Published in final edited form as:

Chem Biol Interact. 2008 January 30; 171(2): 212–235. doi:10.1016/j.cbi.2007.09.002.

Discrimination of *Bacillus anthracis* and Closely Related Microorganisms by Analysis of 16S and 23S rRNA with Oligonucleotide Microarray

Sergei G. Bavykin^{1,*}, Vladimir M. Mikhailovich², Vladimir M. Zakharyev², Yuri p. Lysov², John J. Kelly³, Oleg S. Alferov¹, Igor M. Gavin¹, Alexander V. Kukhtin^{1,**}, Joany Jackman^{4,***}, David A. Stahl⁵, Darrell Chandler^{1,**}, and Andrei D. Mirzabekov^{1,2}

¹Center for Environmental and Security Science and Technology, Argonne National Laboratory, Argonne, Illinois, 60439, USA

²Engelhardt Institute of Molecular Biology, Moscow 117984, Russia

³Department of Biology, Loyola University Chicago, Chicago, IL, 60626, USA

⁴Georgetown University, School of Medicine, Department of Biochemistry and Molecular Biology, Washington, DC 70001, USA

⁵Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98195-2700, USA

Abstract

Analysis of 16S rRNA sequences is a commonly used method for the identification and discrimination of microorganisms. However, the high similarity of 16S and 23S rRNA sequences of *Bacillus cereus* group organisms (up to 99-100%) and repeatedly failed attempts to develop molecular typing systems that would use DNA sequences to discriminate between species within this group have resulted in several suggestions to consider *B. cereus* and *B. thuringiensis*, or these two species together with *B. anthracis*, as one species. Recently, we divided the *B. cereus* group into seven subgroups, Anthracis, Cereus A and B, Thuringiensis A and B, and Mycoides A and B, based on 16S rRNA, 23S rRNA and *gyrB* gene sequences and identified subgroup-specific makers in each of these three genes. Here we for the first time demonstrated discrimination of these seven subgroups, including subgroup Anthracis, with a 3D gel element microarray of oligonucleotide probes targeting 16S and 23S rRNA markers. This is the first microarray enabled identification of *B. anthracis* and discrimination of these seven subgroups in pure cell cultures and in environmental samples using rRNA sequences. The microarray bearing perfect match/mismatch (p/mm) probe pairs was specific enough to discriminate single nucleotide polymorphisms (SNPs) and was able to identify targeted organisms in 5 minutes. We also demonstrated the ability of the microarray to determine subgroup affiliations for *B. cereus* group isolates without rRNA sequencing. Correlation of these seven subgroups with groupings based on multilocus sequence typing (MLST), fluorescent amplified fragment length polymorphism analysis (AFLP) and multilocus enzyme electrophoresis (MME) analysis of a wide spectrum of different genes, and the demonstration of subgroup-specific differences in toxin profiles, psychrotolerance, and the ability

*Corresponding author. Tel.: +1 (630) 252-3980; sbavykin@anl.gov.

**Current Address: Akonni Biosystems, Frederick, Maryland 21701, USA

***Current Address: Applied Physics Laboratory, Johns Hopkins University, Laurel, Maryland 20723, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

to harbor some plasmids, suggest that these seven subgroups are not based solely on neutral genomic polymorphisms, but instead reflect differences in both the genotypes and phenotypes of the *B. cereus* group organisms.

Keywords

B. anthracis; 16S rRNA; 23S rRNA; microarray; SNP; environmental sample

1. Introduction

Bacillus anthracis, the causative agent of the highly infectious disease anthrax, belongs to the *Bacillus cereus* group, which also contains six other closely related species: *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and *Bacillus medusa* [1-6]. *B. anthracis*, which in recent years has become known as a biological weapon and terrorist tool, can be isolated from the environment. For example, *B. anthracis* is periodically detected in sewer water and in soil, among animals and staff in agricultural settings and in zoos, and epizootics among bison are rather common in Canada. While there has been a general decrease in the number of *B. anthracis* outbreaks in the U.S., Canada and Europe, anthrax still is a significant problem in West Africa, Spain, Greece, Turkey, Albania, Romania and Central Asia [7,8].

Related organisms in the *B. cereus* group are common in samples of food, water or soil [2,9-15], and some of the non-anthrax *B. cereus* group bacteria can cause human infections and illness [2,10,16] which can be rather severe [17-19]. For example, *B. cereus* is a ubiquitous soil bacterium and opportunistic human pathogen, with six recognized types of clinical infections and disease: (i) local infections, particularly of burns, traumatic or post surgical wounds, and eye; (ii) bacteremia and septicemia; (iii) central nervous system infections, including meningitis, abscesses, and shunt associated infections; (iv) respiratory infections; (v) endosub-circuititis and perisub-circuititis; and (vi) food poisoning, characterized by toxin induced emetic and diarrheagenic syndromes (2). In the food industry, *B. cereus* and *B. weihenstephanensis* are important as food spoilage organisms, easily contaminating raw foods and processing equipment, including equipment used in dairies and paper mills [2,9-11,14,16,19-22].

B. thuringiensis is widely used as a biological insecticide, accounting for more than 90% of the biopesticides employed worldwide [23,24]. Despite a long safety record, however, there is increasing public concern about the potential pathogenicity of *B. thuringiensis* due to sporadic but sometimes severe infections caused by this species, including diarrhea, food poisoning, corneal ulcer, cellulites, and burn/war wound infections. Like *B. cereus*, *B. thuringiensis* produces a variety of virulence factors, which include phospholipase C, hemolysin and enterotoxins [23,25-34], and diarrheal enterotoxin production was detected in some commercial *B. thuringiensis*-based insecticides [35]. For these reasons, many crop customers require agricultural companies to control for the presence of *B. thuringiensis* in their products. There is also evidence that specific strains of *B. cereus* and *B. thuringiensis* are associated with periodontitis and other oral infections [36].

B. mycoides and *B. pseudomycoides* are ubiquitous in soil and water, and there are toxigenic strains within each of these species [37-43]. Recent findings indicated that *B. mycoides*, like *B. thuringiensis*, may produce pesticidal metabolites [44]. New genomic data indicated that *B. medusa* is a variant of *B. thuringiensis*, suggesting that *B. medusa* may possess or acquire pathogenic potential. Therefore, detection and surveillance of all microorganisms comprising the *B. cereus* group is of significant interest in many aspects of world-wide

public health microbiology, from confirming acts of bioterrorism to disease surveillance and outbreak prevention.

Besides traditional microbiological techniques, a variety of molecular biological methods have been described for the selective identification of *B. anthracis*. Most of them use specific genes or proteins as targets for recognition and discrimination from related microorganisms [45-48]. Genomic methods target unique gene sequences located on plasmids or in bacterial chromosomes. These methods include: microarray detection of genomic markers [33,49-51]; direct testing of plasmid pXO1 DNA with specific probes [52]; real-time multiplex PCR strand specific detection of CAP-C gene on the pXO2 plasmid [53]; ribotyping of 16S/23S rRNA genes [54-56] and their internal transcribed spacers (16S-23S rRNA ITS) [49,57,58]; detecting variations in *gyrA-gyrB* intergenic spacer regions [57] and in *rpoB* gene [59]; identification markers in *gyrA* and *gyrB* genes [60,61] and in other chromosomal sequences [62]; analysis of putative genome-integrated prophages [63]; detection of single nucleotide and repetitive element polymorphisms [64-66]; AFLP analysis of plasmid and chromosomal sequences [67-69]; MME of chromosomal genes [70], PCR-ELISA [71]; and on chip PCR amplification of anthrax toxin genes [72]. Utilization of genomic markers located in single-copy genes for identification of *B. anthracis* and discrimination of it from related microorganisms is complicated by horizontal gene transfer (HGT), which affects up to 32% of bacterial genomes [73-75], and by other mechanisms of genome reorganization [76]. Toxin genes are the subject of HGT particularly often [77]. On the other hand, discriminative identification of *B. cereus* group members other than *B. anthracis* using genomic markers was impossible until very recently [18,58,69,70,78] due to the lack of a genomic-based classification of this group. Currently, rRNA gene sequences are commonly used in microbial systematic [79,80]. Bacterial genomes usually contain 10-15 copies of rRNA genes, which are involved in translation, a fundamental cell function, and which have coevolved with ribosomal proteins. These features would seem to make rRNA genes the least likely of all genes to experience interspecies HGT [81]. In addition, GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) and Ribosomal Database Project II, release 9 (RDP II, r. 9) (<http://rdp.cme.msu.edu/index.jsp>) currently contain more than 300,000 bacterial rRNA sequences including several hundred belonging to *B. cereus* group organisms. However, it has been considered impossible to use rRNA gene sequences for *B. cereus* group classification and discrimination due to the high level of rRNA homology (more than 95%) among *B. cereus* group organisms [78,82-84].

Recently, we reported that the *B. cereus* group may be divided into seven subgroups (Anthraxis, Cereus A and B, Thuringiensis A and B, and Mycoides A and B) based on 16S rRNA, 23S rRNA and *gyrB* gene sequences and each subgroup may be characterized with specific “signatures” (genomic markers) [85]. We also demonstrated that the currently-defined species *B. cereus*, *B. thuringiensis* and *B. mycoides* actually represent mixtures of genetically distinct bacterial groups [85]. Here we have utilized rRNA markers in a 3D gel element oligonucleotide microarray to demonstrate for the first time that *B. anthracis* may be unambiguously discriminated from the most closely related organisms (subgroups Cereus A and B), as well as from bacteria comprising other, more divergent subgroups of the *B. cereus* group. This rRNA-based microarray was also capable of identification and discrimination of all seven subgroups within the *B. cereus* group.

2. Materials and methods

2.1. Bacterial strains

Seven reference strains belonging to the *B. cereus* group, *B. anthracis* str. Ames, *B. anthracis* str. Sterne, *B. thuringiensis* str. B8, *B. cereus* str. NCTC9620, *B. cereus* str. T, *B. thuringiensis* str. 4Q281, and *B. mycoides* str. ATCC10206 were obtained as a generous gift

from Dr. John Ezzell, U.S. Army Medical Research Institute of Infection Diseases, Fort Detrick, Frederick, MD, USA. Four other reference organisms, *B. cereus* NCTC11143, *B. cereus* ATCC43881, *B. mycoides* ATCC6462 and *B. subtilis* B-459, were obtained from the National Collection of Type Cultures and Pathogenic Fungi (NCTC), London, UK, and American Type Culture Collection (ATCC), Rockville, MD, respectively. Three other non-reference organisms, *B. cereus* NCTC3329, *B. cereus* HER1414 and *B. mycoides* ATCC23258 were also obtained from NCTC and ATCC. *B. thuringiensis* B8, related to subgroup Cereus A, apparently represents a misclassification, as it does not produce protein crystals or contain any *cry* genes that are a fundamental characteristics of *B. thuringiensis* [85].

2.2. Oligonucleotide microarray design and construction

DNA microarrays were constructed with eleven pairs of oligonucleotide probes (ps1 through ps20, ps27 and ps28) (Table 1) targeting 16S rRNA sequence “signatures” (Fig. 1A) and four pairs of oligonucleotide probes (ps21 through ps24 and ps 29 through ps 32) (Table 2) targeting 23S rRNA sequence “signatures” (Fig. 1B) using 3D gel element microarray platform [86]. Probes ps25 (BCG1) and ps26 (BSG1) targeting the *B. cereus* group and the *B. subtilis* group, respectively, were selected previously [87]. Each probe was 20 nucleotides in length. Oligonucleotides were synthesized on an automatic DNA/RNA synthesizer (Applied Biosystems 394) using standard phosphoramidite chemistry. 5'-Amino-Modifier C₆ (Glen Research, Sterling, VA) was linked to the 5'-end of the oligonucleotides. A micromatrix containing 100- by 100- by 20 µm polyacrylamide gel pads fixed on a glass slide and spaced 100 µm from each other was manufactured by photopolymerization [88], and activated as described earlier [89]. Six nl of individual 1mM amino-oligonucleotide solutions was applied to each gel pad containing aldehyde groups [88,90] according to the procedure described earlier [89].

