterns) of amplified 16S rDNA from genomic DNA directly extracted from bioaerosol samples at 10 and 600 m and from the bacterial cultures of bioaerosol samples collected at 10 and 600 m, which were cultivated in TS media containing 0 %, 3 %, 10 %, and 15 % NaCl. A 40 % (upper side) to 60 % (lower side) denaturing gradient was used.

Fig. 5 Phylogenetic tree including the partial sequences of 16S rDNA amplicons excised from the DGGE gel shown in Fig. 4. The tree was calculated from a dissimilarity matrix of ca. 409 bp (*Escherichia coli* numbering 421 to 848) alignment using a neighbor-joining algorithm. The accession number of each reference sequence is also given. Sample information (NaCl concentrations in the culture medium or genomic DNA directly extracted from the bioaerosol sample) is shown in parentheses.

Bootstrap values larger than 50 % (after 1,000 resampling) are indicated on the branches.

Table 1. Phylogenetic affiliation of sequences contained in the DGGE bands.

| DGGE band No. *1 | Sampling location *2 | Sample conditon *3 | Length (bp) | Category | GenBank accession no. | Closest relative | Similarity (%) *4 |
|---|---|---|---|---|---|---|---|
| SDd-1<br>SAd-2, 3, 5, 6, 10 | 10m and 600m | <15%NaCl<br>direct extracted DNA | 553 | Gram- positive group | AB511910 | *Bacillus* sp. Z clade | 100 |
| SAd-4 | 600m | 10%NaCl | 409 | Gram- positive group | AB511911 | *B. mojavensis* | 99.4 |
| SDd-7<br>SAd-8, 9, 11, 13 | 10m | <15%NaCl<br>direct extracted DNA | 544 | Gram- positive group | AB511912 | *B. cereus* | 100 |
| SAd-12 | 10m | 10%NaCl | 563 | Gram- positive group | AB511913 | *B. subtilis* | 100 |

***1 Numbers of the bands in Fig. 4 refer to the numbering of the SAd or SDd series.**

***2 Height above ground.**

***3 Cultures cultivated with NaCl at concentrations of 0%, 3%, 10% and 15%, and genomic DNA directly extracted from the bioaerosol samples.**

