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Bacillus-Dominant Airborne Bacterial Communities Identified During Asian Dust Events

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Abstract

Asian dust (AD) events have received significant attention due to their adverse effects on ecosystems and human health. However, detailed information about airborne pathogens associated with AD events is limited. This study monitored airborne bacterial communities and identified AD-specific bacteria and the potential hazards associated with these bacteria during AD events. Over a 33-month period, 40 air samples were collected under normal atmospheric conditions (non-AD events; $n = 34$) and during AD events ($n = 6$). The airborne bacterial communities in the air samples collected during non-AD events (non-AD sample) and AD events (AD sample) were evaluated using both culture-dependent and culture-independent methods. The bacterial diversity increased significantly, along with the 16S rRNA gene copy number, in AD samples ($p < 0.05$) and was positively correlated with PM_{10} concentration. High throughput sequencing of the 16S rRNA gene revealed that the relative abundance of the phylum Firmicutes increased substantially in AD samples (44.3 ± 5.0%) compared with non-AD samples (27.8 ± 4.3%). Within the phylum Firmicutes, AD samples included a greater abundance of *Bacillus* species (almost 23.8%) than non-AD samples (almost 13.3%). Both culture-dependent and culture-independent methods detected common predominant species closely related to *Bacillus cereus* during AD events. Subsequent multilocus sequence typing (MLST) and enterotoxin gene assays confirmed the presence of virulence factors in *B. cereus* isolates from AD samples. Furthermore, the abundance of *bceT*, encoding enterotoxin in *B. cereus*, was significantly higher in AD samples ($p < 0.05$). The systematic characterization of airborne bacterial communities in AD samples in this study suggests that *B. cereus* pose risks to public health.

Keywords Asian dust · Airborne bacterial community · *Bacillus cereus* · Bioaerosol

Introduction

Asian dust (AD) describes the clouds of fine dust particles that are lifted from the deserts in Mongolia and northern China by intense seasonal winds [1–3]. Although East Asia (China, Japan, and Korea) is the main area affected by this natural phenomenon, AD particles are also found in parts of North America and Europe, across the Pacific Ocean [1, 4–6]. Microorganisms (including viruses, bacteria, and fungi) associated with mineral-dust particles, known as "bioaerosols" [7, 8], are transported over long distances. This long-range transport has raised global concern regarding adverse health effects because the number of patients suffering from respiratory and cardiovascular disorders tends to increase during AD events [1, 7, 9]. Furthermore, intratracheal instillation of AD particles in animals has also been shown to produce symptoms of microbial infections [10, 11], suggesting a possible risk of microbial infection from bioaerosols. In view of the significant

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increase in the number of airborne microorganisms in the environment during AD events, elucidation of the biological characteristics of these particles, including identification of newly transported microorganisms, diversity of the microbial communities, and proportions of specific taxa, is critical for human health [5–7]. A previous ground-level study of the atmosphere reported that *Bacillus* species are dominant bioaerosols in all East Asian countries during AD events [3–5, 12–14]. Some species of this gram-positive genus can be transported for long distances by wind by forming spores that protect the cells from extreme heat and/or dry environments [5, 7, 13]. Although recent studies have highlighted the potential adverse health effects of the inhalation of some pathogenic *Bacillus* species, such as *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus cereus* [14–16], few studies have identified and investigated *Bacillus* virulence in AD bioaerosols, and this aspect needs to be further investigated [14, 16].

Although microbial communities, including communities of potential pathogens, can be characterized using various biotechnological methods, there is no readily available method for detailed identification and characterization of unknown bacteria from environmental samples [17, 18]. Metagenomic analysis using high throughput sequencing provides a comprehensive overview of environmental microbial communities [8, 19, 20]. However, this approach sacrifices taxonomic resolution, making this method unsuitable for identification of virulence factors [20–22]. Quantitative polymerase chain reaction (qPCR), multilocus sequence typing (MLST), and virulence gene assays have powerful discriminating capabilities to further identify specific genes and loci in bacteria. These methods can be used to determine the associations among target genotypes by comparing these genotypes with previously reported virulence factors [17, 20, 23]. However, these techniques require prior knowledge of the target organisms, which is not the case for most environmental samples.

Multiphasic examination of environmental samples using high throughput sequencing and bacterial cultivation compensates for the shortcomings of each method [24, 25]. This approach has proven useful in the characterization of microbial community structures and potential hazardous microbes in soil [26], activated sludge [27], feces [28], and aerosols [5, 29]. Therefore, it may be possible to investigate in detail the diversity of bacteria present in the inflow of bioaerosols during AD events by using a combination of culture-dependent and culture-independent molecular methods. The objective of this study was to investigate the changes in airborne bacterial abundance, diversity, and populations during AD events and determine the AD-specific bacteria using culture-dependent and culture-independent molecular methods. Potential airborne pathogens associated with AD events were also investigated in detail by joint use of high throughput sequencing, MLST, gene assays, and qPCR methods. This study provides systematically comprehensive insights into the airborne bacterial communities and potential pathogens in AD samples versus non-AD samples.