2.3. Sample preparation and hybridization with microarray

Total RNA was isolated from frozen cell pellets by bead beating followed by phenol-chloroform extraction as previously described [91]. Isolated RNA was fragmented and labeled with direct magnesium-sodium periodate method and excess of the Lissamine rhodamine B ethylenediamine (LissRhod) (Molecular Probes, Eugene, OR) was removed with butanol treatment as described earlier [92].

Samples of labeled and fragmented nucleic acids from whole cells were obtained as described earlier [87,92] with some modifications. 50 µl of silica suspension containing 40 µl of dry silica was applied to a 25-mm-long sterile disposable centrifuge filter unit containing an Anopore membrane filter (aluminum dioxide) with a diameter of 6.5 mm and a pore size of 0.2 µm (VectaSpin Micro Centrifuge Filter, catalog # 6830-021) (Whatman, Fairfield, NJ) and centrifuged in an Eppendorf 5415C micro-centrifuge for 30 sec at 14,000 rpm (~14,000 g). Silica in the column was washed once with 500 µl of diethylpyrocarbonate-treated (DEPC) H₂O (pipetting of silica was followed by centrifugation at the same conditions). 2.5×10^8 cells were pretreated in 25 µl of 100 mg/ml freshly-diluted lysozyme at 37° C for 5 min, diluted with 550 µl of a mixture (9:4) of L buffer (4.5 M guanidine thiocyanate (GuSCN) and 10 mM EDTA pH 8.0) and B buffer without MgCl₂ (4 M GuSCN, 135 mM Tris-HCl pH 6.4, 3.5 % (wt/vol) Triton X-100 and 17.5 mM EDTA) (9), suspended carefully to lyse the cells by pipetting, amended with 7 µl 5M MgCl₂, and applied to the silica mini-column. (In general pre-treatment is necessary for gram-positive cells only). All manipulations with silica mini-column, except the labeling-fragmentation step, were carried out at room temperature. The column was washed with 500 µl of L-B buffer (4.34 M GuSCN, 46 mM Tris-HCl pH 6.4, 65 mM EDTA 1 % (wt/vol) Triton X-100 (twice), 500 µl of 75% ethanol (twice) and 500 µl of 100% ethanol (twice).

The silica column containing nucleic acids was sealed at the bottom with a cap from a 1.5 ml micro-centrifuge tube and preheated in a sand bath at 95° C for 3 min. to remove traces of ethanol. To prepare labeling cocktail, 1220 µl 25 mM sodium phosphate (pH 7.0) was applied into an ampoule containing 1 mg of lissamine-rhodamine B ethylenediamine (LissRhod) (Molecular Probes, Inc., Eugene, OR). The dye was carefully suspended and then centrifuged at 14,000 g for 30 sec. to precipitate insoluble components of the dye mixture. 732 µl of supernatant were mixed with 30 µl of 150 mM 1,10-phenanthroline-HCl (OP) (Fluka, Ronkonkoma, NY) and 30 µl of 15 mM CuSO₄, and 132 µl of labeling cocktail was preheated for 30 sec. at 95°C. 18 µl of 33 mM H₂O₂ (Sigma-Aldrich, Milwaukee, WI, cat#: H1009) was added to the cocktail immediately before application of 150 µl of hot cocktail mixed with H₂O₂ to the preheated mini column. The mini-column was sealed at the top to prevent evaporation and was incubated in the sand bath for 10 min at 95° C. The reaction was stopped by removing the column from the sand bath and adding 20 µl of freshly prepared stop solution (2.6M sodium acetate, pH 5.2 and 70 mM EDTA) and 540 µl of cold 100% ethanol. After 5-min incubation at room temperature, nucleic acids were precipitated on the column by centrifugation for 30 sec at 14,000 g. Excess fluorescent label was removed by successive washing of the column with 500 µl of 75% ethanol (twice) and 100% ethanol (once). For elution of labeled product, the silica in the column was suspended with 45-60 µl of 10 mM sodium carbonate (pH 8.5), the column was sealed from the top, placed into a micro-centrifuge tube, incubated for 2 min at 95°C and centrifuged for 30 sec at 14,000 g. Elution was performed twice. Yield of labeled nucleic acids was about 20-50 µg.

35 µl of hybridization solution containing 1-3 µg fragmented and labeled RNA, 1M guanidine thiocyanate (GuSCN), 5mM EDTA, and 40mM HEPES (pH 7.5) was passed through a 0.22 µ filter to remove particulates, then heated at 95°C for 3 min and placed on ice. 30 µl of the hybridization solution was added to a hybridization chamber (Grace Biolabs, Bend, OR), and the hybridization chamber was affixed to a microarray. The microarray was allowed to hybridize for an appropriate time at room temperature in the dark. After hybridization, the chamber and hybridization solution were removed from the microarray, and the microarray was washed twice for 10 sec each with 100 µl washing buffer (0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 6 mM EDTA, and 1% Tween 20).

After hybridization the microarrays were analyzed in washing buffer with a custom made wide-field-high-aperture fluorescence microscope (GOI, St.Petersburg, Russia) equipped with a cooled CCD camera (Princeton Instruments, Acton, MA), a thermal table, and XY positioners [93,94]. Exposition time during the image capturing was 0.5-2.0 sec. Alternatively, microarrays were washed for 2-3 sec. in H₂O to remove washing buffer from the microarray surface and dried with dust-free compressed gas. Dry microarrays were analyzed with CCD camera equipped portable reader (Aurora Photonics, Lake Barrington, IL) with image capturing time 10 sec.

All hybridization experiments were performed 2-4 times. The signal intensity of each gel element on the microarray was calculated with MicroChip Imager software (Aurora Photonics, Lake Barrington, IL) as follows: the software set up an inner frame to define the area of the gel element and a larger outer frame covering the area immediately surrounding the gel element which was defined as the background for that gel element. The signal intensity of the gel element was calculated by subtracting the signal intensity per pixel in the outer frame (i.e. the background) from the signal intensity per pixel in the inner frame (i.e. the gel element) and multiplying the difference by the number of pixels within the inner frame. The average (a) and average deviation (Ad) for p/mm ratios were calculated between replicate hybridizations. Average deviation was calculated using equation $Ad = (\sum |x - a|)/n$, where "x" is value, "a", denotes average and "n" indicates the number of replicate

experiments. In our experiments summarized in Tables 3-5, Ad for p/mm signal ratios did not exceed 10% of the average, unless specifically indicated.

For identification of single nucleotide polymorphisms, signal ratios were calculated based on experimental data and compared to theoretical values. The regression coefficient was calculated using the formula $y = Ax + k$, where x , y and k represent theoretical value, experimental value and error, respectively. Error is random deviation from linear rule. Based

on data table, A was calculated as
$$A = \left(\sum_{i=1}^N x_i y_i \right) / \left(\sum_{i=1}^N x_i^2 \right),$$
 where x_i and y_i are values and N is size of data table.

3. Results

3.1. Design of subgroup-specific probes

Analysis of the 16S rRNA, 23S rRNA and *gyrB* gene sequences revealed subgroup-specific “signatures” in each of these three genes. Each “signature” represented a set of single- or double nucleotide variations (differences) in gene sequence and differentiated one subgroup from all others [85]. We also found that discrimination of subgroups within the *B. cereus* group using rRNA sequences would require single base mismatch discrimination (Fig. 1). The microarray contained pairs of probes including one, two or sometimes three subgroup-specific single- or double-nucleotide differences. For example, pair ps1/ps2 was selected for identification of 16S rRNA sequence difference A/G (position 77) that is unique for subgroup Thuringiensis B (Fig. 1A). In this pair probe ps2 targeted nucleotide G that is specific for Thuringiensis B, and probe ps1 recognized nucleotide A that is present in all six other subgroups (Table 1 and Fig. 1A). Another pair, ps3/ps4, targeted two subgroup-specific variations, T/C (position 90) and T/A (position 92). Probe ps4 identified 16S rRNA sequences containing C at position 90 and A at position 92, and probe ps3 corresponded to T at position 90 and T at position 92 (Table 1 and Fig. 1A). In a similar manner, pair ps11/ps12 targeted three subgroup-specific differences, A/C (position 189), T/G (position 200) and G/C (position 208) (Table 1 and Fig. 1A). In addition, some redundancy was included in the probe set: pair ps5/ps6 targeted the same two subgroup-specific differences as pair ps3/ps4, but the targeted sequence for ps5/ps6 was shifted several nucleotides in comparison with pair ps3/ps4.

The microarray (Fig. 2) included three pairs of probes for identification of bacteria from subgroup Thuringiensis B (ps1/ps2, ps3/ps4 and ps5/ps6), and one pair, ps7/ps8, for identification of organisms from subgroups Thuringiensis A and B, Anthracis, and Cereus A and B. However, ps7/ps8 did not identify organisms from subgroups Mycoides A and B (Table 1). Among five pairs for identification of Mycoides B subgroup bacteria, pair (ps9/ps10) targeted variations A/C (position 189) and T/G (position 200); pair ps13/ps14 identified variations T/G (position 200) and G/C (position 208); pair ps15/ps16 recognized variation G/C (position 208), and pair ps19/ps20 detected variations T/C (position 1036) and A/G (position 1045) (Table 1 and Fig. 1A). Finally, pair ps17/ps18, targeting variation C/A at position 1015 discriminated subgroups Anthracis, Cereus A, and Mycoides B from subgroups Cereus B and Thuringiensis A and B, (Table 1 and Fig. 1A). The last pair, ps27/ps28, recognized microorganisms of subgroup Mycoides A, discriminating them from organisms from all other subgroups, using A/G (position 133) as a target (Table 1, Fig. 1A). The combined application of these probes provided a basis for distinguishing subgroup Anthracis from all other subgroups except Cereus A.

Most bacteria of subgroup Cereus A revealed strain-specific sequence variations [85], although some isolates in this subgroup had 16S rRNA sequences identical to subgroup

Anthraxis (Fig. 1A). Organisms within subgroup *Cereus* A do not have any subgroup-specific differences in their 16S rRNA (Fig. 1A), but do differ from subgroup Anthracis in 23S rRNA sequence (Fig. 1B). Therefore, we designed probe pairs ps 21/ps22 and ps 23/ps24 (Table 2) for the discrimination of subgroups Anthracis and *Cereus* A using sequence differences located respectively at position 1559 (G/A) and insertion G at position 1218-1219 in the 23S rRNA molecule (Table 2 and Fig. 1B). As another example of using 23S rRNA subgroup-specific variations for subgroup identification, we also demonstrated discrimination of subgroups *Mycoides* A and *Mycoides* B with probe pairs ps29/ps30 and ps31/ps32 that recognize 23S subgroup-specific substitutions AG/GA at positions 346 and 347, and T/A at position 174, respectively (Table 2 and Fig. 1B).

3.2. Identification strategy for subgroups and reference microorganisms

To demonstrate the ability of the microarray to differentiate all seven subgroups of *B. cereus* group, we selected ten reference organisms, *B. anthracis* Ames and *B. anthracis* Sterne (subgroup Anthracis), *B. cereus* NCTC11143 and *B. thuringiensis* B8 (subgroup *Cereus* A), *B. cereus* T and *B. cereus* NCTC9620 (subgroup *Cereus* B), *B. cereus* ATCC43881 (subgroup Thuringiensis A), *B. thuringiensis* 4Q281 (subgroup Thuringiensis B), *B. mycoides* ATCC6462 (subgroup *Mycoides* A), and *B. mycoides* ATCC10206 (subgroup *Mycoides* B), whose subgroup affiliations were previously determined in accordance with their 16S rRNA and 23S rRNA gene sequences [85]. Our objectives were to determine if we could discriminate organisms from these closely related subgroups, to determine if we could achieve discrimination of bacteria whose rRNA sequences differed by only one base, and to determine if we could discriminate *B. anthracis* from all other closely related organisms in the *B. cereus* group. For these purposes we fabricated 16S rRNA microarrays for unambiguous identification of subgroups *Cereus* B, Thuringiensis A, Thuringiensis B, *Mycoides* A, and *Mycoides* B. This microarray may also identify subgroups Anthracis and *Cereus* A, and discriminate them from five above-mentioned subgroups, but may not discriminate subgroups Anthracis and *Cereus* A each from other (Fig. 2 and Table 1). Therefore, our 16S rRNA targeted microarray contained “subgroup *Mycoides* B-specific” probe Set #1, “Thuringiensis B-specific” probe Set #3, “Thuringiensis A and B-specific” probe ps8, “*Mycoides* A-specific” probe ps28 and probe pair ps17/ps18 that discriminates subgroups *Cereus* B, *Mycoides* A, Thuringiensis A and B from subgroups Anthracis, *Cereus* A and *Mycoides* B, respectively (map of microarray on Fig. 2).

