J Forensic Sci, May 2006, Vol. 51, No. 3
doi:10.1111/j.1556-4029.2006.00132.x
Available online at: www.blackwell-synergy.com

Lori Merrill,[1] *M.S.; John Dunbar,*[1] *Ph.D.; Jennifer Richardson,*[1] *B.S.; and Cheryl R. Kuske,*[1] *Ph.D.*

Composition of *Bacillus* Species in Aerosols from 11 U.S. Cities

ABSTRACT: A PCR-based heteroduplex assay was used to determine the presence and composition of *Bacillus* species in 11,059 Environmental Protection Agency $PM_{2.5}$ aerosol samples from 11 U.S. cities. The assay differentiated three groups: Type A containing *Bacillus anthracis* and very closely related, often pathogenic, *Bacillus cereus* and *Bacillus thuringiensis* strains; Type B containing other *B. cereus* and *B. thuringiensis* strains; and a third group of more-distantly related *Bacillus* species. Eight of the 11 cities were positive for *Bacillus* species in 50% or more of the samples, and the percent of aerosol samples that contained the HD Type A group ranged from 3% to 32%. Cities from the eastern half of the United States generally contained a higher frequency and broader diversity of *Bacillus* species than the western half of the United States. Positive samples were detected throughout the year. These results have implications for pathogen detection in environmental samples, understanding the natural evolution of new pathogenic strains, and incidence of infection caused by strains of the *B. cereus* subgroup.

KEYWORDS: forensic science, environmental detection of bacteria, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus cereus*, aerosol, heteroduplex, PCR detection

The ability to sensitively and specifically detect bacterial pathogens in the environment is critical for forensic and surveillance activities related to biothreat agent detection and for tracking disease epidemiology. Assays designed to detect pathogenic bacteria in environmental samples must be able to distinguish pathogens from related bacterial species that may also be present naturally in a wide variety of environmental sample types. In clinical samples, such as blood or tissue, much research and testing has been devoted to determining the specificity of detection methods before routine use. Similarly, in environmental settings, differentiation of the pathogen from closely related species that may be present in a sample is of greatest importance but may be difficult to achieve because of the lack of information on the presence, frequency of occurrence, and composition of normal microflora.

Detection of *Bacillus anthracis* in environmental samples is complicated by its similarity to *Bacillus cereus* and *Bacillus thuringiensis* strains, which are widespread and frequently present in soil, water, and air. The high degree of similarity among the three species is evident from identical or nearly identical ($>99\%$) rRNA gene sequences (1–3), multilocus enzyme electrophoresis patterns (4), spore protein composition determined by mass spectrometry (5), and more recently, genome sequence similarity (6–9). Analysis of amplified fragment length polymorphism (AFLP) patterns has shown that *B. anthracis*, *B. cereus*, and *B. thuringiensis* isolates are members of a least 10 major strain clusters (10,11). One cluster, referred to as AFLP Branch F (11), or AFLP group 1 (6,7), contains all tested *B. anthracis* isolates (over 200) and a small number of closely related *B. cereus* and *B. thuringiensis* strains that are predominantly food-borne pathogens.

The potential for cross-reaction of *B. cereus* and *B. thuringiensis* (or strains from other species) in PCR assays designed to detect *B. anthracis* in environmental samples can be evaluated in two ways. First, specific *B. anthracis* genetic assays can be used to directly measure the rate of false-positive assays. Because the failure rate of different assays is expected to vary and cannot be predicted *a priori*, widespread testing of numerous assays would be required to estimate average outcomes for any particular location. Second, a general assay for detection of *B. anthracis* and close relatives can be applied to assess the prevalence of potentially interfering organisms at a given location. This approach provides the broadest, most enduring scope of information because it is applicable to many types of detection methods, including both DNA- and protein-based methods. We adopted the latter approach, using a heteroduplex assay based on the 16S rRNA gene that differentiates groups of related *Bacillus* species (12), to evaluate the presence and composition of *Bacillus* species in urban aerosols from 11 U.S. cities.

Materials and Methods

Aerosol Samples

$PM_{2.5}$ aerosol samples were generously provided by the U.S. Environmental Protection Agency (EPA) air quality monitoring network. Each sample consisted of a 47-mm-diameter Teflon filter containing respirable particulates ($\leq 2.5\,\mu$m diameter) collected continuously over an *c.* 23 h interval (for standard EPA methods used see http://www.epa.gov/ttn/amtic/). A total of 11,059 daily filters were obtained from three to five sampling locations in each of 11 major U.S. cities (Table 1). The sample sets we received from each city differed in sample collection schedule, calendar period of samples sent to us, number of sampling locations in a city, and relative area sampled by each city's samplers (Table 1), because of variations in city-specific requirements for aerosol monitoring. Sample collections spanned 9–45 months during 1998–2001. Blank quality control filters (i.e., filters transported to the field and weighed in the EPA laboratories, but not used for aerosol collection) were also provided for seven of the 11 cities. In most cases, samples were stored for 1 year or more at 4°C by state

[1]Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545.

A portion of this work has been presented in abstract and poster form at the American Society for Microbiology Annual Meeting, 2003.

Received 2 Aug. 2005; and in revised form 6 Nov. 2005; accepted 6 Nov. 2005; published 21 April 2006.