Materials and Methods

Sample Collection

In total, 40 urban air samples were collected on different days between April 2011 and November 2013, including 6 AD days and 34 non-AD days, in Seodaemun-gu, Seoul. In 2011, nine bioaerosol samples were collected from the rooftop (approximately 20 m from the ground) of the Seoul air monitoring station (37° 61′ 31″ N, 126° 93′ 01″ E). In 2012 and 2013, the rooftop (approximately 20 m from the ground) of Engineering Building No. 3, Yonsei University, Seoul (37° 33′ 42″ N, 126° 56′ 07″ E) was the site of collection of 31 bioaerosol samples. Three of the AD samples were collected in 2011 (May 1, May 3, and May 4), and the other three AD samples were collected in 2013 (March 1, March 9, and May 23). No AD events occurred in Korea in 2012. The occurrence of AD was identified based on the "Asian Dust Occurrence Reports" from the National Institute of Environmental Research (NIER) and Korea Meteorological Administration (https://web.kma.go.kr/eng/index.jsp) of South Korea. The two sampling sites were approximately 10 km apart and distant from any industrial and agricultural activities. Atmospheric environmental information during this period (PM_{10} concentration, wind speed, humidity, temperature, and geochemical elements) was provided by Korea Meteorological Administration. Atmospheric environmental parameters were measured at the Seoul air monitoring station, which was in the same local area. Atmospheric environmental parameters such as PM_{10}, SO_2, NOx, and CO levels were measured hourly, and all of these data for the same sampling period were chosen for further analysis. To track the transport pathways of air masses during AD events and non-AD events, 72-h back trajectories were calculated using the NOAA HYbrid Single Particle Lagrangian Integrated Trajectory (HYSPLIT) model (http://www.arl.noaa.gov/HYSPLIT.php). The location of the starting point of the backward trajectory was the sampling location of this study at altitudes of 500, 1000, and 1500 m above ground level (AGL).

A high-volume total suspended particulate (TSP) sampler (Thermo Scientific Inc., Franklin, MA, USA) was used to collect the bioaerosol samples for 24 h at an airflow rate of approximately 500 L/min on track-etched polycarbonate membrane filters (8 in. × 10 in.; 0.2 μm pore size; Whatman, GE). The membrane filters were autoclaved prior to sampling, and the filter holder was washed with 70% ethanol at each sampling time point. After sampling, each filter was cut into

four pieces within 1 h. One piece was stored in a sterile petri dish at 4 °C for bacterial cultivation, while the other three pieces were stored at − 20 °C for subsequent DNA extraction.

DNA Extraction and Bacterial Cultivation

All DNA was extracted from six AD event samples and 34 non-AD event samples within 1 week after completion of sampling by employing the method described by Radosevich et al. [30]. In brief, the three pieces of filter prepared for each sample were cut into 30–40 small strips and placed in a sterile 50-mL conical tube containing 45 mL of phosphate-buffered saline + 0.5% Tween-20 (PBST). The conical tube was shaken horizontally for 15 min at maximum power in a Well Block Combi-shaker mixer (VWR International Ltd., Westchester, PA, USA) and sonicated using an ultrasonic processor VCX130 for 10 min at power level 9 on ice (Sonics & Materials Inc., Newtown, CT, USA). Subsequently, the conical tube was vortexed for 1 min, and the suspension was poured into a clean conical tube. After centrifugation, the final biomaterial pellets were obtained. Genomic DNA was extracted from the pellets using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), and the extracts were stored at − 80 °C until further analysis.

The 40 air samples (6 AD samples and 34 non-AD samples) collected were cultivated within 24 h after completion of sampling from the remaining piece of each filter, which was cut into small pieces (1.5×1.5 cm^2) that were then placed on Luria-Bertani (LB) agar (Difco TM), blood agar (Becton Dickinson), and chocolate agar (Becton Dickinson) plates. The prepared plates were incubated at 37 °C for 16 h. A total of 215 (40 from AD samples and 175 from non-AD samples) bacterial isolates were obtained from the 6 AD and 34 non-AD bioaerosol samples, and the species were identified using 16S rRNA gene sequencing with the universal fD1 (5'-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5'-ACGGCTACCTTGTTACGACTT-3′) primers to obtain full-length 16S rRNA gene products. The PCR cycling conditions and sequencing processes have been described previously [31, 32]. For bacterial species identification, the 16S rRNA gene reads of the isolated bacteria were matched with the EzTaxon public database (http://www.ezbiocloud.net) with a cutoff of > 99% sequence identity [33].

Estimation of Airborne Bacterial Abundance

The total bacterial 16S rRNA gene copies from all 40 bioaerosol samples were measured by employing the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The volume of each reaction mix was 20 μL, containing 1× SYBR Master Mix (Bio-Rad, Hercules, CA, USA), primer sets (300 nM each), and tenfold serial dilutions of template DNA. The primers targeting the bacterial 16S rRNA genes have been previously described by Harms et al. [34]. A total of 1×10^1 to 1×10^7 copies/reaction of PCR products from *Escherichia coli* W3110 were used as the standard DNA templates to generate a standard curve for quantifying the 16S rRNA gene. Previously described thermal cycling conditions [34] were used. Gene copy numbers (per m^3) were calculated as described by He et al. [35]. For each qPCR run, triplicate reactions were performed, together with negative controls (containing sterile water as a template). Melting curve generation and quantitative analysis were conducted using iCycler iQ analysis software following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

High Throughput Sequencing Targeting the Bacterial 16S rRNA Gene in Bioaerosol Bacterial Communities

Forty airborne bacterial communities, including 6 AD samples and 34 non-AD samples, were analyzed using Illumina MiSeq sequencing. For this analysis, the V4 regions of the 16S rRNA gene were amplified using the 515F (5′-GTGCCAGCMGCCGCGG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers [36], which include the Illumina flow cell adapter sequences. The reverse primer contained a 12-bp barcode, facilitating multiplexed sequencing of partial 16S rRNA genes. The total volume of each PCR mix was 25 μL, containing 3 μL of template DNA, each primer at 10 μM, and 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA). Amplification was conducted with a C1000TM thermal cycler (Bio-Rad, Hercules, CA, USA) as follows: 3 min at 94 °C, followed by 35 cycles at 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 90 s, and a final extension step at 72 °C for 10 min. Then, the amplicons were purified, and the Illumina libraries were sequenced by separate Illumina MiSeq runs using a 2×150-bp paired-end protocol by Macrogen (Seoul, South Korea). Negative controls (no template and template from unused filters) were examined for all steps of the process after DNA extraction to check for contamination.