3.2.1. Identification of subgroups and reference microorganisms with 16S rRNA gene sequences

3.2.1.1. Identification of subgroup *Mycoides* B—The 16S rRNA of *B. mycoides* ATCC10206, related to subgroup *Mycoides* B [85] (Fig. 1A), forms perfect duplexes with probes ps10, ps12, ps14, ps16 and ps20 (Set #1, “*Mycoides* B-specific”), with probes ps1, ps3 and ps5 (Set #4), and with probes ps17 and ps27. At the same time, this organism includes mismatches with probes ps9, ps11, ps13, ps15 and ps19 (Set #2), ps2, ps4 and ps6 (Set #3) and with probes ps18 and ps28 (Table 1, Fig. 1A and 2). For this reason, *Mycoides* B isolates hybridized with the microarray revealed fluorescent signals from Sets #1, Set #4, and probes 17 and 27 that were higher than the signals from Set #2, Set #3, and probes 18 and 28 (Fig. 2). Pair ps7/ps8 recognized *B. mycoides* ATCC10206 as a “not Thuringiensis A, B” organism revealing signal from ps7 stronger than ps8 one, but because both probes have one additional mismatch with this target (Table 1), pair ps7/ps8 signals were too weak and were seen only with significant overexposure (not shown).

3.2.1.2. Identification of subgroup Thuringiensis B—*B. thuringiensis* 4Q281, affiliated to subgroup Thuringiensis B [85], forms a perfect match with all probes from Set #3 (“Thuringiensis B-specific”), with probes ps13, ps15 and ps19 from Set #2, and with

probes ps8 (“Thuringiensis A, B-specific”), ps18 and ps27 (Fig. 2). Each probe in pairs ps9/ps10 and ps11/ps12 included one additional mismatch with this isolate (Table 1), but also recognized this target as a “not *Mycoides* B” organism and, despite the additional mismatch, produced pronounced signals (Fig. 2).

3.2.1.3. Identification of subgroup *Thuringiensis* A—The combination of probe pair ps7/ps8 and probes ps1 through ps6 (Set #4 and “*Thuringiensis* B-specific” Set #3) (Table 1, Fig. 2) unambiguously identified subgroup *Thuringiensis* A and discriminated it from subgroup *Thuringiensis* B. Hybridization of 16S rRNA of *B. cereus* ATCC43881, which is affiliated with subgroup *Thuringiensis* A [85] revealed perfect matches with probe ps8 (specific to subgroups *Thuringiensis* A and B), with probes ps1, ps3 and ps5 (Set #4, “not *Thuringiensis* B”) and with probe ps27 (Fig. 2). Therefore, hybridization of 16S rRNA from bacteria of the *B. cereus* group with probes from region 77-92 (Fig. 1A, Table 1) was used to discriminate microorganisms of subgroup *Thuringiensis* B from bacteria from all other subgroups, and it was especially important for the discrimination between subgroups *Thuringiensis* A and *Thuringiensis* B.

3.2.1.4. Identification of subgroups *Anthraxis* and *Cereus* A—*B. anthracis* Ames (subgroup *Anthraxis*) and *B. cereus* NCTC11143 (subgroup *Cereus* A) [85] (Fig. 1A) form perfect matches with probe Set #2 and Set #4, with probes ps7, ps17 and ps27 (Fig. 2 and Table 1). Probe ps17 is specific for subgroups *Anthraxis*, *Cereus* A, and *Mycoides* B, forming perfect duplexes with 16S rRNA from *B. anthracis* Ames, *B. cereus* NCTC11143 and *B. mycoides* ATCC10206 (subgroup *Mycoides* B), and mismatches with all other reference microorganisms (Fig.2 and Table 1). In contrast, probe ps18 contains a mismatch (at position 1015) for *B. anthracis* Ames, *B. cereus* NCTC11143 and *B. mycoides* ATCC10206, and is a perfect match with all other references microorganisms (Table 1 and Fig. 1A). Discrimination of *B. anthracis* Ames and *B. cereus* NCTC11143 from *B. mycoides* ATCC10206 was based on a “perfect” signal for probe ps17 (compare with ps18) in combination with “mismatch” signals for probe Set #1 (“*Mycoides* B-specific”) (Fig. 2 and Table 1).

3.2.1.5. Identification of subgroup *Cereus* B—Identification of *B. cereus* T, which is related to subgroup *Cereus* B [85], was established based on perfect match signals for probes ps7, ps18, ps27, and for probe Sets #2 and #4 (Fig. 2 and Table 1). Specifically, pair ps17/ps18 provided differentiation of subgroup *Cereus* B from subgroups *Anthraxis* and *Cereus* A (Fig.2 and Table 1).

3.2.1.6. Identification of subgroup *Mycoides* A—*B. mycoides* ATCC6462 forms perfect duplexes with probes ps28 (“*Mycoides* A-specific”), ps1, ps3, ps5 (Set #4), ps15 and ps19 (Fig. 2 and Table 1). Each probe in pairs ps7/ps8, ps9/ps10, ps11/ps12, ps13/ps14 and ps17/ps18 forms additional mismatches with this isolate (Table 1) and for this reason revealed weak signals or no signals after hybridization (Fig. 2).

3.2.2. Identification of subgroups and reference microorganisms with 23S rRNA gene sequences

Although subgroups *Anthraxis* and *Cereus* A could not be differentiated with 16S rRNA sequences (Fig. 2), utilization of subgroup-specific variations in 23S rRNA genes helped to resolve this problem (Fig. 3A and Table 2). Here we also demonstrated differentiation of isolates from subgroups *Mycoides* A and *Mycoides* B as an example of the usefulness of 23S rRNA variations for identification of different subgroups (Fig. 3B and Table 2).

3.2.2.1. Differentiation of subgroup Anthracis from subgroup Cereus A—

Organisms that belong to subgroup Cereus A contain 16S rRNA sequences that are identical to subgroup Anthracis or that differ by strain-specific sequence variation only [85] (Fig. 1A). Thus, we used 23S rRNA sequences to differentiate subgroups Cereus A and Anthracis. The 23S rRNA sequences of *B. thuringiensis* B8 and *B. cereus* NCTC11143 differ from *B. anthracis* Ames at three sites, Y/C (594), insertion G (1218-1219) and G/A (1559) (Fig. 1B). We designed two pairs of probes, ps21/ps22 and ps23/ps24 to target site 1559 and insertion 1218-1219, respectively. Probes ps21 and ps23 formed a perfect duplex with the 23S rRNA of subgroup Anthracis but not subgroup Cereus A 23S rRNA (Table 2 and Fig. 1B). Probes ps22 and ps24 provided complementary information; they have a mismatch with subgroup Anthracis 23S rRNA and are complementary with Cereus A 23S rRNA (Table 2 and Fig. 1B).

To validate the discriminative feasibility of 23S rRNA probes ps21/ps22 and ps23/ps24, we used them for testing of reference microorganisms from subgroup Anthracis (*B. anthracis* Ames and *B. anthracis* Sterne) and subgroup Cereus A (*B. cereus* NCTC11143 and *B. thuringiensis* B8), as well as for determination of subgroup affiliation for organisms *B. cereus* HER1414 and *B. cereus* NCTC3329, whose rRNA genes were not sequenced. We analyzed hybridization signals from these two probe pairs only when 16S rRNA targeted probes revealed a pattern typical for organisms of subgroups Anthracis and Cereus A. *B. anthracis* Ames and *B. cereus* NCTC11143 revealed this pattern (Fig. 2), as did *B. anthracis* Sterne, *B. thuringiensis* B8, *B. cereus* HER1414 and *B. cereus* NCTC3329 (Fig. 3A, ps18/ps17 are shown only). 23S rRNA probe pairs ps21/ps22 and ps23/ps24 successfully discriminated reference isolates related to subgroup Cereus A (*B. cereus* NCTC11143 and *B. thuringiensis* B8) from reference microorganisms of subgroup Anthracis (*B. anthracis* Ames and *B. anthracis* Sterne), demonstrating hybridization signal ratios for these two pairs 0.9, 0.4 (ps21/ps22, Cereus A), 1.8, 2.2 (ps21/ps22, Anthracis), and 0.4, 0.4 (ps23/ps24, Cereus A), 1.7, 1.7 (ps23/ps24, Anthracis), respectively (Fig. 3A). The same two pairs, ps21/ps22 and ps23/ps24, helped to identify isolates *B. cereus* HER1414 and *B. cereus* NCTC3329, whose rRNA genes have not yet been sequenced, as belonging to subgroup Cereus A (Fig. 3A).

3.2.2.2. Differentiation of subgroups Mycoides A and Mycoides B—Two reference microorganisms *B. mycoides* ATCC6462 (subgroup Mycoides A) and *B. mycoides* ATCC10206 (subgroup Mycoides B) were differentiated effectively with 16S rRNA probes (Fig. 2), as was *B. mycoides* ATCC23258 (Fig. 3B, probe pair ps27/ps28 is shown only), whose rRNA genes have not yet been sequenced. Here we demonstrated that these three isolates may be also differentiated with 23S rRNA probes (Fig. 3B, Table 2).

3.3. Identification of single nucleotide polymorphism sites

Single nucleotide polymorphism or SNP is a DNA sequence variation occurring when a single nucleotide – A, T, C, or G - in the genome differs between members of species or between different copies of multicopy genes. We hypothesized that SNP sites could be identified in multicopied rRNA genes with our microarray technology.

3.3.1. SNP in the sites of subgroup-specific differences in the isolates with sequenced rRNA—

Complete sequencing of the genome of several isolates of *B. anthracis* and *B. cereus* indicated that organisms from the *B. cereus* group may contain at least ten copies of the rRNA operon [78]. Comparative analysis revealed that a SNP is rather rare event in rRNA sequences in general, but, nevertheless, may be found even at some sites of subgroup-specific differences, for example at positions 594 and 1559 of 23S rRNA (Fig. 1B) in *B. anthracis* AMES, *B. cereus* ATCC10987 and *B. cereus* ATCC14579 [85]. Our

recent sequencing [85] indicated that *B. cereus* T, *B. thuringiensis* 4Q281 and *B. cereus* NCTC 9620 also have a SNP in their 23S rRNA genes at site 1559 with G:A ratios equal to 1.5:1, 1:1 and 1:3.5, respectively (Fig. 4). At the same time, *B. anthracis* Ames and *B. mycoides* ATCC10206 have G at this site, and *B. thuringiensis* B8 has A in this position [85]. We demonstrated the possibility of recognizing polymorphisms in this site by using a pair of probes, ps21/ps22 (Table 2). For these probes the perfect-mismatch (p/mm) ratios for *B. cereus* T, *B. thuringiensis* 4Q281 and *B. cereus* NCTC9620 were intermediate (1.0, 0.72 and 0.64, respectively) between *B. anthracis* Ames, or *B. mycoides* 10206 (1.8 and 1.5, respectively) and *B. thuringiensis* B8 (0.45) (Fig. 4).