***4 Similarity value between each isolate and the closest relative in databases.**

1

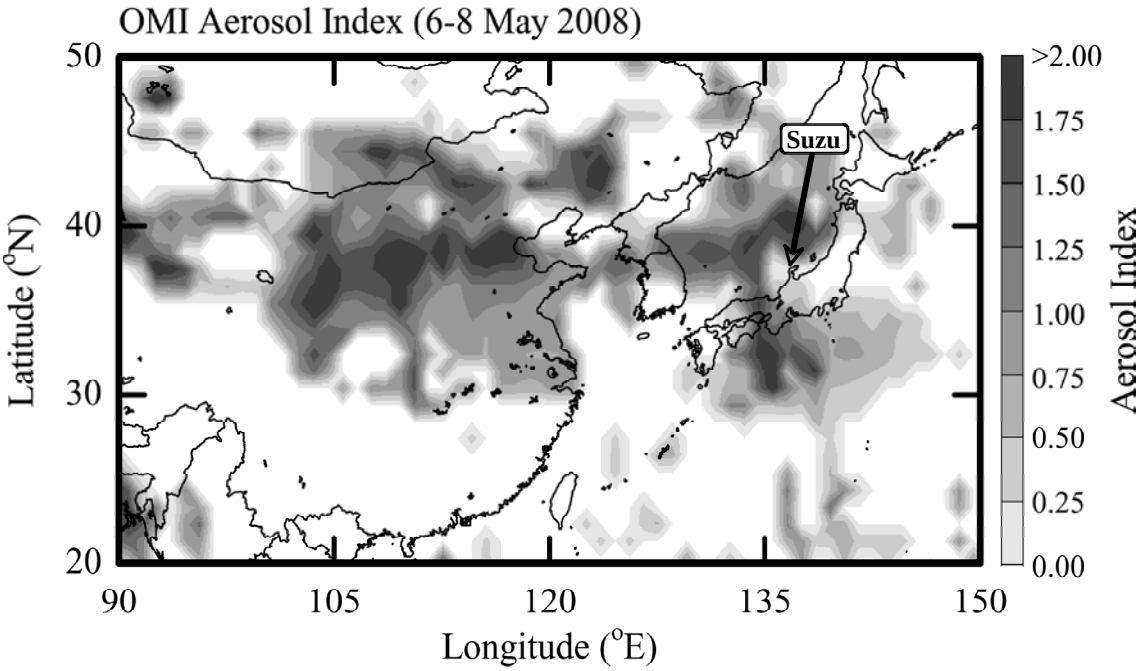
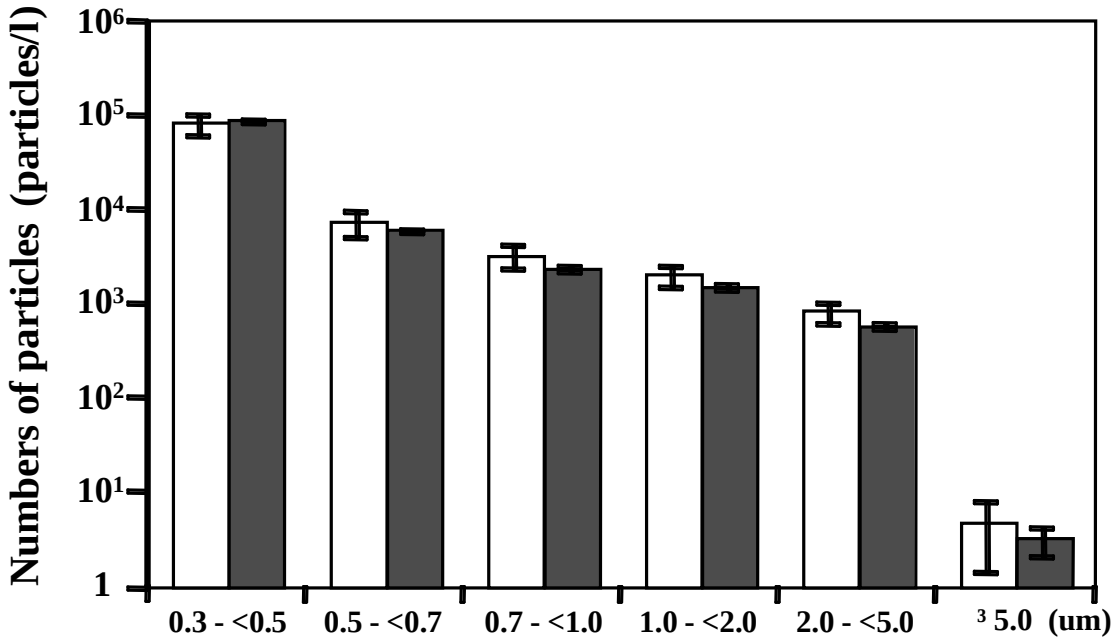



Fig. 1 T. Maki et al.

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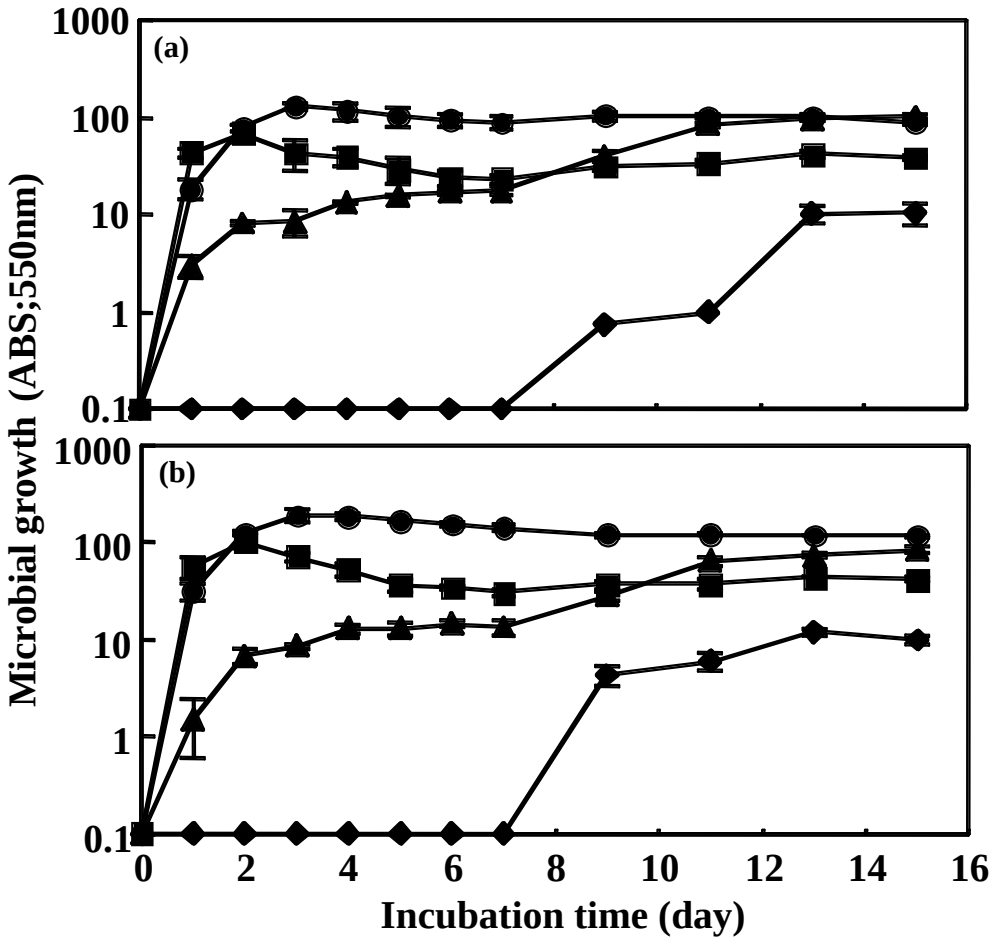