In this work, sequence analysis was performed with the Mothur package v. 1.31, adopted from the Schloss SOP [37]. Illumina MiSeq paired-end sequences were sorted by the barcodes in the paired reads using a Python script. We performed quality filtering of the MiSeq reads as described by Kozich et al. [38]. For subsequent analyses, after trimming and removal of low-quality sequences using the SILVA reference database, the remaining sequences were aligned and classified into operational taxonomic units (OTUs; 97% identity). We selected representative sequences for each OTU and used the RDP classifier to assign taxonomic data to each representative sequence [39]. After obtaining the OTUs, we assessed the alpha diversity, including the OTUs, Chao1 index, and inverse Simpson index, using Mothur. Non-metric

multidimensional scaling (NMDS) was performed using the vegan package in R to visualize the differences in taxonomic structure between AD and non-AD samples.

The following method was employed to potentially assess candidate human pathogenic bacterial sequences: first, 16S rRNA gene sequences of known pathogenic bacterial genera (Table S1) were retrieved from previously existing databases or previous studies [21, 40, 41] based on taxonomic classification of OTUs. Second, a retrieved sequence from MiSeq was regarded as belonging to a potential pathogenic bacterium if the best BLAST hit (blastn) for the sequence shared an identity of ≥ 97% with one of the known genera [40–42]. Subsequently, the sequences isolated from blastn analysis were classified once again (identity > 98%) using EzTaxon [33] to validate the identities of these sequences as those of acknowledged pathogenic bacteria. Only those candidate sequences that passed the BLAST hit and EzTaxon screening processes were designated as potential pathogenic bacteria.

MLST and Virulence Assays Based on Cultivation Method

MLST was carried out on 76 *B. cereus* isolates (17 AD isolates and 59 non-AD isolates) to determine their sequence types and identify the relatedness of these sequence types to those within the *B. cereus* MSLT database, as described by Priest et al. [43]. Internal fragments of seven housekeeping genes, namely, *glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi*, were sequenced using previously described primers [43]. The PCR conditions for amplification were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at a recommended annealing temperature for each primer set (Table S2), and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min [43]. The seven allele sequences of the locus were assigned allele numbers by comparison with the existing *B. cereus* database on the MLST website (http://pubmlst.org/bcereus). New allele numbers were assigned to the alleles that could not be identified in the database. The seven allele numbers define a sequence type (ST) number for each allele combination. The concatenated sequences for the seven gene fragments of all the STs were constructed and downloaded using the *B. cereus* database of the MLST website [43].

To validate potential virulence based on the MiSeq and MLST outcomes, an enterotoxin gene assay was carried out with 76 isolates of the *B. cereus* group. In this study, the presence of the enterotoxin genes encoding the hemolysin BL (HBL) complex (*hblC*, *hblD*, *hblA*, and *hblB*), non-hemolytic enterotoxin (NHE) complex (*nheA*, *nheB*, and *nheC*), and enterotoxin T (*bceT*) was tested. The toxin profiles for the *B. cereus* group have been relatively well characterized in the clinical field using the multiplex enterotoxin gene PCR assay [44]. Fragments of the enterotoxin genes (HBL complex, NHE complex, and enterotoxin T) were amplified from the isolates of the *B. cereus* group and sequenced as described previously [44]. The primers used in this study for amplification and sequencing are listed in Table S2.

Quantification of the Abundances of Virulence Factors

Because *B. cereus* was identified as the predominant AD-specific virulent candidate in this study and because the *bceT* gene is a known virulence factor in *B. cereus* [45], we used this species for quantitative examination of the presence of potential virulence factors. The abundance of the *bceT* gene in each bioaerosol sample was also measured using the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The primers and thermal cycling conditions targeting the *bceT* gene have been described by Shannon et al. [45]. A total of 1×10^1 to 1×10^7 copies/reaction of PCR products from the *B. cereus* strain KACC 11240 were used as standard DNA templates to generate a standard curve for quantifying the *bceT* gene. For each qPCR run, triplicate reactions were performed, together with negative controls (containing sterile water as a template). Melting curve generation and quantitative analysis were conducted using iCycler iQ analysis software, following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

Results

Correlation Among PM_{10}, Bacterial Abundance, and AD Events

The average PM_{10} of 40 air samples collected over a 33-month period was 73.3 ± 53.6 μg/m^3 (Fig. 1). Seven of the samples had extraordinarily high PM_{10} values: three samples from March 2011 (193.46, 242.8, and 279.6 μg/m^3), two samples from March 2013 (139.12 and 150.22 μg/m^3), and two samples from May 2013 (103.29 and 95.83 μg/m^3). Among these seven samples, five samples were determined as representing AD events by NIER (Fig. 1). In contrast, only one sample was determined as representing an AD event (March 2013; 131.5 μg/m^3) from the remaining 33 air samples. During AD events, the relative humidity and temperature ranged from 35% to 51% and 9.5 to 15.5 °C, respectively (Table S3). These values were significantly different from those of the non-AD events (relative humidity = 44–67%, temperature = 8.1–32.4 °C). Other parameters, such as SO_2, NO_2, CO, O_3, sunshine, and wind speed, of the AD and non-AD events were relatively constant.

