Phylogenetic diversity and vertical distribution of a halobacterial community in the atmosphere of an Asian dust (KOSA) source region, Dunhuang 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. We have analyzed bioaerosol samples collected at 10 and 800 m above the ground within the KOSA source area, Dunhuang City, China. The samples were studied by epifluorescent microscopy, revealing the presence of bacterial cells attached to mineral particles. The microorganisms in the bioaerosol samples were able to grow in media containing up to 20% NaCl, suggesting that bacteria tolerant to high salinities remain viable in the atmosphere. Phylogenetic analysis using 16S rDNA sequences revealed that halobacterial communities in the bioaerosol samples collected at both 10 and 800 m above the ground comprised a few bacterial species related to Bacillus pumilus and Staphylococcus spp. The active mixing processes of the boundary layer presumably transports viable halotolerant bacteria into the free atmosphere, where the long-range atmospheric transport of desert dust is frequently observed.

Keywords Asian dust · Bioaerosol · Halophilic bacteria · Halotolerant bacteria · KOSA · NaCl

Introduction

Asian desert dust (KOSA) that originates in desert regions of northern China, such as the Gobi and the Takla Makan Deserts, can carry desert aerosols (Duce et al. 1980; Iwasaka et al. 1983) and possibly impact ecosystem and human health in the downwind environments of Korea and Japan (Iwasaka et al. 1988). Both the mineral dust as well as the microbial communities that are transported by dust storms have been the focus of considerable attention in recent years (Prospero et al. 2005). Bioaerosol particles,
such as viruses, bacteria, fungi, and pollen, as well as plant and animal debris (Jones and Harrison 2004) are reported to negatively influence plant and animal health and cause diseases, such as asthma (due to allergens) or other illnesses (Griffin 2007; Griffin and Kellogg 2004; Kellogg and Griffin 2006). Bioaerosol research has developed very rapidly during the last two decades (Grinshpun and Clark 2005; Mancinelli and Shulls 1987), but accumulated data of microbial communities in the atmosphere are primarily limited to the Caribbean area and the Americas, which are most affected by dust storms originating in the Sahara and Sahel regions of North Africa (Griffin et al. 2006; Kellogg et al. 2004). Only a few studies have focused on the microorganisms traveling from the KOSA source areas towards Japan and Korea (Wu et al. 2004; Yeo and Kim 2002), but the physiological characteristics and microbial species compositions of the atmospheric bioaerosols in the KOSA source area have not yet been clarified.

The ability of a microorganism to survive in the atmosphere is a function of its ability to withstand desiccation, extreme temperature, oxygen limitation, and exposure to ultraviolet (UV) radiation (Alan and Harrison 2004; Imshenetsky et al. 1978). Halobacteria, such as halotolerant bacteria and halophilic bacteria, are known to tolerate conditions of high salinity and are resistant to stresses, such as high pH, extreme temperature, or desiccation (Rothschild and Mancinelli 2001; Russell 1989). As such, the halobacterial community represents a good model system for studying atmospheric travel of bioaerosols across hundreds to thousands of kilometers at low to extreme altitudes (Echigo et al. 2005; Okamoto et al. 2004).

Previous studies on long-range bacterial transports by dust storms have targeted only culturable bacteria (Griffin et al. 2003, 2006; Kellogg et al. 2004). However, it is recognized that 90–99% of bacteria in natural environments cannot be cultivated by traditional methods and that many are viable but unculturable (Olsen and Bakken 1987). A culture-independent method, consisting of a denaturing gradient gel electrophoresis (DGGE) analysis of bacterial 16S rRNA genes PCR-amplified from DNA directly extracted from environmental samples (Muyzer et al. 1993), demonstrated that the composition of bacterial species were influenced by the dynamics of environmental factors in terrestrial (Lorenz et al. 2006) or aquatic environments (Suehiro et al. 2006). However, little information is available on desert dust transport of unculturable bacteria.