3.3.2. Identification and quantification of “polymorphic” sites in mixtures—To demonstrate the ability of our microarray to identify SNP sites in individual bacterial strains we performed hybridizations of mixtures of rRNA samples isolated from pure cultures and compared them with hybridization images of homogeneous rRNA samples from the same isolates. For these purposes we used the 16S rRNA of *B. thuringiensis* B8 (subgroup Cereus A) and *B. thuringiensis* 4Q281 (subgroup Thuringiensis B) and a slightly modified 16S rRNA microarray (Fig. 5). *B. thuringiensis* B8 has a 16S rRNA sequence that is completely identical to *B. anthracis* and differs from *B. thuringiensis* 4Q281 by five subgroup-specific differences (Fig. 1A). Both organisms shared hybridization signals with probe Sets #1 and #2, but generated different signals for probe pairs ps3/ps4, ps5/ps6, ps7/ps8 and ps17/ps18. *B. thuringiensis* B8 forms perfect duplexes with probes ps3, ps5, ps7 and ps17, but *B. thuringiensis* 4Q281 forms perfect duplexes with probes ps4, ps6, ps8 and ps18 (Fig. 5, Table 1 and 3). Therefore, we selected probe pairs ps3/ps4, ps5/ps6, ps7/ps8 and ps17/ps18 as reference pairs for testing of the microarray for its ability to differentiate quantitatively these two organisms in mixtures to imitate polymorphism estimation in sites 90, 92, 192 1015 of 16S rRNA (Fig. 1A). We also performed this experiment with mixtures of *B. anthracis* AMES/*B. cereus* NCTC9620 and *B. anthracis* AMES/*B. thuringiensis* 4Q281 (not shown). Probe pair signal ratios for mixtures were calculated using actual signal intensities from microarray hybridizations (experimental ratios) and using predicted intensities based on data from homogeneous sample hybridizations and the proportions of individual isolates in the mixtures (theoretical ratios) (Table 3). Results for all above mentioned mixtures revealed good correlation between experimental and theoretical data. For probe pairs with strong hybridization signals (ps5/ps6, ps7/ps8, ps17/ps18) the regression coefficient for experimental and theoretical data was 0.96, and the differences between experimental and theoretical ratios did not exceed 10% of the experimental ratio (Table 3). However, this difference may be more pronounced, for pairs like ps3/ps4, where both probes produce signals of low intensity (Fig. 5F, Table 3).

3.4. Influence of hybridization conditions on mismatch recognition

The time required for hybridization and the amount of labeled sample are important parameters in microbial diagnostics. We estimated changes in perfect-mismatch ratios of hybridization signals for selected probe pairs with the amount of *B. mycoides* ATCC10206 labeled RNA varying from 1 to 5 micrograms and the hybridization time varying from 5 min to 18 hours (Table 4). Images were captured with stationary microscope. For this experiments we selected both 16S and 23S rRNA probe pairs containing one (ps17/ps18, ps27/ps28 and ps31/ps32) or two mismatches (ps3/ps4, ps5/ps6 and ps14/ps13) (Tables 1, 2 and 4). Results indicated that p/m ratio is significantly decreased with decreased hybridization time and amount of labeled sample (Table 4). The best recognition (i.e. highest p/m ratios) was obtained after 18 hour microarray hybridization with 1 microgram of labeled sample. However, all six selected pairs demonstrated correct perfect-mismatch recognitions and therefore revealed correct bacteria recognition even after 5 min of hybridization at room temperature and with labeled sample value as small as 2.5 microgram.

The p/mm ratio was minimal (1.37) for pair ps5/ps6 (Table 4), but nonetheless provided correct recognition assuming that average deviation in this experiment did not exceed 10% of p/mm ratio values between different microarrays.

3.5. Influence of environmental admixtures on p/mm signal ratio

We tested the influence of environmental contaminants on microarray hybridization. To maximize inhibition activity, we did not use pre-filtration to remove organic debris present in the collected samples. Instead, 10 ml of ground water samples collected from the bottom of wells in Waterfall Forest Preserve (Darien, IL) were mixed with 3.52×10^8 *B. mycoides* ATCC10206 cells and precipitated by centrifugation. The pellets, which included microbial cells together with debris, had an average volume of 100 microliters which exceeded the volume of pure bacterial cells approximately 20 fold. These “environmental” samples and control pure *B. mycoides* ATCC10206 cells were used for microarray sample preparation in accordance with our standard Silica mini-column labeling-fragmentation procedure and 3 micrograms of labeled samples were hybridized for 1 hour with the microarray. Hybridization images were captured with portable reader. Results indicated that environmental contamination decreased p/mm pair ratios only slightly, 1.3 fold on average (Table 5), and did not affect bacterial recognition.

3.6. Cross-hybridization of selected probes with non-target bacteria

Subgroup-specific differences in rRNA sequences for bacteria from the *B. cereus* group are few and they are localized. This creates difficulties in the selection of probes specific to individual subgroups of bacteria. For example, the sequences of two of the twenty probes on the microarray (ps17 and ps20) (Table 1) selected for identification of *B. cereus* group reference microorganisms also match the 16S rRNA sequences of a number of bacteria that belong to other groups of the genus *Bacillus*. The use of probes targeting the entire *B. cereus* group and for some other groups may resolve this problem. To demonstrate this we used probes ps25 and ps26, respectively. Probe ps25 matches all known sequences of *B. cereus* microorganisms and probe ps26 targets the *B. subtilis* group (*B. subtilis*, *B. amiloliquifaciens*, *B. lentimorbus*, *B. popilliae*, and *B. atrophaeus*). Hybridization analysis indicated that these probes effectively differentiated *B. subtilis* B-459 from *B. anthracis* AMES, *B. cereus* T, *B. mycoides* ATCC10206, and *B. thuringiensis* 4Q281 (Fig. 6). Thus, the *B. cereus* group probes can be used as an internal check to validate probes ps17 and ps20.

4. Discussion

4.1. Molecular typing of *B. cereus* group as a tool for identification and discrimination of *B. anthracis* and related microorganisms

A wide spectrum of DNA typing systems developed recently have not been successful in discriminating between *B. cereus* and *B. anthracis*, or *B. cereus* and *B. thuringiensis*, or *B. cereus*, *B. weihenstephanensis* and *B. mycoides* [18,58,69,70,78]. These systems included 16S and 23S rRNA genes, 16S-23S rRNA ITS and *gyrB* gene analysis [49,55,58,61,95-102], MEE [36,70,103-105], MLST [78,105-110] and AFLP [18,62,67,69,97] among others. These findings resulted in several suggestions to consider *B. cereus* and *B. thuringiensis* [103], or these two bacteria together with *B. anthracis* [36,58,67,70], as one species.

Our recent findings [85] indicated that the *B. cereus* group could be divided into several genomic groups based on 16S and 23S rRNA sequences, and that genomic group Anthracis, which unifies all strains of *B. anthracis*, could be unambiguously discriminated from other members of the *B. cereus* group based on these sequences. This grouping was confirmed by analysis of *gyrB* gene sequences and indicated that the genomogroups defined by rRNA and

gyrB sequences are inconsistent with the seven currently defined species [85]. We found that three of the largest and most widely distributed *B. cereus* group species (*B. cereus*, *B. thuringiensis* and *B. mycoides*), which had been defined based on morpho-physiological criteria [9], actually represent mixtures of microorganisms related to several genetically distinct bacterial genomogroups [85]. We called such groups “subgroups” and at present time have identified seven of them: Anthracis, Cereus A and B, Thuringiensis A and B, and Mycoides A and B [85]. We also performed molecular typing and identified subgroup-specific genomic markers in 16S rRNA, 23S rRNA and *gyrB* sequences [85]. Our grouping results confirm AFLP-based findings that *B. cereus* and *B. thuringiensis* species reveal a high degree of polymorphism, suggesting that these two species may be polyphyletic [69,97]. Our findings as well as many others mentioned above, demonstrated that current taxonomy, which divides the *B. cereus* group into seven species (*B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. medusa* [1-6]) needs serious corrections.

The *B. cereus* group was divided into seven subgroups based on sequences of three different evolutionarily conserved genes (16S rRNA, 23S rRNA, and *gyrB*) which were analyzed by two different methods: identification of “subgroup-specific signatures” and phylogenetic tree construction. Phylogenetic analysis was performed for each of these three genes separately with four different methods of phylogenetic tree construction: minimum evolution, neighbor joining, UPGMA, and maximum parsimony [85]. Therefore, we demonstrated the existence of these seven subgroups by several genomic tests, which independently confirmed the presence of seven new genomic taxons within the *B. cereus* group. Several studies by other laboratories have produced phylogenetic trees for *B. cereus* group organisms using a variety of target genes and phylogenetic tools. For example, *B. cereus* group organisms have been compared based on 16S rRNA sequences [97], 16S-23S rRNA ITS sequences [49,98], and *gyrB* sequences [61]. In addition, AFLP-based phylogenetic tree construction [18], MLST based on six chromosomal genes [107], seven housekeeping gene fragments [108], and several other targets [17,78,99,105,106,110] have been used to compare *B. cereus* group organisms. When our results were compared to these studies by looking at specific strains that were analyzed in common (see Table 6), we found good agreement between our seven subgroups and clades found in all of these studies [17,18,61,78,97-99,105-111]. For example, an MLST-based phylogenetic tree constructed for the concatenated sequences of six chromosomal genes produced four main branches bearing clades: A; B-II, III, IV; C-I, II and D-I, II [107], which contained isolates found in four our subgroups Anthracis (clade A), Cereus A (clades B-II, B-III and B-IV), Cereus B (clades C-I and C-II), and Thuringiensis B (clades D-I and D-II), respectively. Similarly, an MLST-based tree generated using concatenated sequences of seven housekeeping gene fragments [108] produced clades “Anthracis”, “Cereus I”, “Tolworthi”, “Kurstaki” and “Others”, which included organisms from our subgroups Anthracis, Cereus A, Cereus B, Thuringiensis B and Mycoides A, respectively. In the same manner, phylogenetic grouping based on distance analysis of 16S-23S rRNA ITS sequences [49] revealed groups I, V, VI and VII, which correspond to our subgroups Anthracis/Cereus A, Thuringiensis B, Mycoides A and Mycoides B, respectively, as well as a phylogenetic tree branch bearing groups II, III and IV which contain isolates related to subgroup Cereus B.

Our seven subgroups also correspond to some phenotypic properties of *B. cereus* group organisms. For example, organisms from different subgroups differ in toxin profiles: *B. cereus* BCRC 17039 (synonym of *B. cereus* NCTC 11143, affiliated to subgroup Cereus A) showed toxin pattern VII; *B. cereus* BCRC 10603 (synonym of *B. cereus* ATCC14579, subgroup Cereus B) demonstrated toxin pattern I; and *B. mycoides* BCRC 10604 (synonym of *B. mycoides* ATCC6462, subgroup Mycoides A) demonstrated toxin pattern IV [113]. Similarly, several lines of evidence suggest that significant phenotypic differences exist

between subgroups Mycoides A and Mycoides B. First, von Stetten et al [100] developed PCR primers that could discriminate psychrotolerant and mesophilic strains of the *Bacillus cereus* group based on 16S rDNA sequences. Comparison of their primer sequences with the subgroup specific mutations identified in our previous study [85] revealed that the psychrotolerant primers matched sequences from subgroup Mycoides A and the mesophilic primers matched sequences from subgroup Mycoides B. We also found that the last subgroup, Mycoides B, included *B. pseudomycoides* [85], which recently was described as a new species [5]. *B. pseudomycoides* revealed mesophilic phenotype and genetically was rather far from psychrotolerant strains of *B. mycoides* and *B. weihenstephanensis* [5]. In addition, Hendriksen et al. [101] found distinct toxin profiles in strains identified by PCR as psychrotolerant (90 isolates of *B. weihenstephanensis* and 86 strains of *B. mycoides*-like *B. weihenstephanensis*) and strains identified by PCR as mesophilic (10 strains of *B. mycoides*). These data indicate that subgroups Mycoides A and Mycoides B differ in toxin profiles, and support our previous suggestion that psychrotolerance is an essential and unique feature of subgroup Mycoides A, as well as that *B. weihenstephanensis* represents one of the *B. mycoides* strains affiliated to subgroup Mycoides A [85]. The *B. cereus* subgroups may also differ in the presence of certain types of plasmids. For example, extensive analysis of *B. cereus* group isolates with sequenced rRNA genes indicated that plasmids harboring *cry* genes have never been found in subgroups Anthracis, Mycoides A and Mycoides B [85], but may be present in all of the other four subgroups (S. Bavykin, unpublished). The fact that our seven subgroups, which are based on rRNA and *gyrB* sequences, correlate well with groupings based on AFLP, MME and MLST analysis of a wide spectrum of different genes, and the demonstration of subgroup-specific differences in toxin profiles, psychrotolerance, and the ability to harbor some plasmids, suggest that these seven subgroups are not based solely on neutral genomic polymorphisms, but instead reflect differences in both the genotypes and phenotypes of the *B. cereus* group organisms. Further investigations that we are performing now will indicate the final number of subgroups and will group them in genomospecies, in accordance with genetic principles [79].