The bacterial abundance in the bioaerosol samples was monitored by 16S rRNA gene-targeted qPCR (Fig. 1a). The average bacterial abundance was significantly higher

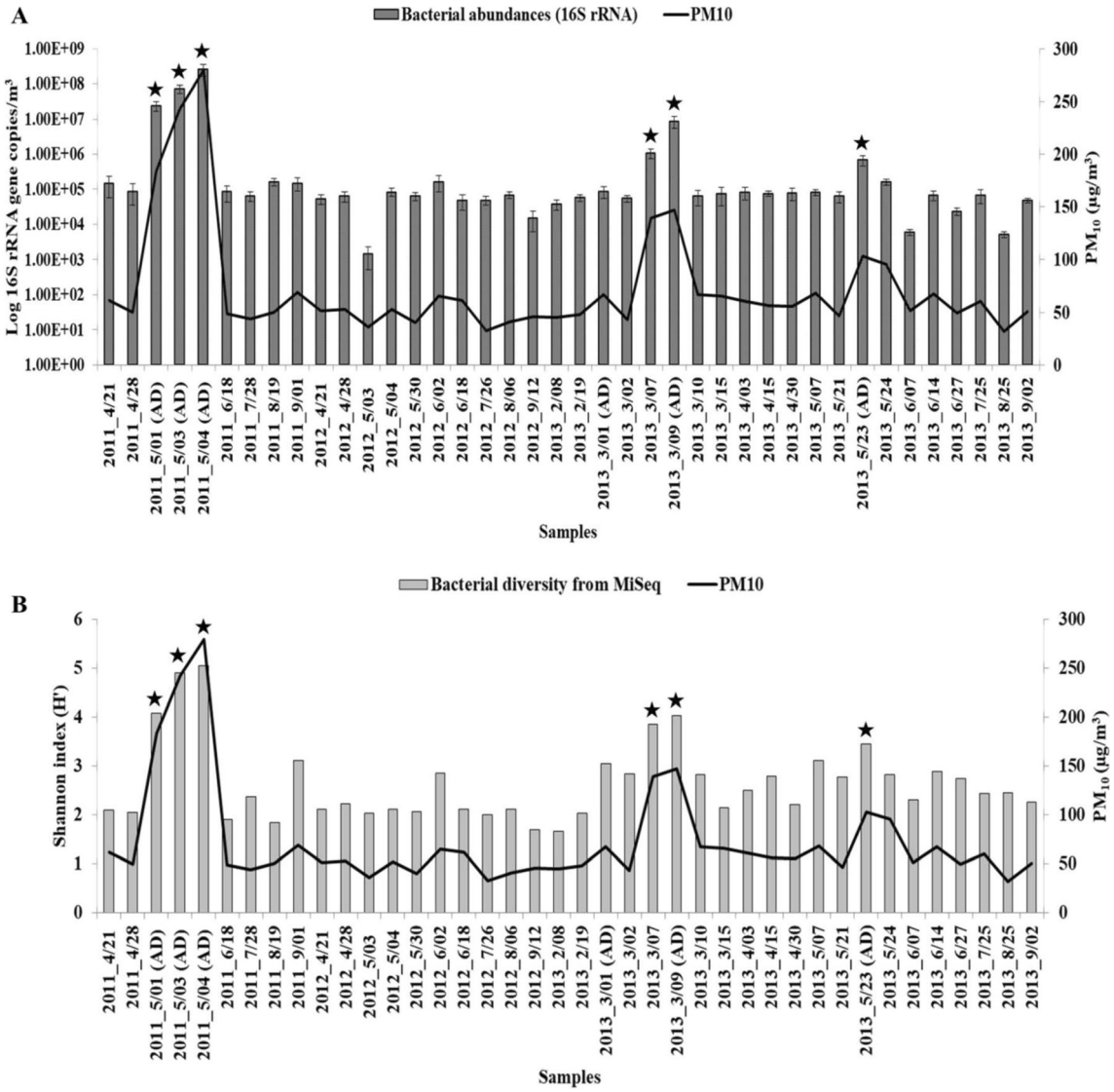

Fig. 1 Total abundances of airborne bacteria (**a**) and bacterial diversity (**b**) in the air samples during AD events ($n = 6$) and non-AD events ($n = 34$). The qPCR results are expressed in log scale to visualize the low detection levels. A single asterisk indicates a p value < 0.05 in the t test performed by SAS v.9.2

($p < 0.05$) in the AD samples (5.65×10^7 gene copies/m^3, SD = 1.42×10^6) than in the non-AD samples (6.87×10^5 gene copies/m^3, SD = 1.42×10^4). In addition, the bacterial abundance in the AD samples exhibited a high correlation with PM_{10} concentration (Fig. 1a). In contrast to the PM_{10} values, which were approximately 2–50 times higher in AD samples than non-AD samples, 16S rRNA gene copy numbers were, on average, 100-fold higher in AD samples than in non-AD samples. The bacterial diversity of the air samples collected during AD events and non-AD events was also evaluated by determination of the Shannon (H'), Chao1, and inverse Simpson indexes (Fig. 1b and Table 1). The highest degree of bacterial diversity was detected in AD samples ($H' = 4.14 \pm 0.64$, Chao1 = 858 ± 121, inverse Simpson = 104 ± 46), and bacterial diversity (H') was positively correlated with PM_{10} concentration. However, most of the bacterial diversity parameters were lower for non-AD samples than for AD samples (H' (2.88 ± 0.97), Chao1 (523 ± 216), and inverse Simpson (44 ± 29)) (Table 1).