In this study, bioaerosol samples were collected in August 2007 at heights of 10 and 800 m above the ground in Dunhuang City on the eastern border of the Takla Makan Desert (Fig. 1). The viabilities of halobacterial communities in the bioaerosol samples were evaluated with the NaCl amendment assay using culture media supplemented with different NaCl concentrations. The bacterial species identified in the microbial cultures with NaCl amendment and in the genomic DNA of environmental bioaerosol samples were analyzed by PCR-DGGE analysis of 16S rDNA.

**Experiment**

**Sampling**

Bioaerosol sampling was performed on the campus of the Dunhuang City Meteorological Department (40.0°N, 94.5°E) on August 16, in 2007 (Fig. 1). Dunhuang City is located on the eastern border of the Takla Makan Desert, which is the source area of KOSA traveling towards Japan and Korea. The bioaerosol sample was collected 800 m above the ground using a tethered balloon (Yamada et al. 2005), while that collected 10 m above ground was collected from the roof of a building. The collection procedure at each altitude consisted of an air pump with a 0.45-μm membrane filter that sampled 0.7 m³ of air in the course of 1 h during the day. The 1-h sampling time was a maximum length of time that the air pump could function at 800 m before the battery ran down. Prior to sampling, the filters were autoclaved with a filter holder (In-Line Filter Holder, 47 mm; Millipore, Tokyo, Japan). Environmental factors, such as temperatures and particle numbers in the atmosphere, were determined using a thermometer (EX-501: EMPEX Instruments, Tokyo, Japan) and a particle counter (KR-12A; RION, Tokyo, Japan), respectively (Fig. 2). The mean temperatures of the atmosphere at 800 m and below 20 m were 14.7 and 22.5°C, respectively, and the mean relative humidity (RH) values were 92.1 and 56.3%, respectively. Within 1 h 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 wash solution was used for the observation of KOSA particles under the epifluorescent microscope and for the determination of the bacterial species composition by the PCR-DGGE analysis. The eluted wash solution was also utilized as the cultivation spike in media containing different NaCl concentrations to investigate the viabilities of halobacteria.

**Epifluorescence microscopy**

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-phenylindole) at a final concentration of 0.5 μg/mL for 15 min and filtered through 0.2-μm pore-sized membrane filters stained with Sudan Black (Porter and Feig 1980; Russell et al. 1974). The mineral particles on the filters and the bacterial cells attached to the mineral particles were observed using
Physiological experiments

To investigate the viabilities of halotolerant bacteria, we inoculated 1 ml of the wash solution into 19 ml of Tryptic Soy Broth (TS) media supplemented with NaCl to final concentrations of 0, 3, 10, and 20% (w/v). The TS medium comprised 17 g trypticasepeptone, 5 g phytonepeptone, 2.5 g K₂PO₄, and 2.5 g glucose in 1 l of pure water. Tryptic Soy Broth medium has often been used for detecting and isolating bacteria from bioaerosol samples (Fabian et al. 2005; Kalogerakis et al. 2005). After the microorganisms in the bioaerosol samples were cultivated in the media at 20°C in the dark, microbial growth was estimated at 550 nm absorbance every 2 days (Fig. 4). After 14 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 milliliter 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 sodium dodecyl sulfate (SDS), proteinase K, and lysozyme as described previously (Maki et al. 2004). The genomic DNAs were purified by phenol–chloroform extraction, chloroform extraction, and ethanol precipitation. A 16S rDNA region (approx. 550 bp) of the extracted genomic DNAs were amplified by PCR using oligonucleotide primers for PCR-DGGE analysis, which were F341-GC (5′- CGC CCG CCG CCC GCG CCC GTC CCG CCG CCC CCG GCC TAC GGG AGG CAG CAG-3′) and R907 (5′-CCG TCA ATT CCA T[G/A] 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), which contained 2 μmol/l dNTPs (TaKaRa, Ohtsu, Japan), 2 nmol/l each of the primers, and 1U of Taq DNA polymerase (TaKaRa). Thermal cycling was performed using a Program Temp Control System PC-700 (ASTEC, Fukuoka, Japan) with a thermal cycling program, as follows: a 5-min denaturing step at 94°C, followed by 34 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C and a final 10-min extension step at 72°C. Amplification was verified by agarose gel electrophoresis (1.5%, w/v, agarose gel).