TABLE 1—*$PM_{2.5}$ samples collected from 11 U.S. cities.*

City	Site	Inclusive Area (km^2)	Frequency	Number of Filters	Number of Months	Calendar Dates
Chicago	Mayfair R & P	160	Daily	947	38	January 1998–December 2001
	SE Chicago		Daily	780	28	
	Northbrook		Daily	923	42	
Seattle	Duwamish	345	Daily	444	15	October 1998–December 1999
	South Park		Daily	359	12	
	Beacon Hill		One in three	157	14	
	North Bend		One in three	98	10	
	Scan Design		One in three	98	10	
Phoenix	TF27	103	Daily	270	12	January 1999–December 1999
	TF31		Daily	309	12	
	TF32		Daily	198	10	
	TF28		One in three	117	12	
DC	McMillian	22	Daily	280	12	July 1999–June 2000
	River T		Daily	267	12	
	Ohio Dr		One in three	87	12	
Dallas	104	91	Daily	260	9	July 1999–June 2000
	105		Daily	360	12	
	106		Daily	345	12	
Houston	133	225	Daily	300	12	July 1999–June 2000
	134		Daily	329	12	
	135		Daily	313	12	
Los Angeles	Azusa	793	Daily	266	9	January 2000–September 2000
	Central LA		Daily	254	9	
	Long Beach		Daily	253	9	
New York	McDans	93	Daily	341	12	January 2000–December 2000
	IS 52		Daily	351	12	
	PS 321		One in three	115	12	
Nashville	Lockeland School	56	Daily	310	12	January 2000–December 2000
	Hillwood School		Daily	298	12	
	Hendersonville		One in three	121	12	
San Diego	01	119	Daily	321	11	July 2000–June 2001
	10		Daily	288	10	
	51		One in three	102	10	
		1,501	Daily, October–March			July 2000–December 2001
San Francisco	San Jose-Tully		One in six, April–September	205	14	
			Daily, October–March			
	Concord		One in six, April–September	193	14	
			Daily, October–March			
	San Francisco		One in six, April–September	207	14	
	San Jose-4th St.		Daily, October–March			
			One in six, April–September	193	14	
			Totals	11,059	506	

air quality monitoring agencies then shipped overnight upon request.

DNA Extraction from Filters

For each sampling location, $PM_{2.5}$ aerosol filters were sorted into monthly lots, for a total of 506 composite monthly samples (excluding blanks). Locations with a daily sampling schedule had a maximum of 31 daily filters per composite monthly sample, and locations with a 1-in-3 days or 1-in-6 days sampling schedule had a maximum of 11 or five daily filters per composite monthly sample, respectively. For each composite monthly sample, filters were cut in half and placed into 50-mL conical tubes with a maximum of 16 filters (32 halves) per tube. To wash material from the filter surfaces, 30 mL of wash buffer (100 mM phosphate buffer (pH 7.4), 0.05% Tween 80, and freshly prepared 10% sodium pyrophosphate) was added to the filters. Tubes were vortexed for 30 s and placed on a rocking shaker for 15 min. Four rounds of vortexing and shaking were performed for a total of 1 h of washing. To concentrate extracted debris, tubes were centrifuged in a swinging bucket rotor at 3700 r.p.m. for 10 min at 5–10°C in a Beckman J2-21centrifuge (Beckman Coulter, Inc., Fullerton, CA). Filters were removed with forceps and the supernatant was removed by pipette. The pellet was suspended in 750 μL of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and transferred to a sterile 2-mL screw cap tube containing 500 mg each of 106 and 500 μm zirconia–silica beads. Cells and spores were disrupted by homogenization for 2 min in a bead mill homogenizer (www.biospec.com), and centrifuged at 12,000 r.p.m. for 4 min to pellet beads and debris. Approximately 500 μL of supernatant was transferred to a sterile 2-mL tube. To increase DNA recovery, 300 μL of sterile TE buffer was added to the bead beater tube, mixed thoroughly by inversion, centrifuged at 12,000 r.p.m. for 4 min, and the second supernatant was combined with the first supernatant. To remove PCR inhibitors, 250 μL of 4 M guanidine thiocyanate and 100 μL of 100 mM phenacyl thiazolium bromide was added to the 800 μL of combined supernatant, and mixed by vortexing (13). The solution was incubated at 37°C overnight (about 18 h), then centrifuged to pellet PCR inhibitors, and the supernatant was transferred to a new sterile tube. The DNA was precipitated with 1 μL GenElute LPA (Sigma-Aldrich, Inc., St. Louis, MO), 1/10 volume 3 M sodium acetate (pH 5.2), and 0.6 volume isopropanol, washed with cold 70% ethanol, and suspended in 50 μL of 10mM Tris pH 8.0. DNA extracts were stored at −20°C.

Single monthly pooled samples did not yield sufficient DNA for reliable quantification plus several PCR assays. Therefore, aliquots of extracts were pooled by site, to yield a total of 25 μL of DNA extract per site for each of the cities. Aerosol DNA was quantified using PicoGreen intercalating dye. Ten microliters of undiluted pooled extract was analyzed in 250 μL of PicoGreen reagent (Molecular Probes, Eugene, OR), according to manufacturer's protocols, using 0.1–10 ng lambda DNA reference standards, in a spectrofluorometer equipped with a 150W Xenon source (Spex FluoroLog-2, Jobin Yvon Inc., Edison, NJ; excitation at 502 nm, emission at 520 nm).