According to the backward trajectory analysis, the air masses of the AD samples picked up dust from the Gobi Desert and passed over Inner Mongolia and China before crossing the Yellow Sea to the sampling points in Seoul,

Table 1 Bacterial diversity index of the AD and non-AD event samples

Diversity index	AD ($n = 6$)	Non-AD ($n = 34$)	
Chao1	858 ± 121	523 ± 216	$p < 0.05$
Inverse Simpson	104 ± 46	44 ± 29	$p < 0.05$

South Korea (Fig. S1). However, the backward trajectories of the non-AD samples indicated origination near the northeast region of China (Shanxi, Inner Mongolia, and Heilongjiang), relatively close to Seoul, and the air masses either passed over the Yellow Sea or North Korea before arriving at the sampling points.

Airborne Bacterial Communities and Potential Pathogens in AD Events

In the non-AD samples, the three most dominant bacterial phyla identified in the urban atmosphere were Firmicutes (27.75 ± 4.32%), β-Proteobacteria (19.55 ± 2.94%), and γ-Proteobacteria (11.72 ± 3.12%). However, the relative abundances of Firmicutes (44.32 ± 5.01%) and Actinobacteria (10.72 ± 1.04%) nearly doubled in AD samples (Fig. 2a). To determine whether the characteristics of bacterial communities are clearly distinguishable based on the microorganisms transported during AD events, we analyzed the differences in bacterial community structures between the AD samples and non-AD samples using NMDS at the phylum level. According to the NMDS analysis, there were marked differences in the airborne bacterial communities between the AD samples and non-AD samples (Fig. S2). The NMDS ordinance clustered the AD samples at the bottom left corner of the plot, indicating a unique bacterial community structure (Fig. S2). Analysis of similarities (ANOSIM) was performed, and an R value of 0.283 ($p < 0.05$) was obtained.

The airborne bacterial communities at the genus level in the AD samples and non-AD samples are shown in Fig. 2b. *Bacillus* (23.84%), *Neisseria* (9.78%), *Nocardioides* (9.07%), *Massilia* (5.78%), and *Modestobacter* (4.86%) were significantly more abundant in AD samples ($p < 0.05$) than in non-AD samples, whereas *Pseudomonas* (10.45%), *Desulfitobacterium* (7.42%), and *Escherichia/Shigella* (5.47%) were more abundant in non-AD samples than in AD samples ($p < 0.05$). Relative abundances of potential pathogens were analyzed by aligning the 16S rRNA sequences to those of acknowledged pathogenic bacteria (Table 2). The total number of sequences associated with potential pathogens was higher in samples from AD samples (0.79 ± 0.29%) than in those from non-AD samples (0.43 ± 0.11%). Among these sequences, many OTUs exhibited high sequence similarity (identity > 0.98) with known pathogenic species belonging to the genera *Bacillus*, *Neisseria*, *Pseudomonas*, *Clostridium*, *Escherichia/Shigella*, *Acinetobacter*, *Ralstonia*, and *Staphylococcus* (Table 2). In particular, the relative abundances of OTUs that shared high similarity (≥ 98%) with known pathogens such as *B. cereus* and *B. licheniformis* in AD samples were 0.37 and 0.24%, respectively, significantly higher than the abundances observed in non-AD samples ($p < 0.05$).

Validation of Potential Virulence Using Culture-Based Methods and qPCR

Overall, 137 out of 215 selected colonies formed on isolation media were identified as *Bacillus* species (Table S4). According to the full-length 16S rRNA gene sequence analysis, *Bacillus* was the predominant genus in both non-AD samples (105 among 175 isolates) and AD samples (32 among 40 isolates). The relative abundance of *Bacillus* was greater in the AD samples (80% of the total isolates) than in the non-AD samples (60% of the total isolates). The increased detection of *Bacillus* in AD samples is consistent with the culture-independent observations from high throughput sequencing analysis (Fig. 2b). *B. cereus* accounted for 42.5% and 33.7% of the *Bacillus* isolates from AD samples and non-AD samples, respectively (Table S4). The abundance of *B. licheniformis* was much lower than that of *B. cereus*, but this species also showed significantly higher abundance in AD samples (17.5%) than in non-AD samples (9.7%).

MLST was subsequently used to identify the toxin genes of the *B. cereus* and *B. licheniformis* isolates. Nearly half of the assigned STs (7 out of 17) for *B. cereus* isolates from AD samples were closely related to those of known loci from known pathogens (ST29-dlv, ST51-dlv, ST72-slv, ST138, ST184, and ST470) (Table 3). This proportion was much higher than that for non-AD samples, which had only 6.8% (4 out of 59) STs in this category, such as ST51-dlv, ST64, ST223, and ST470. Among the assigned STs from AD samples, ST72-slv, ST138, and ST184 were identified in isolates from human samples, while only one ST223 was identified in the same category from non-AD samples. The other STs were identified for the first time in this study. The MLST profiles of *B. cereus* isolates from AD samples were highly diverse compared with those from non-AD samples. However, *B. licheniformis* did not match any known human infectious STs in the MLST DB.

The subsequent enterotoxin gene assay analysis revealed significantly higher frequencies of the toxin genes in *B. cereus* isolates from the AD samples ($p < 0.05$) compared with those from the non-AD samples (Table 4). qPCR showed that the number of *bceT* genes ranged from 8.26×10^4 to 4.32×10^3 gene copies/m^3 in AD samples (Fig. 3), significantly higher ($p < 0.05$) than the value observed in non-AD samples (3.42×10^3 to 1.42×10^2 gene copies/m^3). The abundance of the *bceT* gene was not only positively correlated with the relative abundances of the potential *Bacillus* pathogens (Fig. 3) but was also highly correlated with PM_{10} concentrations greater than 100 μg/m^3 ($R^2 = 0.80$) in the AD samples (Fig. S3). Furthermore, the ratio of the *bceT* gene and the relative abundance of *Bacillus* were significantly higher in AD samples than in non-AD samples (Fig. S4).