Unique chromosomal sequences, as well as anthrax toxin genes, are the well established genetic markers used in genomic methods of *B. anthracis* identification and discrimination from related organisms of *B. cereus* group [33,49-53,57,59-62,64-66]. However, recently a number of publications revealed the presence of plasmids with high levels of homology (up to 99.6%) to *B. anthracis* plasmids pXO1 and pXO2 in a number of non-anthrax isolates from the *B. cereus* group, such as *B. cereus* ATCC10987, *B. cereus* F4810/72, *B. cereus* G9241, *B. thuringiensis* HD73, *B. thuringiensis* 97-27, *B. cereus* D-17, *B. cereus* ATCC43881, *B. thuringiensis* ATCC33679, as well in several isolates from outside the *B. cereus* group, such as *B. circulans* CBD118 and *B. sp.* CBD119 (related to *B. luciferences*) [17,78,106,114-118]. Therefore, traditional targets used for of *B. anthracis* identification must be supplemented with new genomic markers. From this point of view, multicopied rRNA genes, as standardized targets commonly used for most bacteria classification and discrimination, represent a highly useful target for emergent *B. cereus* organism detection. In addition, the presence of 20,000 – 50,000 ribosomes in one bacterial cell [119] makes rRNA a valuable target for *B. cereus* organism identification without preliminary PCR amplification. The goal of this study was to confirm the existence and demonstrate the practical usefulness of previously developed genetic markers for identification and discrimination of all seven subgroups of the *B. cereus* group.

4.2. Discrimination of subgroups in *B. cereus* group with rRNA probes

A set of 16S/23S rRNA targeted oligonucleotide probes was designed in the current study (Fig. 1 and 2, Table 1 and 2) and these probes were immobilized within an oligonucleotide microarray. This microarray enabled us for the first time to differentiate all seven subgroups

in the *B. cereus* group each from other (Fig. 2 and 3), including subgroup Anthracis that contains all *B. anthracis* isolates whose rRNA was sequenced [85] (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>; <http://rdp.cme.msu.edu/index.jsp>). We successfully tested our microarray with a set of previously identified [85] reference strains representing all seven subgroups. Using our microarray we also identified subgroup affiliation for *B. cereus* HER1414 and *B. cereus* NCTC3329 (Cereus A) (Fig. 3A) and *B. mycoides* ATCC23258 (Mycoides A) (Fig. 3B), whose rRNA genes have not been sequenced yet.

Previous studies have shown that 16S rRNA sequences of organisms from subgroups Anthracis and Cereus A, e.g., *B. anthracis* Sterne and *B. cereus* NCTC11143, have 99.9-100% similarity and do not contain subgroup-specific differences [83,85]. However, we demonstrated that these organisms, as well as other isolates that belong to Anthracis and Cereus A subgroup, may be differentiated using subgroup-specific signatures located in their 23S rRNA sequences (Fig. 3A and Table 2).

4.3. Perfect match - mismatch discrimination

This study also indicated that our 3D gel-based oligonucleotide microarray format [86] was able to discriminate microbial strains which differed by only one base in their rRNA molecules based on hybridization signal ratios between pairs of perfect match and mismatched probes. For example, using pairs ps7/ps8 and ps17/ps18 we discriminated *B. cereus* NCTC11143 (Cereus A) from *B. cereus* T (Cereus B) and *B. cereus* T from *B. cereus* ATCC43881 (Thuringiensis A) (Fig. 2), strains that differ from each other in their 16S rRNA gene sequences by only one substitution (A/C(1015) and T/C(192), respectively) (Fig. 1A and Table 1). Successful one base mismatch discrimination was also demonstrated for 16S rRNA targeted probe pairs ps1/ps2, ps15/ps16, ps27/ps28 (Table 1 and Fig. 2) and for pairs ps21/ps22, ps23/ps24 and ps31/ps32 related to 23S rRNA (Table 2 and Fig. 3). The ability of our technology to discriminate single base differences suggests that organisms affiliated to the same subgroup, but differing by one nucleotide strain-specific mutation [85], may be also differentiated with our 3D microarray technology.

4.3.1. Efficacy of perfect-mismatch paired microarray for emergent detection

—One of the obstacles for nucleic acid detection with oligonucleotide microarrays is the considerable difference between A-T and G-C pair stability that results in sequence-dependent intensity of fluorescent signals from different oligonucleotide probes of the same length. Due to this sequence dependent hybridization stability, mismatch-forming duplexes can be more stable than perfect duplexes of the same length but with different sequences. Therefore, perfectly matched oligonucleotide probes may produce signals of lower intensity than other probes containing a mismatch with the target. Sequence dependent hybridization impacts the time required for efficient hybridization of all probes, and as a result most microarray hybridization protocols hybridize for at least several hours [120]. However, microarrays constructed with p/mm pairs in which the mismatch is located in the middle of the probe effectively overcome the problem of sequence dependent hybridization stability because within a p/mm pair a perfectly matched sequence should always produce a duplex that is more stable than one containing mismatch. For those cases in which both members of the p/mm probe pair generate signals of equal intensity, these probes can simply be eliminated from further consideration. The p/mm pair strategy has been widely used in microarray technology for one base mismatch discrimination and as a control for nonspecific hybridization [121,122]. In our case, we used p/mm probe pairs to discriminate single- or double-nucleotide variations in rRNA sequences and differentiate one subgroup from all others. Use of p/mm pairs permitted us to dramatically decrease hybridization time to as little as 5 minutes while still enabling perfect-mismatch discrimination (Table 4). In

addition, we have recently developed a free radical based Silica mini-column method for microarray sample preparation that requires only 1 minute for labeling-fragmentation of nucleic acids [92]. Therefore, our methods can produce highly-purified end-labeled nucleic acids samples with easy-controlled fragment length, in roughly 15 minutes starting from whole bacterial cells. In addition, these methods can be utilized for both DNA and RNA and should be easily automated. Therefore, combination of the 3D gel-based p/mm paired microarray with free radical-based method of sample preparation may be effectively used in development of detectors for emergent bacteria identification.

4.3.2. SNP sites quantification—We have demonstrated above that our microarray technology enabled us to discriminate single nucleotide variations in rRNA sequences. At the same time, variation of p/mm ratios in some sites of subgroup-specific differences at different organisms of the same subgroup suggested that several copies of rRNA genes (total number of rRNA operons may vary from 13 to 15 in *B. cereus* group [78]) may contain SNPs. Our recent analysis of the complete genome sequenced of *B. cereus* group isolates confirmed this. SNPs were found at positions 594 and 1559 of 23S rRNA in several isolates [85]. In current study we demonstrated *in vivo* (Fig. 4) and in artificial mixtures (Fig. 5 and Table 3) that our microarray platform was able to identify the presence of SNPs when single nucleotide substitutions were present in at least in 1 of 5 copies, with an accuracy of p/mm ratio not smaller than 10% (Table 3). Moreover, based on the data presented in Fig. 5 it is easy to hypothesize that it would be possible to identify individual species within mixed communities using a database of hybridization images of individual *B. cereus* group organisms.

4.3.3. Bacterial identification in environmental samples—Analysis of nucleic acids in environmental samples generally requires removal of organic matter (e.g. humic acids), debris, etc., because many environmental contaminants can inhibit reactions employed for analysis. Membrane filtration with a pre-filter that accumulates insoluble environmental contaminants helps but does not completely prevent PCR inhibition due to co-elution of compounds such as humic substances, metal ions, etc., that are concentrated on the filter along with targeted bacteria [123]. Our successful testing of our microarray with environmental samples without pre-filtration in presence of organic contaminants (Table 5), which exceeded volume of pure bacterial cells approximately 20 fold, indicated that even at this extreme condition our microarray technology in combination with our previously developed free-radical based Silica mini-column sample preparation method [87,92], worked properly and has a significant potential for direct analysis of environmental samples without preliminary cell culturing or PCR amplification.

4.4. Hierarchical approach in microarray construction

In the current study we demonstrated that our microarray unambiguously discriminated *B. anthracis* from all other members of the *B. cereus* group based on 16S/23S rRNA sequences, and we demonstrated discrimination of seven subgroups within the *B. cereus* group (Anthraxis, Cereus A and B, Thuringiensis A and B, and Mycoides A and B) that had previously been identified only theoretically based on “subgroup-specific signatures” [85]. Subgroup discrimination was achieved using probe pairs selected in accordance with these subgroup-specific markers. However, the limited number of subgroup-specific differences between organisms within the *B. cereus* group made it difficult to select subgroup-specific probes that did not match any organisms from closely related, non-*B. cereus* group species. To address this difficulty, the microarray included probes ps25 and ps26, which were specific for the *B. cereus* group and the *B. subtilis* group, respectively, and which successfully discriminated organisms from these two groups (Fig. 6). The combination of group-specific probes ps25 and ps26 with probe pairs specific for the *B. cereus* subgroups

represents a hierarchical probe design strategy that offers significant advantages over an “endpoint” strategy that focuses on designing specific probes for each individual target. The “endpoint” approach requires the design of probes that are absolutely unique for each individual target (Fig. 7A). The requirement for absolutely unique probes is labor-consuming and necessitates regular renovation of probe sets in response to newly described organisms. The “endpoint” strategy also generates only “endpoint” information (Fig. 7B). Alternatively, “hierarchical” probe design (Fig. 7C) allows one to concentrate probe selection efforts on the key nodes and, in practice, produces more reliable identification assuming perfect signals in a chain of nodes as a major requirement of correct group identification (Fig. 7D). The “hierarchical” approach also does not necessitate frequent re-design of probes characterizing each node, but in fact calls for doing this rather rarely (in response to considerable changes in structure of phylogenetic tree only) in comparison with the “endpoint” approach. Therefore, the hierarchical approach we have taken combining probe pairs differentiating closely-related species/isolates (“determinative” probe set) with specific (non-paired) probes targeting particular nodes on the target-related branch of the phylogenetic tree (“phylogenetic” probe set) should significantly improve the reliability of microarray-based microbial identification. Recently, we successfully tested a hierarchically organized “phylogenetic” probe set targeting “all life”, eubacteria, *B. subtilis* group and *B. cereus* group cells with human, *Escherichia coli*, *B. subtilis* and *B. thuringiensis* targets [87]. From this point of view, further development of hierarchically complicated bacterial phylogenetic trees, where each particular branch consists of dozens of nodes, will lead us to development of highly reliable microarray-based microbial diagnostics. Therefore, combination of high multiplicity of rRNA targets in bacterial cells with simultaneous using of “phylogenetic” and “discriminative” probe sets provide highly-sensitive and highly-reliable identification of *B. anthracis* and related *B. cereus* group organisms. The developed subgroup-specific markers may be useful for *B. anthracis* and other *B. cereus* group pathogens identification in terroristic and environmental events, food outbreaks and bacterial control in agriculture, where *B. thuringiensis* strains are used as a bio-pesticide, as well as in basic studies of molecular mechanisms of toxin actions and their distribution among *B. cereus* group isolates.

Acknowledgments

We express our gratitude to Gennadiy Yershov; Boris Chernov; Julia Golova; Eduard Timofeev, Irina Taran, Sergey Surzhikov and Ann Gemmell for microarray fabrication and Alexey Chernyi for helpful consultations in analysis of rRNA sequences and hybridization images. We are also grateful to John Ezzell, who kindly provided us with reference strains of microorganisms, Wentso Liu and Jodi Flax for their help in the early stages of this project.