PCR Assays

Each aerosol sample was tested in a PCR assay with primers designed to universally detect members of the bacteria domain, using the 27F/787Rb primer set (14,15), to determine if the sample could support PCR. Twenty-five microliters of PCR assays contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 μM each primer, 0.94 U of Ampli*Taq* LD polymerase (Perkin-Elmer, Boston, MA), 5 μg BSA (Roche Boehringer Mannheim, Boulder, CO), and 1 μL (*c*. 200 pg) aerosol DNA. Cycling conditions were as follows: 4 min denaturation at 94°C; annealing at 55°C for 45 s, incubation at 72°C for 60 s, and 94°C for 30 s; for 45 cycles, and a final cycle of 55°C for 45 s and 72°C for 5 min.

16S rRNA gene fragments *c*. 465 bp in length were amplified for *Bacillus*-specific heteroduplex analysis using primers Bac629F (5′-AGGGTCATTGGAAACTGGG, *E. coli* positions 629–647, Wilson, K. H. personal communication) and Bac1091R (5′-AACCCAACATCTCACGAC, *E. coli* positions 1091—1073) (12). This primer set amplified 16S rRNA gene sequences primarily from Gram-positive species, in particular members of the *Bacillus–LactoBacillus–Staphylococcus* subgroup (12). Each 50 μL PCR reaction contained 30 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 μM each primer, 1.875 U of Ampli*Taq* LD polymerase (Perkin-Elmer), 10 μg BSA (Boehringer Mannheim) and 1 μg of aerosol DNA. Cycling conditions were as follows: 3 min denaturation at 94°C; 50 cycles of 50°C for 10 s, 72°C for 70 s, and 94°C for 10 s; and a final cycle of 50°C for 45 s and 72°C for 5 min. Two PCR assays were performed for each aerosol sample. Reaction volumes were combined and precipitated using sodium acetate and isopropanol as described. Pelleted PCR products were dried using a Vacufuge (Eppendorf, Westbury, NY) for 20 min. The pellet containing the PCR amplicon was suspended in 10 μL TE (10 mM Tris, pH 7.4, 1 mM EDTA). Amplified PCR products were separated by electrophoresis on 2% agarose gels and visualized under ultraviolet light following staining with ethidium bromide.

Heteroduplex Assay

Heteroduplex assays were performed on the PCR amplicons as previously described (Fig. 1; (12)). The method uses a single-stranded fluorescent DNA probe (464 nucleotides in length) derived from the *B. anthracis* 16S rRNA gene. The hybridization probe is bound to the mixture of *Bacillus* 16S rRNA amplicons in an environmental sample. Homo- and heteroduplexes are then separated using an acrylamide gel in an ABI 377 DNA sequencer. Elution profiles contain the probe homoduplex (at 461 base pair equivalents, (bpe)), a one SNP heteroduplex with *B. anthracis* and *B. cereus* strains containing identical 16S rRNA sequences at 467 bpe (designated HD Type A), and a two SNP heteroduplex at 470 bpe representing other *B. cereus* and *B. thuringiensis* strains

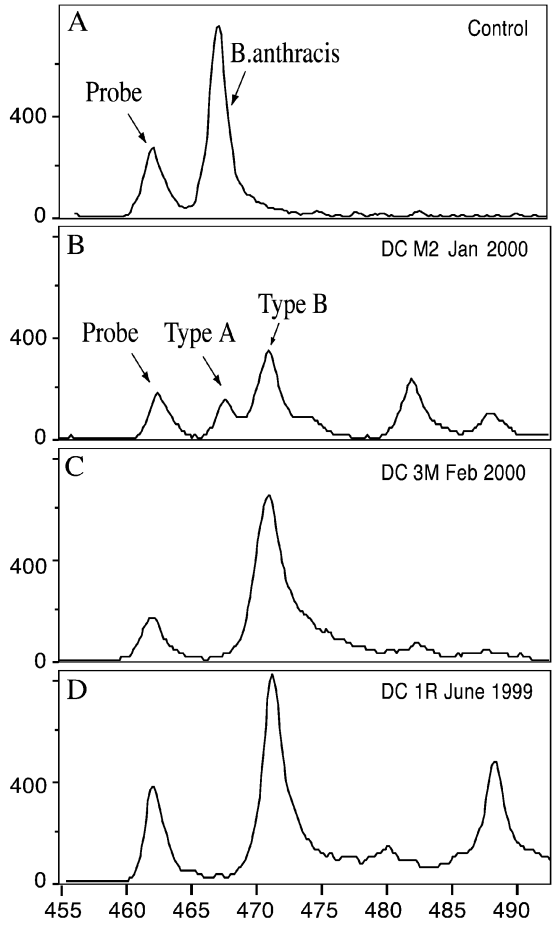

FIG. 1—*Representative heteroduplex profiles. (A) Control showing the homoduplex probe and the Type A heteroduplex comprised of probe hybridized to the PCR-amplified 16S rRNA gene from Bacillus anthracis, which contains one mismatch relative to the probe. (B–D) Examples of heteroduplex profiles from aerosol DNAs. The Types A and B heteroduplex peaks are present in (B) sample, but only the Type B peak is present (C and D) samples. Negative control profiles of the amplified probe alone always showed the homoduplex peak and never contained the Type A, Type B, or other heteroduplex peaks. X-axis is base pair equivalents (bpe). Y-axis is fluorescence.*

(designated HD Type B). The heteroduplex assay also detects more distantly related *Bacillus* species as slower migrating peaks, between 473 and 489 bpe, in the profile.