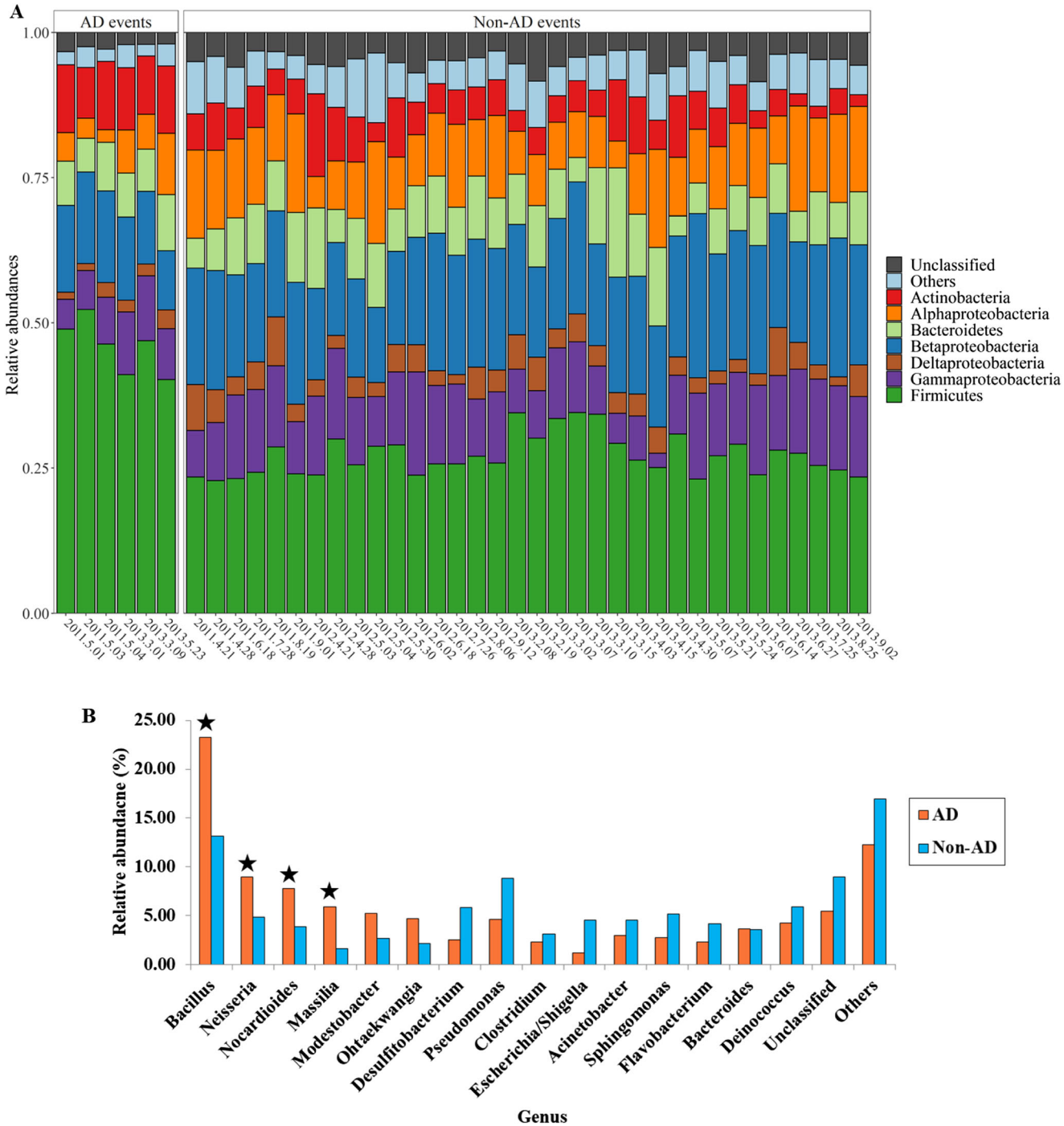

Fig. 2 Relative abundances of airborne bacterial communities at the phylum level (**a**) and genus level (**b**) between AD events ($n = 6$) and non-AD events ($n = 34$). A single asterisk indicates a p value < 0.05 in the t test performed using SAS v.9.2

Discussion

In this study, we confirmed increases in bacterial abundance and diversity in AD samples in Korea. In addition, the airborne bacterial populations were clearly distinguishable between AD and non-AD samples. Based on the 16S rRNA gene copy numbers and bacterial diversity, we estimated that the abundance and diversity of airborne bacteria were significantly affected by PM_{10} concentrations and the occurrence of AD events compared with seasonal changes during non-AD events (Fig. 1). In particular, for days when the PM_{10} was greater than 100 μg/m^3, the R^2 value for the 16S rRNA gene copy number was 0.94, and H' was 0.76 (Fig. S3). Previous studies have also demonstrated different magnitudes of increase in bacterial abundance (both CFU/m^3 and gene copies/m^3), diversity, and PM_{10} in AD samples [4–6, 12–14,

Table 2 Relative abundances of identified potential pathogens associated with human health based on the 16S rRNA gene