This research was supported by Defense Advanced Research Project Agency under Interagency Agreement AO-E428 and by the Russian Human Genome Program grant 5/2000.

References

1. Sneath, PHA. Endospore-forming Gram-positive rods and cocci. In: Holt, JG., editor. Bergey's Manual of Systematic Bacteriology. The Williams & Wilkins Co.; Baltimore, MD: 1986. p. 1104-1139.
2. Drobniowski FA. *Bacillus cereus* and related species. Clin Microbiol Rev. 1993; 6:324–338. [PubMed: 8269390]
3. Turnbull PCB. Definitive identification of *Bacillus anthracis* — a review. J Appl Microbiol. 1999; 87:237–240. [PubMed: 10475956]
4. Lechner S, Mayr R, Fransis KP, Pruss BM, Kaplan T, Wiebner-Gunkel E, Stewart GS, Scherer AB. *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. Int J Syst Bacteriol. 1998; 48:1373–1382. [PubMed: 9828439]
5. Nakamura LK. *Bacillus pseudomycoides* sp. nov. Int J Syst Bacteriol. 1998; 48:1031–1034.

6. Delaporte M. Description de *Bacillus medusa* n.sp. C R Acad Sc Paris. 1969; 269:1129–1131.Serie D
7. Hugh-Jones M. 1996-97 global anthrax report. J Appl Microbiol. 1999; 87:189–191. [PubMed: 10475945]
8. Dragon DC, Elkin BT, Nishi JS, Ellsworth TR. A review of anthrax in Canada and implications for research on the disease in northern bison. J Appl Microbiol. 1999; 87:208–213. [PubMed: 10475950]
9. Granum, PE. *Bacillus cereus*. In: Doyle, MP., et al., editors. Food Microbiology: Fundamentals and Frontiers. 2nd. ASM Press; Washington, D.C.: 2001. p. 373-381.
10. Rajkowski, KT.; Smith, JL. Update: Food Poisoning and Other Diseases Induced by *Bacillus cereus*. In: Hui, YH.; Pierson, MD.; Gorham, JR., editors. Foodborne Disease Handbook. 2nd. Vol. I. Marcel Dekker, Inc.; New York, Basel: 2001. p. 61-76.
11. Schoeni JL, Wong MCL. *Bacillus cereus* food poisoning and its toxins. J Food Protect. 2005; 68:636–648.
12. Sorokin A, Kandelon B, Guilloux K, Galleron N, Wackerow-Kouzova N, Ehrlich SD, Bourguet D, Sanchis V. Multiple-locus sequence typing analysis of *Bacillus cereus* and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of psychrotrophic strains. Appl Environ Microbiol. 2006; 72:1569–1578.
13. Damgaard, PH. Natural occurrence and dispersal of *Bacillus thuringiensis* in the environment. In: Charles, JF.; Delecluse, A.; Nielsen-Le Roux, C., editors. Entomopathogenic Bacteria: from Laboratory to Field Application. Kluwer Academic Publishers; Dordrecht, Boston, London: 2000. p. 23-40.
14. Stenfors LP, Mayr R, Scherer S, Granum PE. Pathogenic potential of fifty *Bacillus weihenstephanensis* strains. FEMS Microbiol Lett. 2002; 215:47–51. [PubMed: 12393199]
15. Hendriksen NB, Hansen BM, Johansen JF. Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in a sandy loam. Antonie Van Leeuwenhoek. 2006; 89:239–249. [PubMed: 16710635]
16. Kotiranta A, Lounatmaa K, Haapasalo M. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect. 2000; 2:189–198. [PubMed: 10742691]
17. Hoffmaster AR, Ravel J, Rasko DA, Chapman GD, Chute MD, Marston CK, De BK, Sacchi CT, Fitzgerald C, Mayer LW, Maiden MC, Priest FG, Barker M, Jiang L, Cer RZ, Rilstone J, Peterson SN, Weyant RS, Galloway DR, Read TD, Popovic T, Fraser CM. Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proc Natl Acad Sci USA. 2004; 101:8449–8454. [PubMed: 15155910]
18. Han CS, Han CS, Xie G, Challacombe JF, Altherr MR, Bhotika SS, Brown N, Bruce D, Campbell CS, Campbell ML, Chen J, Chertkov O, Cleland C, Dimitrijevic M, Doggett NA, Fawcett JJ, Glavina T, Goodwin LA, Green LD, Hill KK, Hitchcock P, Jackson PJ, Keim P, Kewalramani AR, Longmire J, Lucas S, Malfatti S, McMurphy K, Meincke LJ, Misra M, Moseman BL, Mundt M, Munk AC, Okinaka RT, Parson-Quintana B, Reilly LP, Richardson P, Robinson DL, Rubin E, Saunders E, Tapia R, Tesmer JG, Thayer N, Thompson LS, Tice H, Ticknor LO, Wills PL, Bretin TS, Gilna P. Pathogenomic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis*. J Bacteriol. 2006; 188:3382–3390. [PubMed: 16621833]
19. Lapidus A, Goltsman E, Auger S, Galleron N, Séguens B, Dossat C, Land ML, Broussole V, Brillard J, Guinebretiere MH, Sanchis V, Nguen-the C, Lereclus D, Richardson P, Winker P, Weissenbach J, Ehrlich SD, Sorokin A. Extending the *Bacillus cereus* group genomics to putative food-borne pathogens of different toxicity. Chem Biol Interact. 2007 *ibid*.
20. Ehling-Schultz M, Fricker M, Scherer S. *Bacillus cereus*, the causative agent of emetic type of food-borne illness. Mol Nutr Food Res. 2004; 48:479–487. [PubMed: 15538709]
21. McKillip JL. Prevalence and expression of enterotoxins in *Bacillus cereus* and other *Bacillus* spp., a literature review. Antonie Van Leeuwenhoek. 2000; 77:393–399. [PubMed: 10959569]
22. Pirttijarvi TS, Andersson MA, Scoging AC, Salkinoja-Salonen MS. System., Evaluation of methods for recognising strains of the *Bacillus cereus* group with food poisoning potential among

- industrial and environmental contaminants. *Syst Appl Microbiol*. 1999; 22:133–144. [PubMed: 10188285]
23. Lambert B, Peferoen M. Insecticidal promise of *Bacillus thuringiensis*. Facts and mysteries about a successful biopesticide. *Bioscience*. 1992; 42:112–122.
 24. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev*. 1998; 62:775–806. [PubMed: 9729609]
 25. Burges HD. *Bacillus thuringiensis* in pest control. *Pesticide Outlook*. 2001; 12:90–98.
 26. Hansen, BM.; Salmitou, S. Virulence of *Bacillus thuringiensis*. In: Charles, J.; Delecluse, A.; Nielsen-Le Roux, C., editors. *Entomopathogenic Bacteria: from Laboratory to Field Application*. Kluwer Academic Publishers; Dordrecht, Boston, London: 2000. p. 41–64.
 27. Klier, A. Virulence of *Bacillus thuringiensis*. In: Charles, JF.; Delecluse, A.; Nielsen-Le Roux, C., editors. *Entomopathogenic Bacteria: from Laboratory to Field Application*. Kluwer Academic Publishers; Dordrecht, Boston, London: 2000. p. 485–504.
 28. Whalon ME, Wingerd BA. Bt: Mode of action and use. *Arch Insect Biochem Physiol*. 2003; 54:200–211. [PubMed: 14635181]
 29. Rivera AMG, Granum PE, Priest FG. Common occurrence of enterotoxin genes and enterotoxicity in *Bacillus thuringiensis*. *FEMS Microbiol Lett*. 2000; 190:151–155. [PubMed: 10981706]
 30. Damgaard PH, Granum PE, Bresciani J, Torregrossa MV, Eilenberg J, Valentino L. Characterization of *Bacillus thuringiensis* isolated from infections in burn wounds. *FEMS Immunol Med Microbiol*. 1997; 18:47–53. [PubMed: 9215586]
 31. Perani M, Bishop AH, Vaid A. Prevalence of β -exotoxin, diarrhoeal toxin and specific δ -endotoxin in natural isolates of *Bacillus thuringiensis*. *FEMS Microbiol Lett*. 1998; 160:55–60. [PubMed: 9495012]
 32. Guttman DM, Ellar DJ. Phenotypic and genotypic comparison of 23 strains from the *Bacillus cereus* complex for a selection of known and putative *B. thuringiensis* virulence factors. *FEMS Microbiol Lett*. 2000; 188:7–13. [PubMed: 10867226]
 33. Sergeev N, Distler M, Vargas M, Chizhikov V, Herold KE, Rasooly A. Microarray analysis of *Bacillus cereus* group virulence factors. *J Microbiol Methods*. 2006; 65:488–502. [PubMed: 16242802]
 34. Jackson SG, Goodbrand RB, Ahmed R, Kasatiya S. *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. *Lett Appl Microbiol*. 1995; 21:103–105. [PubMed: 7639990]
 35. Damgaard PH. Diarrhoeal enterotoxin production by strains of *Bacillus thuringiensis* isolated from commercial *Bacillus thuringiensis*-based insecticides. *FEMS Immunol Med Microbiol*. 1995; 12:242–250.
 36. Helgason E, Caugant DA, Olsen IK, Kolsto AB. Genetic structure of population of *Bacillus cereus* and *B. thuringiensis* isolates associated with periodontitis and other human infections. *J Clin Microbiol*. 2000; 38:1615–1622. [PubMed: 10747152]
 37. Mantynen V, Lindstrom K. A rapid PCR-based DNA test for enterotoxigenic *Bacillus cereus*. *Appl Environ Microbiol*. 1998; 64:1634–1639.
 38. Pruss BM, Dietrich R, Nibler B, Martlbauer E, Scherer S. The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. *Appl Environ Microbiol*. 1999; 65:5436–5442.
 39. Beattie SH, Williams AG. Detection of toxigenic strains of *Bacillus cereus* and other *Bacillus spp.* with an improved cytotoxicity assay. *Lett Appl Microbiol*. 1999; 28:221–225. [PubMed: 10196773]
 40. Hsieh YM, Sheu SJ, Chen YL, Tsen HY. Enterotoxigenic profiles and polymerase chain reaction detection of *Bacillus cereus* group cells and *B. cereus* strains from foods and food-borne outbreaks. *J Appl Microbiol*. 1999; 87:481–490. [PubMed: 10583675]
 41. Tsen HY, Chen ML, Hsieh YM, Sheu SJ, Chen YL. *Bacillus cereus* group strains, their hemolysin BL activity, and their detection in foods using a 16S RNA and hemolysin BL gene-targeted multiplex polymerase chain reaction system. *J Food Prot*. 2000; 63:1496–1502. [PubMed: 11079690]