For each hybridization reaction, *c*. 15 ng of single-stranded fluorescent probe was mixed with 2 μL of PCR amplicon from each aerosol sample. NaCl was added to a final concentration of 100 mM. The mixture was heated to 94°C for 4 min, ramped to 60°C at 1°C/s, held at 60°C for 1.5 h, then ramped to 25°C to generate homo- and heteroduplexes. A 1-μL aliquot of the heteroduplex mixture was combined with 1.5 μL of loading buffer (1 μL of 15% Ficoll, 0.25 μL of 25 mM EDTA–3% blue dextran, and 0.25 μL ABI Genescan TAMRA 2500 size standard). The samples were loaded on a 0.48 × MDE gel (FMC Bioproducts, Rockland, ME) containing 0.6 × Tris-Borate-EDTA (10 × stock is 890 mM Tris-OH, 890 mM boric acid, 2 mM EDTA; TBE) buffer. Electrophoresis was performed with an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA) in GeneScan mode at 3000 V, 40°C with 0.6 × TBE buffer for 3.25 h. ABI GeneScan analytical software version 3.1 was used for data collection and fragment analysis. Analysis parameters included the use of a peak detection threshold of 50 fluorescence units, peak minimum half-width of 5 pts, local southern sizing method, and

rightmost split peak correction. Positives were scored based on the presence of fluorescent heteroduplex peaks with heights greater than 50 fluorescent units occurring in profiles between positions 467 and 489 bpe.

Results

DNA Concentration in Aerosol Samples

The DNA concentration in individual composite monthly samples was very low and could not be determined because the concentrations were below the threshold of reliable detection. The DNA quantity was therefore estimated using aliquots of monthly samples pooled across a calendar year. Our DNA yields ranged from 0.34 to 1.22 ng total DNA per pooled monthly sample, and varied considerably by city (compare means, Table 2) and by location within city (compare standard deviations, Table 2). It is important to note that the composition of the extracted DNA is unknown, and probably contains fungal and plant pollen DNA in addition to bacterial DNA.

Calibration of the HD Assay with Bacillus *AFLP Type*

Results from the heteroduplex assay correlated with AFLP genomic fingerprint analysis. In a set of 68 *B. anthracis*, *B. cereus*, and *B. thuringiensis* isolates, all 24 isolates with the Type A peak (467 bpe) were members of AFLP Branch F that contains all *B. anthracis* and a few, mostly pathogenic, *B. cereus* and *B. thuringinsis* isolates (Fig. 2; (11)). The remaining 45 isolates exhibited the Type B peak (470 bpe) and were all members of the other six AFLP branches (Fig. 2).

PCR Assays on the Aerosol Samples

The 506 composite $PM_{2.5}$ aerosol samples were tested for the general presence of bacterial DNA using universal bacterial primers 27F/787R. Seventy-one percent of the samples were positive as indicated by detection of visible PCR amplicons on ethidium bromide-stained agarose gels. To evaluate whether the frequency of positives was related to the amount of particulate matter collected, we used sampler schedule and number of filters per composite sample as crude categorical indicators of particulate loads. In terms of sampler schedules, the percentage of PCR-positive samples for bacterial DNA varied as follows: daily samplers, 70.9% positive (371 samples); 1-in-3-day samplers, 81.3% positive (91 samples); 1-in-6-day samplers, 47.7% positive (44 samples). A more detailed analysis of the percentage of positives as a function of the number of filters per composite sample exhibited no apparent correlation (data not shown). Eighty-six of the city samples that failed to support amplification of a general amplicon using the universal bacterial primers ($n = 147$) gave positives with the *Bacillus* primer set, probably because of differences in the ability of the PCR primer sets to amplify DNA and the greater detection sensitivity of the fluorescent heteroduplex assay used in the latter analyses (12). Combining both sets of data, 88% of the samples tested positive for bacterial DNA. Overall these observations suggest that the presence of detectable bacterial DNA in any aerosol sample is dependent on numerous factors that may be difficult to characterize or predict.

TABLE 2—*Average amount of DNA extracted from pooled monthly Environmental Protection Agency $PM_{2.5}$ filters from 11 U.S. cities.*

City	DNA Per Pooled Monthly Sample*
Chicago	0.57 (0.16)
Seattle	0.95 (0.39)
Phoenix	0.66 (0.24)
DC	0.34 (0.08)
Dallas	1.22 (0.50)
Houston	1.19 (0.93)
Los Angeles	0.43 (0.36)
New York	0.76 (0.52)
Nashville	0.68 (0.32)
San Diego	ND
San Francisco	0.61 (0.39)

*Nanogram DNA (standard deviation), averaged across location.
ND, not determined.