Pathogen candidates	Relative abundances (%)			*p* value
	NGS technology	AD events ($n = 6$)	Non-AD events ($n = 34$)	
B. cereus	MiSeq	0.371 ± 0.098	0.096 ± 0.038	< 0.0001**
B. licheniformis		0.242 ± 0.012	0.058 ± 0.008	0.034*
N. sicca		0.071 ± 0.008	0.033 ± 0.010	0.064
N. lactamica		0.034 ± 0.007	0.021 ± 0.003	0.618
P. fluorescens		0.094 ± 0.003	0.034 ± 0.007	0.055
C. difficile		0.043 ± 0.003	0.034 ± 0.003	0.382
S. flexneri		0.022 ± 0.001	0.020 ± 0.002	0.573
A. baumannii		0.032 ± 0.003	0.047 ± 0.007	0.645
R. pickettii		0.016 ± 0.004	0.012 ± 0.07	0.630
S. aureus		0.056 ± 0.002	0.041 ± 0.002	0.538

A *t* test was performed using SAS v.9.2 to evaluate statistical significance

*$p < 0.05$, **$p < 0.001$

16]. These findings suggest that bacterial abundance and diversity increase in AD samples and that transported dust particles contain various microorganisms that can alter airborne bacterial communities.

The observed changes in the bacterial community at the phylum level in the urban atmosphere confirmed the effect of AD events on bioaerosol properties. A significant increase in the abundance of the phylum Firmicutes in AD samples was consistently reported using metagenomics, DGGE, and 16S rRNA clone library analysis [4–7, 12–14]. Within the phylum Firmicutes, *Bacillus* sequences (45%) not only dominated in the metagenome sequencing database but also constituted 80% of isolates obtained from the AD samples. *Bacillus* species are commonly found in sand and dust storms in the Gobi Desert, which is the suspected source of the bioaerosols detected in the AD samples [3, 5–7, 10, 12, 13]. The relative abundances of *B. cereus*, *B. licheniformis*, *B. subtilis*, and *B. megaterium* have also been reported to increased significantly [3, 4, 14–16, 46] in AD samples. Some *Bacillus* species can form spores that can shield the bacteria from harsh desert environments in the spring, such as extreme temperatures, UV radiation, and hydrophobic conditions, thereby maintaining cell viability for long durations [1, 2, 7, 16]. Therefore, the significant increase in *Bacillus* abundance in bioaerosols observed in AD samples may reflect the resilience of these species to competition in their new habitats.

Our results showed that the relative humidity during AD events varied from 35% to 51%; the temperature varied from 9.5 to 15.5 °C, and the wind speed varied from 3.1 to 4.7 m/s.

Table 3 Sequence types and allelic profiles of MLST for the isolated *B. cereus* between AD events and non-AD events

Isolate ID	Locus (allelic profile for *B. cereus* in MLST)							MLST-based ST[a]	Source
	glp	*gmk*	*ilv*	*pta*	*pur*	*pyc*	*tpi*		
AD1	43	26	35 (2)	40	39	41	30	72-slv	Human/soil/plant
AD2	37	9	14	12	12	14	7	138	Human/soil
AD3	12	8	8	14	9	12	7	184	Human
AD4	11	9	14	29	28 (1)	27	7	51-dlv	Plant
AD5	14 (1)	8	8	10 (1)	8	17	17	29-dlv	Unknown
AD6	43	74	35	42	39	112	30	470-slv	Unknown
AD7	43	74	35	42	39	112	96	470	Unknown
Non-AD1	43	26	35	42	39	41	63	223	Human/soil
Non-AD2	11	9	14	29	28 (1)	27	7	51-dlv	Plant
Non-AD3	33	8	36	19	41	17	27	64	Soil
Non-AD4	43	74	35	42	39	112	96	470	Unknown

Numbers in parentheses represent single-base changes between alleles

slv single locus variant, *dlv* a double locus variant

[a] Sequence type (ST) determined by using the MLST website (http://pubmlst.org/bcereus/)

Table 4 Detection of enterotoxin genes as virulence factors from the isolated *B. cereus* strains between AD events and non-AD events by using PCR

Enterotoxin gene assay for *B. cereus*		Number of isolates (%)		*p* value
		AD versus non-AD		
		AD ($n = 17$)	Non-AD ($n = 59$)	
HBL complex (hemolysin)	*hblC*	15 (88.2%)	37 (62.7%)	$p < 0.05$
	hblD	16 (94.1%)	40 (67.8%)	
	hblA	15 (88.2%)	36 (61.0%)	
	hblB	15 (88.2%)	37 (62.7%)	
NHE complex (non-hemolytic enterotoxin)	*nheA*	14 (82.4%)	43 (72.9%)	
	nheB	13 (76.5%)	36 (61.0%)	
	nheC	16 (94.1%)	41 (69.5%)	
bceT		16 (94.1%)	31 (52.5%)	

In addition, there were significant differences in temperature and relative humidity between the AD samples and non-AD samples ($p < 0.05$). AD samples usually occur most frequently in spring, when midlatitude Asian deserts dry quickly due to a sharp increase in temperature and a decrease in relative humidity (40~50%) [1, 46–48]. Because suspension of terrestrial bacteria is one of the major sources of airborne bacteria, environmental conditions that facilitate this suspension also increase the diversity of airborne bacteria. Low relative humidity facilitates suspension because dry particles are lighter than wet particles, and high-speed winds also facilitate suspension because these winds have the energy required to lift particles into the air [47, 48]. Therefore, these environmental conditions may have affected the abundance, diversity, composition, and survival of airborne bacteria, because these conditions can provide selective advantages to some bacteria found in desert sand [46–48].