The DGGE analysis was performed on a 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× 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) (Fig. 5). Several bands on the gels were excised for sequencing. The excised gel pieces were transferred to PCR tubes, and the PCR amplicons (approx. 550 bp) were purified by phenol–chloroform extraction and chloroform extraction followed by ethanol precipitation. The nucleotide sequences were determined using a Dye Deoxy Terminator Cycle Sequencing kit (ABI, Foster City, USA) and a DNA autosequencing system (model 373A; ABI) 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 (Table 1). A phylogenetic tree including all sequences was constructed according to the neighbor-joining algorithmic method using TreeViewPPC (Saitou and Nei 1987) (Fig. 6).

Nucleotide sequence accession numbers

The DDBJ accession numbers for the partial 16S rRNA gene sequences are AB455151 to AB455158.

Results

Environmental factors and epifluorescence microscopy

The atmosphere at 800 m above the ground in Dunhuang City included fewer aerosol particles of every diameter category than that below 20 m above the ground. The size distributions of the particle numbers were similar between the atmosphere at 800 m and that below 20 m above the ground (Fig. 2), indicating that atmospheric constituents were well mixed. At the heights of 800 m and below 20 m, there were high concentrations of particles between 0.3 and 0.5 μm in diameter, with more than 91,000 particles/l, which represents about 90% of the total number of particles greater than 0.3 μm. In addition, particles between 0.5 and 2.0 μm in diameter were present at concentrations one order of magnitude lower than those between 0.3 and 0.5 μm, ranging from 2100 to 9200 particles/l. Relatively large particles greater than 2.0 μm were also detected at heights of 800 m and below 20 m, and the total concentrations ranged from 800 to 1150 particles/l.
Epifluorescence microscopy after DAPI staining revealed that the mineral particles collected at a height of 800 m were relatively large particles with white–blue self-fluorescence and a diameter of more than 5 μm (Fig. 3). The bacterial particles stained with DAPI, which were observed as small particles with bright-blue fluorescence, were located on the mineral 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.

Physiological culture

When the bioaerosol samples were cultivated in TS media supplemented with different NaCl concentrations, microbial growth in media containing 0 and 3% NaCl was rapid within 3 days of incubation and with absorbances that fluctuated over 1200 (approx. 5.0 × 10^8 to approx. 1.0 × 10^9 cells/ml) during the experimental period (Fig. 4). In the medium containing 10% NaCl, the microorganisms of both samples (10 m and 800 m) gradually increased to an absorbance of more than 1200 absorbance during 7 days of incubation. The cultures in 20% NaCl showed only weak microbial growth until the sixth day, maintaining low absorbance values below 600 (approximately 3.0 × 10^8 cells/ml) during the experimental period.

DGGE analysis of the bacterial communities

The composition of the bioaerosol samples in terms of bacterial species was compared by PCR-DGGE analysis targeting bacterial 16S rRNA genes (Fig. 5). Analysis of the PCR products amplified from the genomic DNA directly extracted from each bioaerosol sample revealed two common 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. The bacterial community of the microbial cultures cultivated in media containing 0, 3, 10, and 20 NaCl, respectively, was also investigated using PCR-DGGE analysis (Fig. 5). In the media containing 0 and 3% NaCl, the DGGE bands of microbial cultures showed the same banding patterns in the bioaerosol samples collected at 10 and 800 m, suggesting that the bacterial community which grew in low-salinity media had similar species compositions. A few bands in the upper (DAd-1, DAd-3, DAd-7, DAd-12, DAd-16, and DAd-18) and the middle gel segments (DAd-9, DAd-10 and DAd-20) were detected from the microbial cultures at all NaCl concentrations tested, while other bands in the lower segments (DAd-11, DAd-17, and DAd-22) of the gel appeared only in the microbial cultures at 0, 3, and 10% NaCl. The remaining bands (DAd-13, DAd-27, and DAd-29) were specific to the microbial cultures cultured in 10 and/or 20% NaCl and were different from the samples collected at 10 and 800 m. This result indicates that the species composition of the bacterial community in the atmosphere at 800 m, which requires high NaCl concentrations, are different from those in the atmosphere at 10 m.