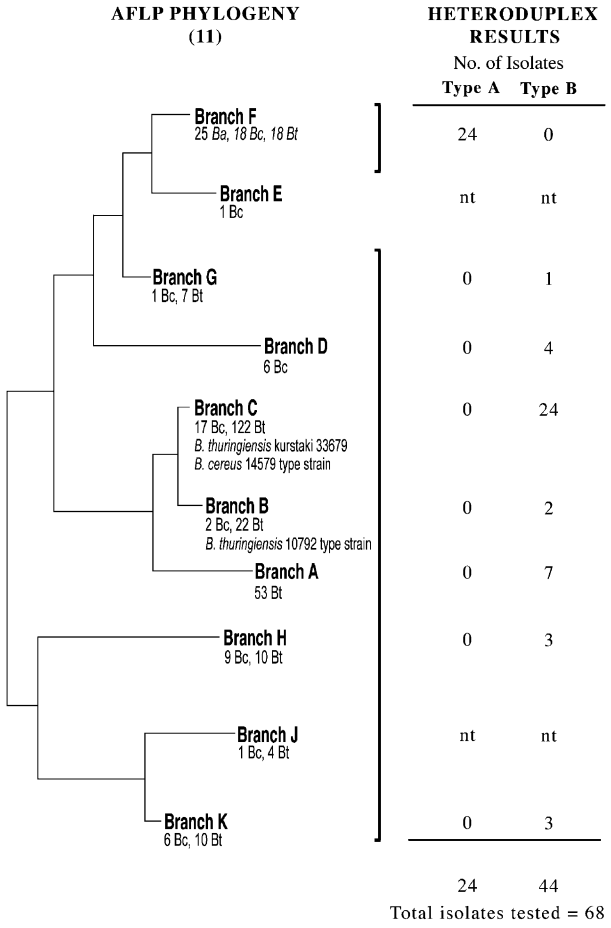

FIG. 2—*Differentiation of* Bacillus anthracis, Bacillus cereus, *and* Bacillus thuringiensis *strains by heteroduplex analysis. The dendrogram shows relationships among strains based on cluster analysis of amplified fragment length polymorphism (AFLP) profiles (11). The numbers below each branch name indicate the number of* B. anthracis *(Ba),* B. cereus *(Bc), and* B. thuringiensis *(Bt) isolates in that cluster. The HD Types A and B strains generate heteroduplex peaks with average locations of 467 and 470 bpe, respectively. All of the tested isolates comprising AFLP Branch F had the HD Type A peak.*

Detection of Bacillus *species in Urban Aerosols*

The percent of pooled monthly aerosol samples in which *Bacillus* species were detected ranged from 19% in San Diego to 91% in Chicago (Fig. 3). Eight of the 11 cities were positive for *Bacillus* species in at least 50% of the their pooled monthly samples. The HD Type A peak (diagnostic of *B. anthracis* and closely related, often pathogenic, *B. cereus* and *B. thuringiensis* isolates) was detected in samples from all of the cities and was present in

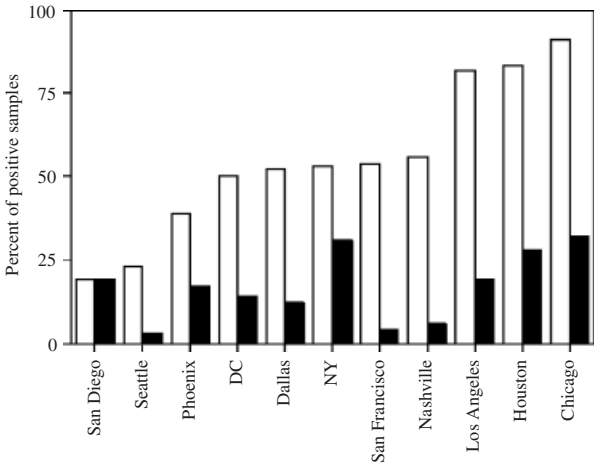

FIG. 3—*Percent of composite monthly samples in each of 11 U.S. cities that were positive for* Bacillus *species (clear) and HD Type A species (black) using the heteroduplex assay.*

3–32% of the samples depending on the city (Fig. 3). Of the 506 samples, 17.8% (90 samples) were positive for HD Type A. The frequency of detection of the HD Type A species was not directly correlated with the presence of *Bacillus* species in general.

Three categories of *Bacillus* species were documented using the heteroduplex assay. The HD Types A and B categories represented *B. anthracis*, *B. cereus*, and *B. thuriengensis* strains as indicated in Fig. 2. The "other" category represented strains from more distantly related *Bacillus* species that generated heteroduplex peaks located between 473 and 489 bpe. Examples of *Bacillus* species in this category include *B. megaterium*, *B. flexus*, *B. amyloliquefaciens*, *B. pseudomegaterium*, and *B. subtilis* (12). Figure 4 illustrates the frequency of positive detection for the three HD types, with data pooled by calendar year quarter (January–March, April–June, July–September, October–December), and averaged over the multiple sampling locations in each city. Considerable variation in the composition of *Bacillus* species was observed among the cities.

Geographic location of a city only partially predicted the occurrence of *Bacillus* species in the city air. In very general terms, *Bacillus* species were detected more frequently and displayed a broader diversity (Types A, B, and other peaks) in the eastern cities (Chicago, New York, DC, Nashville, Houston) where the climate is generally wetter than the western cities in more arid regions (San Diego, Phoenix, Dallas). For example, in aerosol samples from Chicago and New York, all three categories of *Bacillus* species were routinely detected, whereas only the HD Type A strains were detected in aerosol samples from San Francisco. A notable exception to this trend was Los Angeles. The HD Type B peak was frequently detected in the eastern cities, and with the exception of Los Angeles, rarely detected in the western cities. This indicates that although geographic location may be important in determining the city aerosol composition, individual cities can vary significantly, possibly because of differences in the presence and types of urban vegetation and "green space," local weather patterns, location of air monitoring samplers, and other factors.

In all of the cities except San Diego, *Bacillus* species were detected in some of the aerosol samples throughout the year. The frequency of positive samples did not exhibit consistent seasonal patterns across the cities, which is to be expected as seasonal climate trends differ greatly across the cities surveyed. In Chicago, we obtained 45 months of aerosol data that provided the opportunity to compare year-to-year variability and seasonal trends across multiple years (Fig. 4). Generally, all three HD types were present in 20–80% of the samples comprising each quarter. In most quarters (13 of 15), *Bacillus* species from outside the *B. cereus* group (light gray bars, Fig. 4) and HD Type B *B. cereus/B. thuringiensis* (dark gray bars, Fig. 4), were detected more frequently than the HD Type A group (black bars). The pattern of detection differed in each of the 4 years for which we had samples, and no consistent seasonal pattern was observed.