Although culturable and non-culturable bacterial communities in AD samples have been reported previously [3–6, 14], those investigations did not determine the differences in potential pathogenic bacterial abundance and viability between AD and non-AD samples. In the present study, high throughput sequencing revealed a significant increase in the relative abundance of *B. cereus* during AD events (Table 2). MLST analysis showed that more than 40% of the *B. cereus* isolates from the AD samples contained STs that were comparable to known toxin loci associated with human disease (Table 3). Furthermore, the enterotoxin gene assay (Table 4) and qPCR results (Fig. 3) showed a significant increase in virulent *B. cereus* in the AD samples ($p < 0.05$). Apart from

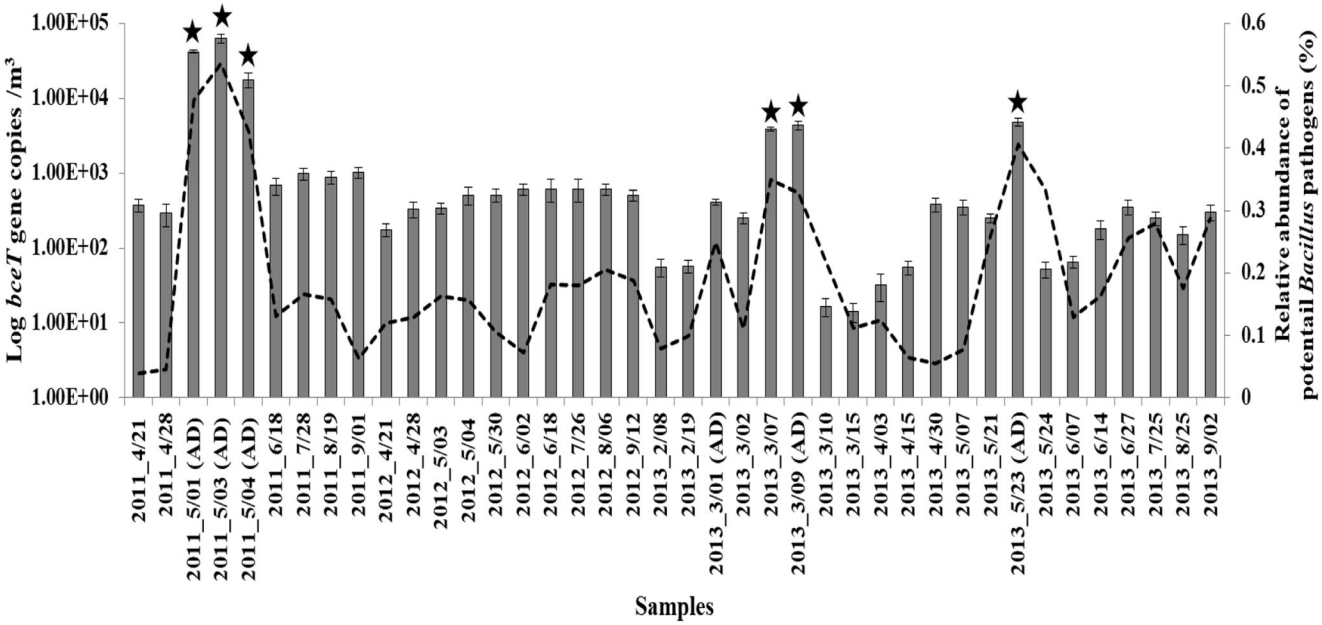

Fig. 3 Abundance of the targeted *bceT* gene of *B. cereus* in the air samples during AD events ($n = 6$) and non-AD events ($n = 34$). Bars and error bars represent the means and standard deviations. The qPCR results are expressed in log scale to visualize the low detection levels. A single asterisk indicates a p value < 0.05 in the t test performed using SAS v.9.2

occasionally causing gastrointestinal illness [49, 50], *B. cereus* is usually considered a non-pathogenic bacterium. However, previous studies have indicated that exposure to *B. cereus* can cause fatal infections with a fulminant course, such as wound infections, septicemia, meningitis, pneumonia, and respiratory infections [49–51]. *B. cereus* can produce biofilms [51, 52], which can play a major role in attachment to dust particles. Because of their protected mode of growth on inert surfaces, biofilm formation may also contribute to *B. cereus* persistence in extreme environments, such as low relative humidity and high UV intensity, by providing a setting in which replication can take place, in addition to survival as spores. Low humidity and the inhalation of airborne dust particles can compromise the integrity of the airway mucosa, which can allow *B. cereus* that may be present in dust to evade the host's innate immune system [51, 53]. Moreover, *B. cereus*, by virtue of its extensive exoenzyme armamentarium, is centrally situated between *Bacillus anthracis*, and *B. cereus* can acquire and harbor *B. anthracis* genes to produce anthrax-like pulmonary infections [49, 50]. These observations suggest that increased exposure to *B. cereus* during AD events may have a strong and direct effect on public health. Therefore, long-term monitoring and further analysis are absolutely necessary to validate the risk of *B. cereus* during AD events.

Conclusion

This study characterized the bacterial abundance and diversity in bioaerosols in AD and non-AD samples using high throughput sequencing, qPCR, MLST, and enterotoxin gene assays. The results suggested the presence of potential bacterial pathogens with known impacts on human health, including *B. cereus*. Although there have been no reports of human infectious diseases associated with exposure to *B. cereus* during AD events, our findings indicate that a significant increase in *B. cereus* abundance in AD bioaerosols may lead to increased risks to public health throughout the vast areas affected by AD events. Therefore, the potential pathogen-associated human risks of AD bioaerosols need to be further examined and verified. In future studies, clinical samples from respiratory, lung, and even gastrointestinal diseases that occur during AD events and non-AD events should be obtained and subjected to analysis to contextualize the pathogen-related information derived from the collected bioaerosol samples.

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