We excised and sequenced 19 bands from the DGGE gel (Fig. 5). Eight phylotypes were obtained after comparing the sequences with each other and with the bacterial 16S rDNA databases (Table 1). All sequences belonged to the Gram-positive bacterial group and clustered with the members of the genus *Bacillus* and the genus *Staphylococcus* (Fig. 6). Moreover, three phylotypes among all eight 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%. These sequences formed an identical cluster with another phylotype (DDd-31 and DDd-33) from the DNA directly extracted from bioaerosol samples. One phylotype consisting of bands DAd-1, DAd-3, DAd-7, DAd-12, DAd-16, and DAd-18 was also related to *B. pumilus* with a high similarity of 99.4%. Three other phylotypes, bands DAd-9, DAd-10, and DAd-20, respectively, obtained from microbial cultures below 10% NaCl, were related to *B. pumilus* with similarities below 98.1% and formed a cluster between the clusters of *Bacillus* and *Staphylococcus* on the phylogenetic tree. The three sequences of DAd-13, DAd-27, and DAd-29 were specific to the microbial cultures of 10 and/or 20% NaCl and were classified into two phylotypes belonging to the genus *Staphylococcus*. Phylotype DAd-27/DAd-29 detected from the microbial cultures of a bioaerosol sample collected at 10 m was identical to *Staphylococcus xylosus* and another phylotype (DAd-13), obtained from the microbial cultures of a bioaerosol sample collected at 800 m, was related to *S. epidermidis* with a high similarity of 99.8%, indicating the difference between the bacterial species with altitude.

**Discussion**

The results from several field investigations (Griffin 2007; Kellogg and Griffin 2006) indicate that microorganisms in the atmosphere are transported around the world and influence ecological systems. In our study, the epifluorescence microscopy analysis detected bacterial cells attached to the surfaces of mineral particles collected at an altitude of 800 m (Fig. 2). As this observation demonstrated microbial transport by atmospheric dispersion, we subsequently investigated bacterial species composition and aspects associated with viability. The vertical distribution of microorganisms was also interest in this KOSA source region (Dunhuang City, China) as bacterial populations vertically carried from the ground area may be transported to other regions, thereby expanding their habitats to other environments.
Microbial growth in bioaerosol samples collected at heights of 10 and 800 m above the ground was observed in media containing NaCl at concentrations ranging from 0 to 20% (Fig. 4). The microbial cultures growing in media with NaCl at concentrations ranging from 0 to 800 m was also similar, suggesting that the aerosol particles of desert-sand origin were vertically well mixed (Fig. 2). In a previous investigation, terrestrial bacteria belonging to the genus Bacillus were isolated at 20,000 m above the ground, supporting the concept of vertical transportion of microorganisms into the Earth’s upper atmosphere (Griffin 2004). Therefore, as a result of active mixing processes in China’s KOSA source region, viable halobacterial cells associated with mineral particles may be transported over vast distances.

The phylogenetic analysis using partial 16S rDNA sequences revealed that the composition of the bacterial species was similar in the bioaerosol samples collected at 10 and 800 m (Fig. 6). In particular, the sequences obtained from the DNA directly extracted from bioaerosol samples were identical in bioaerosol samples collected at both altitudes, and the two phylotypes of these sequences were closely related to a single bacterial species, B. pumilus. The size distribution of aerosol particles at the heights of 10 and 800 m was also similar, suggesting that the aerosol particles of desert-sand origin were vertically well mixed (Fig. 2). In a previous investigation, terrestrial bacteria belonging to the genus Bacillus were isolated at 20,000 m above the ground, supporting the concept of vertical transportion of microorganisms into the Earth’s upper atmosphere (Griffin 2004). Therefore, as a result of active mixing processes in China’s KOSA source region, viable halobacterial cells associated with mineral particles may be transported over vast distances.