Discussion

This study was conducted to evaluate the frequency of occurrence and composition of *Bacillus* species naturally present in urban aerosols. Using PCR followed by a heteroduplex assay, we attempted to answer two basic questions: (1) How frequently are *Bacillus* species, and particularly close relatives of *B. anthracis*, present in urban aerosol samples? and (2) How variable is the background of *Bacillus* species among cities in different geographic regions and across time? Although the sample set of over 11,000 filters from 11 U.S. cities could not be standardized temporally or spatially, which limits comparisons that can be made, this is the first reported survey of *Bacillus* species in urban aerosols over time in cities across the United States. Our survey results demonstrated that a variety of *B. cereus* subgroup species, as well as other *Bacillus* species, are commonly present in urban aerosols across all seasons of the year.

The frequent presence of the HD Type A group strains (comprised of *B. anthracis*, and *B. cereus/B. thuringiensis* food-borne pathogens) in aerosol samples has implications for detection of specific pathogenic *Bacillus* species in environmental samples. The co-occurrence of several closely related strains in environmental samples also suggests the potential for natural evolution of new strains within this group. Recent studies using MEE (4), plasmid gene sequencing (6,7), and AFLP typing methods (10,11) have demonstrated the close genomic relationships among isolates of *B. anthracis*, *B. cereus*, and *B. thuringiensis*, and the suggestion that they are a single species has been made (4). These studies have recently been substantiated by whole genome sequencing of isolates from these species (8,9). Isolates of these three species share considerable DNA sequence similarity across chromosomes and also share plasmid-encoded traits. Members of all three species can be pathogenic although the host and severity of disease differ. *B. anthracis* causes anthrax in animals, *B. cereus* can cause food poisoning and periodontal disease, and *B. thuringiensis* can be an insect pathogen. Recently, bacteria causing symptoms of inhalation anthrax were identified as *B. cereus* (by DNA sequencing, AFLP typing, and standard clinical assays), but were found to possess *B. anthracis* pathogeneicity factors (16). This example illustrates that the ability to cause inhalation anthrax symptoms or carry the anthrax toxin genes may not be exclusively limited to isolates that can be "typed" as *B. anthracis*. Thus, our concept of species and pathogenicity within this group may need substantial revision.

Our environmental survey data and the genomic data described above illustrate the challenge of identifying specific attributes in anthrax-causing strains that are useful for forensic and surveillance detection of biothreat activity in the presence of a myriad of closely related *B. cereus* and *B. thuringiensis* species. When used to test for the presence of particular genes in environmental samples where many closely related species co-occur, DNA-based detection assays are an effective strategy to obtain strong evidence of the presence of those particular genes. However, this approach