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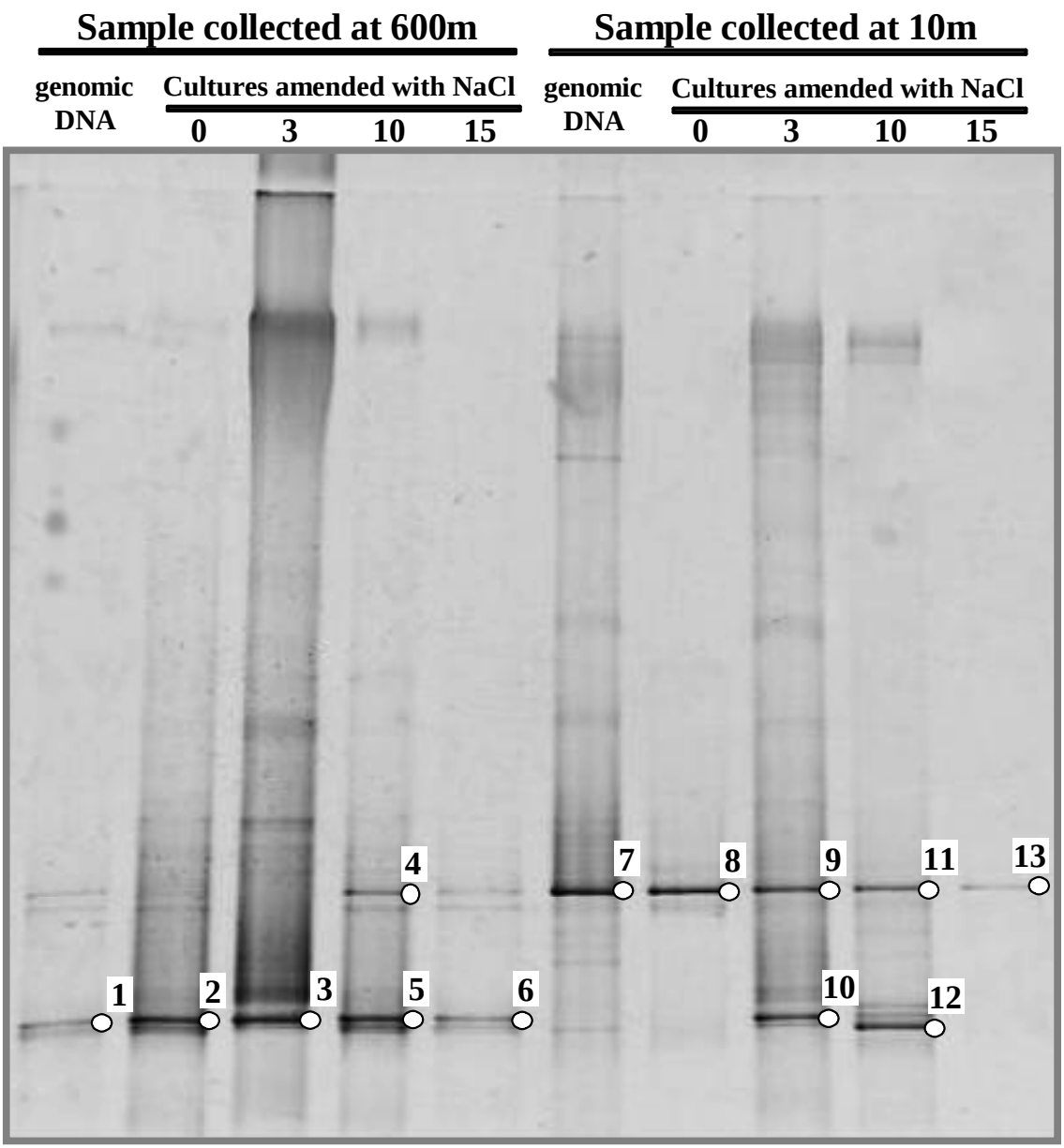