42. Yang IC, Shih DY, Huang TP, Hunag YP, Wang JY, Pan TM. Establishment of a novel multiplex PCR assay and detection of toxigenic strains of the species in the *Bacillus cereus* group. *J Food Prot.* 2005; 68:2123–2130. [PubMed: 16245717]
43. Hendriksen NB, Hansen BM, Johansen JE. Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in sandy loam. *Antonie Van Leeuwenhoek.* 2006; 89:239–249. [PubMed: 16710635]
44. Heins, SD.; Manker, DS.; Jimenez, DR.; Marrone, PG. *Bacillus mycoides* strain for controlling corn rootworms. US Patent 5906818. 1999.
45. Lim DV, Simpson JM, Kearns EA, Kramer MF. Current and developing technologies for monitoring agents of bioterrorism and biowarfare. *Clin Microbiol Rev.* 2005; 18:583–607. [PubMed: 16223949]
46. Dib EG, Dib SA, Korkmaz DA, Mobarakai NK, Glaser JB. Nonhemolytic, nonmotile Gram-positive rods indicative of *Bacillus anthracis*. *Emerg Infect Dis.* 2003; 9:1013–1015. [PubMed: 12967505]
47. Klee SR, Nattermann H, Becker S, Urban-Schriefer M, Jacob D, Appel B. Evaluation of different methods to discriminate *Bacillus anthracis* from other bacteria of the *Bacillus cereus* group. *J Appl Microbiol.* 2006; 100:673–681. [PubMed: 16553722]
48. Levine SM, Tang YW, Pei Z. Recent advances in the rapid detection of *Bacillus anthracis*. *Rev Med Microbiol.* 2005; 16:125–133.
49. Nubel U, Schmidt PM, Reiss E, Bier F, Beyer W, Naumann D. Oligonucleotide microarray based on intergenic transcribed spacer in ribosomal DNA. *FEMS Microbiol Lett.* 2004; 240:215–223. [PubMed: 15522510]
50. Volokhov D, Pomerantzev A, Kivovich V, Rasooly A, Chizhikov V. Identification of *Bacillus anthracis* by multipore microarray hybridization. *Diagn Microbiol Infect Dis.* 2004; 49:163–171. [PubMed: 15246505]
51. Burton JE, Oshota OJ, Silman NJ. Differential identification of *Bacillus anthracis* from environmental *Bacillus* species using microarray analysis. *J Appl Microbiol.* 2006; 101:754–763. [PubMed: 16968287]
52. Hutson RA, Duggleby CJ, Lowe JR, Manchee RJ, Turnbull PCB. The development and assessment of DNA and oligonucleotide probes for the specific detection of *Bacillus anthracis*. *J Appl Bacteriol.* 1993; 75:463–472. [PubMed: 8300447]
53. Lee MA, Brightwell G, Leslie D, Bird H, Hamilton A. Fluorescent detection techniques for real-time multiplex strand specific detection of *Bacillus anthracis* using rapid PCR. *J Appl Microbiol.* 1999; 87:218–223. [PubMed: 10475953]
54. Patra G, Sylvestre P, Ramisse V, Therasse J, Guedson JL. DNA fingerprinting of *Bacillus anthracis*. *Salisbury Medical Bulletin.* 1996;59. Special Supplement No. 87.
55. Henderson I. Fingerprinting *Bacillus anthracis* strains. *Salisbury Medical Bulletin.* 1996;55–58. Special Supplement No. 87.
56. Shangkuan YH, Yang JF, Lin HC, Shaio MF. Comparison of PCR-RFLP, ribotyping and ERIC-PCR for typing *Bacillus anthracis* and *Bacillus cereus* strains. *J Appl Microbiol.* 2000; 89:452–462. [PubMed: 11021577]
57. Harrell LJ, Andersen GL, Wilson KH. Genetic variability of *Bacillus anthracis* and related species. *J Clin Microbiol.* 1995; 33:1847–1850. [PubMed: 7665658]
58. Daffonchio D, Cherif A, Borin S. Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the “*Bacillus cereus* Group”. *Appl Environ Microbiol.* 2000; 66:5460–5468. [PubMed: 11097928]
59. Ko KS, Kim JM, Kim JW, Jung BY, Kim W, Kim IJ, Kook YH. Identification of *Bacillus anthracis* by *rpoB* sequence analysis and multiplex PCR. *J Clin Microbiol.* 2003; 41:2908–2914. [PubMed: 12843020]
60. Hurtle W, Bode E, Kulesh DA, Kaplan RS, Garrison J, Bridge D, House M, Frye MS, Loveless B, Norwood D. Detection of the *Bacillus anthracis gyrA* gene by using a minor groove binder probe. *J Clin Microbiol.* 2004; 42:179–185. [PubMed: 14715750]

61. La Duc MT, Satomi M, Agata N, Venkateswaran K. *gyrB* as a phylogenetic discriminator for members of the *Bacillus anthracis* group. *J Microbiol Methods*. 2004; 56:383–394. [PubMed: 14967230]
62. Radnedge L, Agron PG, Hill KK, Jackson P, Ticknor LO, Keim P, Anderson GL. Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. *Appl Environ Microbiol*. 69:2755–2764.
63. Sozhamannan S, Chute MD, McAfee FD, Fouts DE, Akmal A, Galloway DR, Meteczun A, Baillie LW, Read TD. The *Bacillus anthracis* chromosome contains four conserved, excision-proficient, putative prophages. *BMC Microbiology*. 2006; 6:34. [PubMed: 16600039]
64. Keim P, Van Ert MN, Pearson T, Vogler AJ, Huyih LY, Wagner DM. Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scale. *Infect Genet Evol*. 2004; 4:205–213. [PubMed: 15450200]
65. Van Ert MN, Easterday WR, Huynh LY, Okinaka RT, Hung-Jones ME, Ravel J, Zaneki SR, Pearson T, Simonson TS, U'Ren JM, Kachur SM, Leadem-Dougherty RR, Rhoton SD, Zinser G, Farlow J, Coker PR, Smith KL, Kenefic LJ, Fraser-Liggett CM, Vagner DM, Keim P. Global genetic population structure of *Bacillus anthracis*. *PLoS ONE*. 2007; 5:e46.
66. Cherif A, Borin S, Rizi A, Quzari H, Boudabous A, Daffonchio D. Characterization of a repetitive element polymorphism-polymerase chain reaction chromosomal marker that discriminates *Bacillus anthracis* from related species. *J Appl Microbiol*. 2002; 63:450–462.
67. Kiem P, Kalif A, Schupp J, Hill K, Travis SE, Richmond K, Adair DM, Hugh-Jones M, Kuske CR, Jackson P. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J Bacteriol*. 1997; 179:818–824. [PubMed: 9006038]
68. Jackson PJ, Hill KK, Laker MT, Ticknor LO, Keim P. Genetic comparison of *Bacillus anthracis* and its close relatives using amplified fragment length polymorphism and polymerase chain reaction analysis. *J Appl Microbiol*. 1999; 87:263–269. [PubMed: 10475963]
69. Hill KK, Ticknor LO, Okinaka RT, Asay M, Blair H, Bliss KA, Laker M, Pardington PE, Richardson AP, Tonks M, Beecher DJ, Kemp JD, Kolsto AB, Wong ACL, Keim P, Jackson PJ. Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis* isolates. *Appl Environ Microbiol*. 2004; 70:1068–1080. [PubMed: 14766590]
70. Helgason E, Økstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I, Kolsto AB. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. *Appl Environ Microbiol*. 2000; 66:2627–2630. [PubMed: 10831447]
71. Beyer W, Pocivalsek S, Böhm R. Polymerase chain reaction-ELISA to detect *Bacillus anthracis* from soil samples — limitations of present published primers. *J Appl Microbiol*. 1999; 87:229–236. [PubMed: 10475955]
72. Strizhkov BN, Drobyshev AL, Mikhailovich VM, Mirzabekov AD. PCR amplification on a microarray of gel-immobilized oligonucleotides: detection of bacterial toxin- and drug-resistant genes and their mutations. *BioTechniques*. 2000; 29:844–857. [PubMed: 11056816]
73. Koonin EV, Makarova KS, Aravind L. Horizontal gene transfer in procariotes: quantification and classification. *Annu Rev Microbiol*. 2001; 55:709–742. [PubMed: 11544372]
74. Jain R, Rivera MC, Moore JE, Lake JA. Horizontal gene transfer in microbial genome evolution. *Theor Popul Biol*. 2002; 61:489–495. [PubMed: 12167368]
75. Lawrence JG. Gene transfer in bacteria: speciation without species? *Theor Popul Biol*. 2002; 61:449–460. [PubMed: 12167364]
76. Santoyo G, Romero D. Gene conversion and concerted evolution in bacterial genomes. *FEMS Microbiol Rev*. 2005; 29:169–183.
77. Hacker J, Kaper J. Pathogenicity islands and the evolution of microbes. *Annu Rev Microbiol*. 2000; 54:641–679. [PubMed: 11018140]
78. Rasko DA, Altherr MR, Han CS, Ravel J. Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev*. 2005; 29:303–329.
79. Brenner, DJ.; Staley, JT.; Krieg, NR. Classification of prokaryotic organisms and the concept of bacterial speciation. In: Garrity, GM., editor. *Bergey's manual of systematic bacteriology*, 2nd ed Vol 2, The Proteobacteria; Part A Introductory Essays. Springer; New York, N.Y.: 2005. p. 27-31.

80. Garrity, GM.; Bell, JA.; Lilburn, T. The revised road map to the Manual. In: Garrity, GM., editor. *Bergey's manual of systematic bacteriology*, 2nd ed Vol 2, The Proteobacteria; Part A Introductory Essays. Springer; New York, N.Y.: 2005. p. 159-187.
81. Woese CR. Interpreting the universal phylogenetic tree. *Proc Natl Acad Sci USA*. 2000; 97:8392–8396. [PubMed: 10900003]
82. Ash C, Collins MD. Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-directsequencing. *FEMS Microbiol Lett*. 1992; 94:75–80. [PubMed: 1381695]
83. 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 16S rRNA. *Int J Syst Bacteriol*. 1991; 41:343–346. [PubMed: 1715736]
84. Ash C, Farrow JAE, Wallbanks W, Collins MD. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Lett Appl Microbiol*. 1991; 13:202–206.
85. Bavykin SG, Lysov YP, Zakhariyev V, Kelly JJ, Jackman J, Stahl DA, Cherni A. Use of 16S rRNA, 23S rRNA, and *gyrB* gene sequence analysis to determine phylogenetic relationships of *Bacillus cereus* group microorganisms. *J Clin Microbiol*. 2004; 42:3711–3730. [PubMed: 15297521]
86. Zlatanova J, Mirzabekov AD. Gel immobilized microarrays of nucleic acids and proteins. *Methods Mol Biol*. 2001; 170:17–38. [PubMed: 11357680]
87. Bavykin SG, Akowski JP, Zakhariyev VM, Barsky VE, Perov AN, Mirzabekov AD. Portable system for microbial sample preparation and oligonucleotide microarray analysis. *Appl Environ Microbiol*. 2001; 67:922–928. [PubMed: 11157263]
88. Guschin D, Yershov G, Zaslavsky A, Gemmell A, Shick V, Proudnikov D, Arenkov P. Mirzabekov, Manual manufacturing of oligonucleotide, DNA, and protein microarrays. *Anal Biochem*. 1997; 250:203–211. [PubMed: 9245440]
89. Proudnikov D, Timofeev E. Mirzabekov, Immobilization of DNA in polyacrylamide gel for the manufacture of DNA and DNA-oligonucleotide microarrays. *Anal Biochem*. 1998; 259:34–41. [PubMed: 9606140]
90. Timofeev EN, Kochetkova SV, Mirzabekov AD, Florentiev VL. Regioselective immobilization of short oligonucleotides to acrylic copolymer gels. *Nucl Acids Res*. 1996; 24:3142–3148. [PubMed: 8774893]
91. Stahl DA, Flesher B, Mansfield HR, Montgomery L. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl Environ Microbiol*. 1988; 54:1079–1084. [PubMed: 3389805]
92. Kelly JJ, Chernov BK, Tovstanovsky I, Mirzabekov AD, Bavykin SG. Radical generating coordination complexes as a tool for rapid and effective fluorescent labeling and fragmentation of DNA or RNA for microarray hybridization. *Analyt Biochem*. 2002; 311:103–118. [PubMed: 12470669]
93. Barsky, IYa; Grammatin, AP.; Ivanov, AV.; Kreindlin, EYa; Kotova, EYu; Barsky, VE.; Mirzabekov, AD. Wide-field luminescence microscopes for analyzing biological microarrays. *J Opt Technol*. 1998; 65:938–941.
94. Yershov G, Barsky V, Belgovskiy A, Kirillov Eu, Kreindlin E, Ivanov I, Parinov S, Guschin D, Drobyshev A, Dubiley S, Mirzabekov A. DNA analysis and diagnostics on oligonucleotide microarrays. *Proc Natl Acad Sci USA*. 1996; 93:4913–4918. [PubMed: 8643503]
95. Chen ML, Tsen HY. Discrimination of *Bacillus cereus* and *Bacillus thuringiensis* with 16S rRNA and *gyrB* gene based PCR primers and sequencing of their annealing sites. *J Appl Microbiol*. 2002; 92:912–919. [PubMed: 11972696]
96. Yamada S, Ohashi E, Agata N, Venkateswaran K. Cloning and nucleotide sequence analysis of *girB* of *Bacillus cereus*, *B. thuringiensis*, *B. mycoides*, and *B. cereus* in rice. *Appl Environ Microbiol*. 1999; 65:1483–1490. [PubMed: 10103241]
97. Ticknor LO, Kolsto AB, Hill KK, Keim P, Laker MT, Tonks M, Jackson PJ. Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. *Appl Environ Microbiol*. 2001; 67:4863–4873. [PubMed: 11571195]