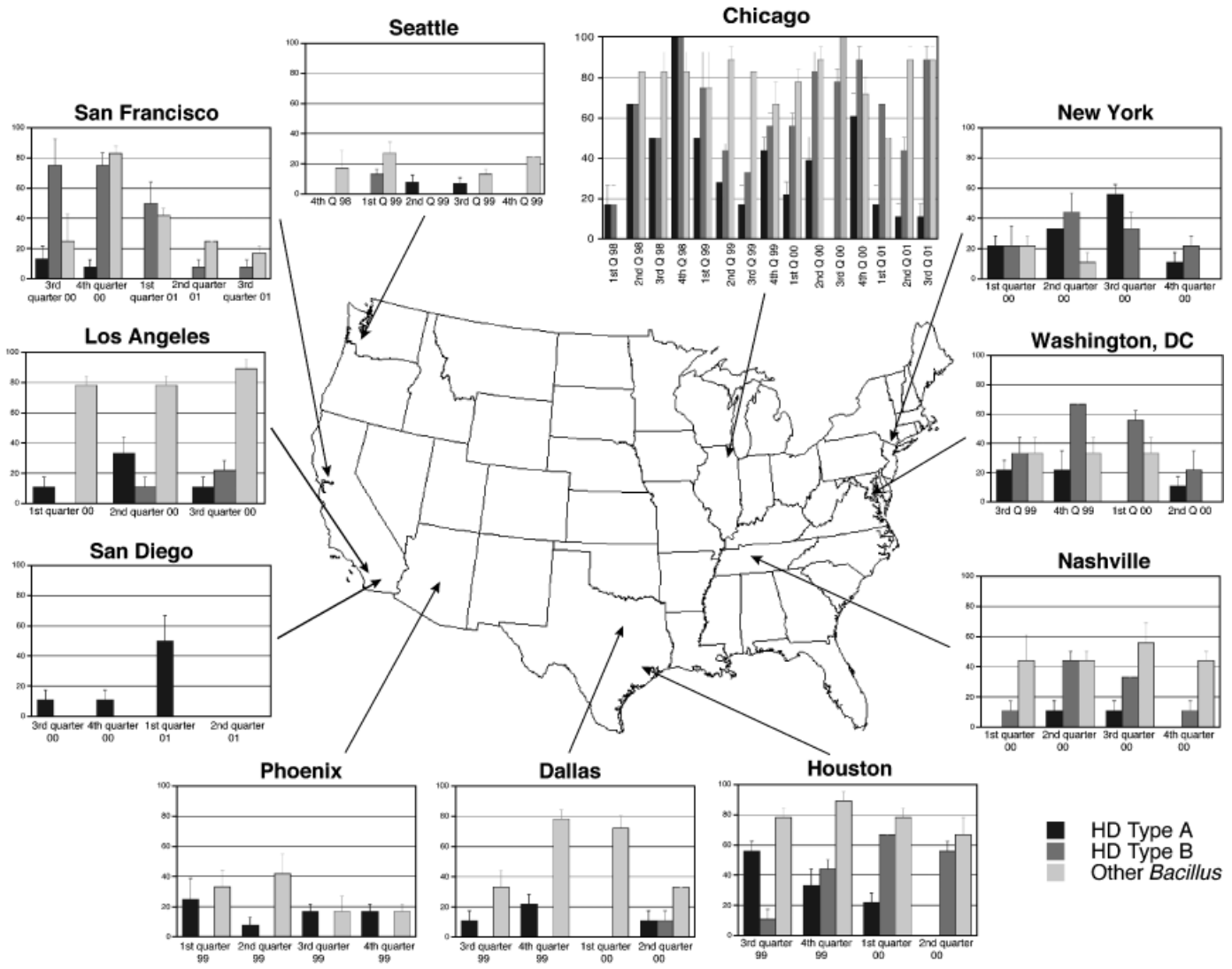

FIG. 4—*Seasonal and geographic variation in detection of* Bacillus *DNA in aerosol samples among sites. Each bar shows the average percentage of positive samples among sites per year quarter. The first quarter (quarter or Q on graphs) is January–March, second quarter is April–June, third quarter is July–September, and fourth quarter is October–December for the years indicated. The number of sites per city ranged from three to five, and each site had three samples per quarter (i.e., one sample representing each month in the 3-month quarter). The error bars show the standard error of the mean for monthly samples at all sites and months in that quarter. HD Type A (black) =* Bacillus anthracis *plus some* Bacillus cereus *and* Bacillus thuringiensis *strains. HD Type B (medium gray) = other* B. cereus *and* B. thuringiensis *in the* B. cereus *group, Other (light gray) = other* Bacillus *species, based on the heteroduplex assay.*

cannot provide information on the individual species carrying those genes. For this reason, obtaining cultured isolates from the environmental sample, where many traits can be tested in a single genome, remains the gold standard for unambiguous identification and determination of pathogenicity.

Detection frequency of *Bacillus* species was highly variable among cities, across seasons of the year, and across locations within a city. Well-defined seasonal and spatial patterns exist for the annual distribution of plant pollen in the air. Different plant species have predictable seasonal cycles of pollination that repeat every year, and they are detected over the same period of weeks each year and then are not detected the remainder of the year. In contrast to this, the amount and composition of fungi and bacteria in the air at any one time can be extremely variable based on local weather conditions, construction activities, traffic patterns, local vegetation, and landscaping/agricultural practices (17–19), and are thus difficult to predict. Seasonal patterns for cultured aerosolized fungal spores (i.e., mold) and bacteria have been detected for some locations (20–23) but not for others (24). Using an Andersen air sampler, Jones and Cookson (25) found significant seasonal variation in culturable fungal and bacterial counts in the large size fraction ($>8\,\mu m$) but not in small size fractions ($<8\,\mu m$), which would be more consistent with the $PM_{2.5}$ respirable fraction samples used in our study. Our observations show that spore-forming *Bacillus* species are present in aerosol samples throughout the year. A repeatable seasonal pattern in detection frequency was not observed for the two cities for which we had multiyear data (Chicago, San Francisco). This suggests that the presence of *Bacillus* species in the air may not depend strongly on seasonal cycles tied to plant growth or senescence and decay. However, fluctuations in the detection frequency may be influenced by wind movement and aerosolization of soil particles in local environments and may be affected by local weather conditions including sunlight intensity, humidity, and rain events. This study measured the presence/absence of *Bacillus* species that represent different phylogenetic groups and did not measure the rel-

ative abundance of *Bacillus* spores or cells in each of the PCR-positive aerosol samples. Thus, any seasonal trends based on the relative abundance of a single group would be missed. Defining reliable patterns and the sources from which aerosol *Bacillus* species originate will require more extensive sampling efforts and inclusion of quantitative measures of relative abundance.

Twelve percent of the aerosol samples did not support PCR amplification of detectable amplicons with the general bacterial primers or the heteroduplex primers. The negative PCR results can result from several different factors, including insufficient template DNA in the reaction, degraded DNA, or presence of inhibitors. The amount of DNA obtained from individual monthly samples was too low to quantify by spectroscopic means (ranging from 0.34 to 1.22 ng—estimated from a pooled year sample) and the very limited sample amounts made it difficult to carry out additional analyses to determine the cause of the negative results. Some samples from this collection were tested for inhibitors by spiking target DNA into the sample, and in general the procedure used to extract and purify the DNA was shown to successfully remove PCR inhibitors (15).

Acknowledgments

For the generous contribution of $PM_{2.5}$ aerosol samples to this study, we thank Lee Ann Byrd of the U.S. Environmental Protection Agency (EPA), regional EPA managers, and the following representatives of local air quality monitoring agencies: Ted Erdman and Bob Day (DC); Mahmood Hossain (CA); Randy Sedlacek and Bev Chenausky (AZ); Edward Michel (TX); Steve Barbosa (CA); Bob La Morte (IL); Dirk Felton (NY); Tracy Daub and Jeffery D. Robbins (CA); Susan Davis (WA); and the Nashville air quality monitoring group (TN). We thank Paul Jackson, Richard Okinaka, Karen Hill, and Lawrence Ticknor for providing DNA from *Bacillus* isolates and AFLP results for calibration of the heteroduplex assay. We thank Erin Williams and Christy Grow for technical help. This study was supported by the U.S. Department of Energy, Chemical & Biological Nonproliferation Program.