Fig. 4 T. Maki et al.

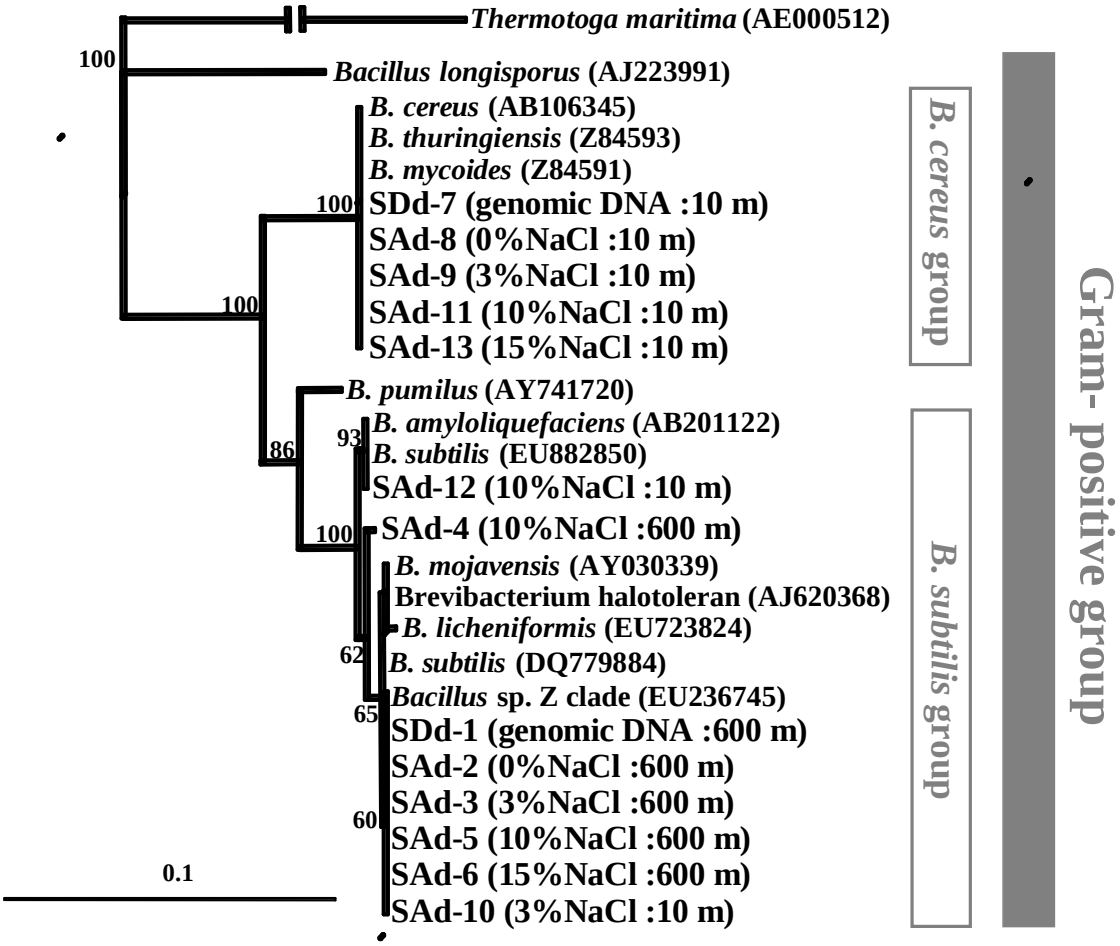


Fig. 5 T. Maki et al.