The phylotypes in cultures grown on media containing 0, 3, and 10% NaCl belonged to the genus Bacillus and were close relatives of, or identical to, B. pumilus (Fig. 6). Species of Bacillus are tolerant of wide ranges of NaCl concentrations. Moreover, phylotypes identified as B. pumilus were also obtained from the DNA directly extracted from bioaerosol samples, suggesting that these phylotypes would dominate in a KOSA-laden atmosphere. One strain of B. pumilus has been identified as a toxin-producing human pathogen (Hoult et al. 2003). This species has been isolated in both open ocean and coastal marine environments (Banerjee et al. 2007; Ivanova et al. 1999).

Fig. 6 Phylogenetic tree including the partial sequences of 16S rDNA amplicons excised from the DGGE gel shown in Fig. 5. The tree was calculated from a dissimilarity matrix of approximately 450 bp (Escherichia coli numbering 422–886) alignment using a neighbor-joining algorithm. The accession number of each reference sequence is given. Sample information (NaCl concentrations in the culture medium or genomic DNA directly extracted from bioaerosol sample) is shown in parentheses. Bootstrap values larger than 50% (after 1000 resampling) are indicated on the branches.

Phenotypic growth in bioaerosol samples collected at heights of 10 and 800 m above the ground was observed in media containing NaCl at concentrations ranging from 0 to 20% (Fig. 4). The microbial cultures growing in media with NaCl at concentrations ranging from 0 to 800 m was also similar, suggesting that the aerosol particles of desert-sand origin were vertically well mixed (Fig. 2). In a previous investigation, terrestrial bacteria belonging to the genus Bacillus were isolated at 20,000 m above the ground, supporting the concept of vertical transportion of microorganisms into the Earth’s upper atmosphere (Griffin 2004). Therefore, as a result of active mixing processes in China’s KOSA source region, viable halobacterial cells associated with mineral particles may be transported over vast distances.

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The two phylotypes with DAd-13, DAd-27, and DAd-29, which were specific to bioaerosols cultured on media containing 10 and/or 20% NaCl, were closely related to species of the genus *Staphylococcus*. These isolates are considered to be the halophilic bacteria and therefore require some concentration of NaCl for growth. These phylotypes were not detected from the DNA directly extracted from bioaerosol samples, indicating that *Staphylococcus* is a less dominant member of the bacterial community in the atmosphere over Dunhuang City and/or in the KOSA source regions. Several members of the genus *Staphylococcus*, which often colonize on the skin or in the oral cavities of humans, are capable of causing human disease (Spellerberg et al. 1998; Zhang et al. 2003) and are known to survive aerosol transport (Wisplinghoff et al. 2003). Related bacterial species, such as *S. xylosus* and *S. epidermidis*, in addition to *B. pumilus*, have been identified in aerosols collected from the northern Caribbean during African dust events (Griffin et al. 2003). The findings of this study indicate that *Staphylococcus* sp. can maintain viability during atmospheric dust transport and easily distribute to habitats.

In this study, we found that a halobacterial community composed of members of the genus *Bacillus* and *Staphylococcus* was present in the atmosphere over Dunhuang City. Based on our results, we believe that active mixing processes of the boundary layer transport viable cells associated with mineral particles from the desert surface to an altitude of 800 m. In particular, we found that *B. pumilus* dominated throughout the boundary layers of the atmosphere. These communities may serve as seeds in downwind ecosystems over vast distances. The NaCl amendment halobacteria technique employed in this study has a potential for use in bacterial transport research geared towards understanding the influence of ecological change through time and the possible effects on human and animal health in downwind environments.

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