References

1. Ash C, Farrow JAE, Dorsch M, Stackebrandt E, Collins MD. Comparative analysis of *Bacillus anthracis*, *Bacillus cereus* and related species on the basis of reverse transcriptase sequencing of ribosomal RNA. Int J Syst Bacteriol 1991;41:343–6.
2. Barns SM, Hill KK, Jackson PJ, Kuske CR. 16S ribosomal RNA sequence-based phylogeny of *Bacillus* species and the *B. cereus* group. Report No.: LA-UR-99-5628, Los Alamos, NM: Los Alamos National Laboratory, 1999.
3. Bavykin SG, Lysov YP, Zakhariev V, Kelly JJ, Jackman J, Stahl DA, et al. Use of 16S rRNA, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of *Bacillus cereus* group microorganisms. J Clin Microbiol 2004;42:3711–30.
4. Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, et al. *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl Environ Microbiol 2000;66:2627–30.
5. Elhanany E, Barak R, Fisher M, Kobiler D, Altboum Z. Detection of specific *Bacillus anthracis* spore biomarkers by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2001;15:2110–6.
6. Pannucci J, Okinaka RT, Sabin R, Kuske CR. *Bacillus anthracis* pXO1 plasmid sequence conservation among closely related bacterial species. J Bacteriol 2002;184:134–41.
7. Pannucci J, Okinaka RT, Williams E, Sabin R, Ticknor LO, Kuske CR. DNA sequence conservation between the *Bacillus anthracis* pXO2 plasmid and genomic sequence from closely related bacteria. BMC Genom 2002;3:34.
8. Read TD, Peterson SN, Tourasse N, Baillie LW, Paulsen IT, Nelson KE, et al. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature 2003;423:81–6.
9. Rasko DA, Ravel J, Okstad OA, Helgason E, Cer RZ, Jiang L, et al. The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. Nucleic Acids Res 2004;32:977–88.
10. Ticknor LO, Kolsto AB, Hill KK, Keim P, Laker MT, Tonks M, et al. Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. Appl Environ Microbiol 2001;67:4863–73.
11. Hill KK, Ticknor LO, Okinaka RT, Asay M, Blair H, Bliss KA, et al. Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis* isolates. Appl Environ Microbiol 2004;70:1068–80.
12. Merrill L, Richardson J, Kuske CR, Dunbar J. Fluorescent heteroduplex assay for monitoring *Bacillus anthracis* and close relatives in environmental samples. Appl Environ Microbiol 2003;69:3317–26.
13. Dunbar J, Kuske CR Inventors. Assignee. Chemical agent for selective removal of PCR inhibiting compounds from environmental DNA extracts. Invention disclosure. No. 2001-108.
14. Kuske CR. Analysis of pathogen backgrounds in soils and EPA aerosol samples, Report No.: LA-UR-04-3191, Los Alamos, NM: Los Alamos National Laboratory, 2004.
15. Kuske CR, Barns SM, Grow CC, Merrill L, Dunbar J. Environmental survey for four pathogenic bacteria and closely related species using phylogenetic and functional genes. J Forensic Sci 2006;51(3):548–58.
16. Hoffmaster AR, Ravel J, Rasko DA, Chapman GD, Chute MD, Marston CK, et al. Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proc Natl Acad Sci USA 2004;101:8449–54.
17. Di Giorgio C, Krempff A, Guiraud H, Binder P, Tiret C, Dumenil G. Atmospheric pollution by airborne microorganisms in the city of Marseilles. Atmospheric Environ 1996;30:155–60.
18. Lighthart B. Microbial aerosols: estimated contribution of combine harvesting to an airshed. Appl Environ Microbiol 1984;47:430–2.
19. Lighthart B. The ecology of bacteria in the alfresco atmosphere. FEMS Microbiol Ecol 1997;23:263–74.
20. Bovallius A, Bucht B, Roffey R, Anas P. Three-year investigation of the natural airborne bacterial flora at four localities in Sweden. Appl Environ Microbiol 1978;35:847–52.
21. Hysek J, Fisar Z, Binek B. Long-run monitoring of bacteria, yeasts and other micromycetes in the air of an industrial conurbation. Grana 1991;29:450–3.
22. Katial RK, Zhang Y, Jones RH, Dyer PD. Atmospheric mold spore counts in relation to meteorological parameters. Int J Biometeorol 1997;41:17–22.
23. Kelly CD, Pady SM. Microbiological studies of air masses over Montreal during 1950 and 1951. Can J Bot 1954;32:591–600.
24. Wright TJ, Greene VW, Paulus HJ. Viable microorganisms in an urban atmosphere. J Air Pollut Control Assoc 1969;19:337–41.
25. Jones BL, Cookson JT. Natural atmospheric microbial conditions in a typical suburban area. Appl Environ Microbiol 1983;45:919–34.

Additional information and reprint requests:
Cheryl R. Kuske, Ph.D.
Bioscience Division, B-N1, M888
Los Alamos National Laboratory
Los Alamos, NM 87545
E-mail: kuske@lanl.gov