98. Cherif A, Borin S, Rizzi A, Ouzari H, Boundabous A, Daffonchio D. *Bacillus anthracis* diverges from related clades of the *Bacillus cereus* group in 16-23S ribosomal DNA intergenic transcribed spacer containing tRNA genes. *Appl Environ Microbiol.* 2003; 69:33–40. [PubMed: 12513974]
99. Daffonchio D, Raddadi N, Merabishvili M, Cherif A, Carmagnola L, Brusetti L, Rizi A, Chanishvili N, Visca P, Sharp R, Borin S. Strategy for identification of *Bacillus cereus* and *Bacillus thuringiensis* strains closely related to *Bacillus anthracis*. *Appl Environ Microbiol.* 2006; 72:1295–1301. [PubMed: 16461679]
100. von Stetten F, Francis KP, Lechner S, Neuhaus K, Scherer S. Rapid discrimination of psychrotolerant and mesophilic strains of the *Bacillus cereus* group by PCR targeting of the 16S rDNA. *J Microbiol Methods.* 1998; 34:99–106.
101. Hendriksen NB, Hansen BM, Johansen JE. Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in sandy loam. *Antonie Van Leeuwenhoek.* 2006; 89:239–249. [PubMed: 16710635]
102. Wunschel D, Fox KF, Black GE, Fox A. Discrimination among the *Bacillus cereus* group, in comparison to *B. subtilis*, by structural carbohydrate profiles and ribosomal RNA spacer region PCR. *Syst Appl Microbiol.* 1994; 17:625–635.
103. Carlson CR, Causant DA, Kolsto AB. Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. *Appl Environ Microbiol.* 1994; 60:1719–1725. [PubMed: 16349267]
104. Helgason E, Causant DA, Lecadet MM, Chen Y, Mahillon J, Lovgren A, Hegna I, Kvaløy K, Kolstø AB. Genetic diversity of *Bacillus cereus*/*B. thuringiensis* isolates from natural sources. *Curr Microbiol.* 1998; 37:80–87. [PubMed: 9662607]
105. Helgason E, Tourasse NJ, Meisal R, Causant DA, Kolsto AB. Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. *Appl Environ Microbiol.* 2004; 70:191–201. [PubMed: 14711642]
106. Rasko DA, Ravel J, Okstad OA, Helgason E, Cer RZ, Jiang L, Shores KA, Fouts DE, Tourasse NJ, Angiuoli SV, Kolonay J, Nelson WC, Kolsto AB, Fraser CM, Read TD. The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. *Nucl Acids Res.* 2004; 32:977–988. [PubMed: 14960714]
107. Ko KS, Kim JW, Kim JM, Kim W, Chung S, Kim IJ, Kook YH. Population structure of the *Bacillus cereus* group as determined by sequence analysis of six Housekeeping genes and the *plcR* gene. *Infect Immun.* 2004; 72:5253–5261. [PubMed: 15322020]
108. Priest FG, Barker M, Baillie LWJ, Holmes EC, Maiden MCJ. Population structure and evolution of the *Bacillus cereus* group. *J Bacteriol.* 2004; 186:7959–7970. [PubMed: 15547268]
109. Vassileva M, Torii K, Oshimoto M, Okamoto A, Agata N, Yamada K, Hasegawa T, Ohta M. Phylogenetic analysis of *Bacillus cereus* isolates from severe systemic infections using multilocus sequence typing scheme. *Microbiol Immunol.* 2006; 50:743–749. [PubMed: 16985296]
110. Tourasse NJ, Helgason E, Okstad OA, Hegna IK, Kolsto AB. The *Bacillus cereus* group: novel aspects of population structure and genome dynamics. *J Appl Microbiol.* 2006; 101:579–593. [PubMed: 16907808]
111. Ehling-Schultz M, Svensson B, Guinebretiere MH, Lindback T, Andersson M, Schulz A, Fricker M, Christiansson A, Granum PE, Martlbauer E, Nguyen-The C, Salkinoja-Salonen M, Scherer S. Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. *Microbiology.* 2005; 151:183–197. [PubMed: 15632437]
112. Bavykin SG, Lysov YP, Zakhariev V, Kelly JJ, Jackman J, Stahl DA, Cherni A. Erratum. *J Clin Microbiol.* 2006; 44:2676.
113. Yang IC, Shih DY, Huang TP, Huang YP, Wang JY, Pan TM. Establishment of a novel multiplex PCR assay and detection of toxigenic strains of the species in the *Bacillus cereus* group. *J Food Prot.* 2005; 68:2123–2130. [PubMed: 16245717]
114. Berry C, O'Neil S, Ben-Dov E, Jones AF, Murphy L, Quail MA, Holden MT, Harris D, Zaritsky A, Parkhill J. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. israelensis. *Appl Environ Microbiol.* 2002; 68:5082–5095. [PubMed: 12324359]

115. Van der Auwera GA, Andrup L, Mahillon J. Conjugative plasmid pAW63 brings new insights into the genesis of the *Bacillus anthracis* virulence plasmid pXO2 and of the *Bacillus thuringiensis* plasmid pBT9727. *BMC Genomics*. 2005; 6:103. [PubMed: 16042811]
116. Ehling-Schultz M, Fricker M, Grallert H, Rieck P, Wagner M, Scherer S. Cereulide synthetase gene cluster from emetic *Bacillus cereus*: Structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin pXOI. *BMC Microbiology*. 2006; 6:20. [PubMed: 16512902]
117. Luna VA, King DS, Peak KK, Reeves F, Heberlein-Larson L, Veguilla W, Heller L, Duncan KE, Cannons AC, Amuso P, Cattani J. *Bacillus anthracis* virulent plasmid pXO2 genes found in large plasmids of two other *Bacillus* species. *J Clin Microbiol*. 2006; 44:2367–2377. [PubMed: 16825351]
118. Pannucci J, Okinaka RT, Sabin R, Kuske CR. *Bacillus anthracis* pXO1 plasmid sequence conservation among closely related bacterial species. *J Bacteriol*. 2002; 184:134–141. [PubMed: 11741853]
119. Bremer, H.; Dennis, PP. Modulation of chemical composition and other parameters of the cell by growth rate. In: Neidhardt, FC.; Ingraham, JL.; Low, KB.; Magasanik, B.; Schaechter, M.; Umberger, HE., editors. *Escherichia coli and Salmonella typhimurium: cellular and molecular biology*. Vol. 2. American Society for Microbiology; Washington, D.C.: 1987. p. 1527-1542.
120. Vitko, J., Jr; Franz, DR.; Alper, M.; Biggins, PDE.; Brandt, LD.; Bruckner-Lia, C.; Burge, HA.; Ediger, R.; Hollis, MA.; Laughlin, LL.; Mariella, RP., Jr; McFarland, AR.; Schaudies, RP. Sensor systems for biological agent attacks. *The National Academies Press*; Washington, D.C.: 2005. Nucleic acid sequence-based identification for detect-to-warn applications; p. 84-104.
121. Mei R, Hubbell E, Bekiranov S, Mittmann M, Christians FC, Shen MM, Lu G, Fang J, Liu WM, Ryder T, Kaplan P, Kulp D, Webster TA. Probe selection for high-density oligonucleotide arrays. *Proc Natl Acad Sci USA*. 2003; 100:11237–11242. [PubMed: 14500916]
122. Brodie EL, DeSantis TZ, Parker JPM, Zubietta IX, Piceno YM, Andersen GL. Urban aerosol harbor diverse and dynamic bacteria populations. *Proc Natl Acad Sci USA*. 2007; 104:299–304. [PubMed: 17182744]
123. Reynolds KS, Gerba CP, Pepper IL. Rapid PCR-based monitoring of infectious enteroviruses in drinking water. *Wat Sci Tech*. 1997; 35(11-12):423–427.

Fig. 1.

Positions of subgroup-specific sequence differences in the 16S rRNA (A) and 23S rRNA (B) genes of seven *B. cereus* subgroups and corresponding sequences of reference microorganisms. The 16S rRNA and 23S rRNA sequences of *B. anthracis* Sterne (GenBank accession no. AF 176321 and AF 267877, respectively) in 5' to 3' orientation have been used as the consensus sequences. Vertical lines denote nucleotides identical to the consensus sequence. Numbers indicate distance from 5'-end of consensus rRNA sequence. Principle of pair probe's design is shown below sequences on Fig. 1A. R = G, or A; Y = T, or C.

Fig. 2. Identification of *B. cereus* subgroup reference microorganisms with 16S rRNA oligonucleotide microarray. Bulk RNA from reference microorganisms was isolated, fluorescently labeled with LissRhod, and hybridized with a microarray bearing 20 b oligonucleotides. Positions of the probes and targeted subgroups (in rectangles) are shown in the upper right corner. Reference organisms and their subgroups (in parentheses) are indicated under each hybridization image captured with stationary microscope. For more details about probe design and probe abbreviations, see Table 1.

Fig. 3. Discrimination of subgroups Anthracis and Cereus A (A), or Mycoides A and Mycoides B (B) isolates with 23S rRNA probes. Image capture was carried out with stationary microscope for *B. anthracis* Sterne, *B. thuringiensis* B8 and *B. cereus* HER1414, and with portable reader for all other isolates (A, B). Probe signal ratio is shown on the right site of the images. For more details about probe design and probe abbreviations, see Table 2.

	ps21/ps22	ps21/ps22 signal ratio	site 1559 in 23S rRNA	G/A ratio in site 1559
<i>B. anthracis</i> Ames (Anthraxis)		1.80	G	-
<i>B. mycoides</i> ATCC10206 (Mycoides B)		1.50	G	-
<i>B. cereus</i> T (Cereus B)		1.00	R	1.5
<i>B. thuringiensis</i> 4Q281 (Thuringiensis B)		0.72	R	1
<i>B. cereus</i> NCTC9620 (Cereus B)		0.64	R	0.3
<i>B. thuringiensis</i> B8 (Cereus A)		0.45	A	-

Fig. 4.
Identification of single-base polymorphisms in reference microorganisms using hybridization of fluorescently labeled total RNA from *B. cereus* group bacteria to probes targeting the 23S rRNA. Images were captured with stationary microscope. R = G or A.

Fig. 5.

Identification of “polymorphic” sites imitated in mixtures. Samples of *B. thuringiensis* B8 and *B. thuringiensis* 4Q281 labeled total RNA were hybridized with microarray separately (A, B) or mixed in proportions 1 to 2 (C), or 1 to 5 (D). Positions of the probes and targeted subgroups (in rectangles) are shown on scheme (E). Images were captured with stationary microscope. For probe signal ratio in pairs selected for SNP quantization see Table 3. Correlation between actual and predicted data is indicated on chart (F). Values of theoretical (x) versus experimental (y) signal ratios for four selected pairs obtained for two different mixtures, 1 to 2 and 1 to 5 (Table 3) determine each data point (blue rhombuses) position on the chart. Approximation line is described by equation $y = Ax + k$, where A and k are regression coefficient and error, respectively. One of the data point obtained for pair ps3/ps4 (rhombus in upper right corner) was not representative due to low hybridization signals. Calculations performed without these data revealed volumes of A to be equal to 0.96. The error values for all other pairs, where differences between experimental and theoretical ratios did not exceed 10% of the experimental ratio (Table 3), were 0.04 or less.

Microorganism	<i>B. cereus</i> group	<i>B. subtilis</i> group	Probe signal ratio
<i>B. anthracis</i> Ames (Anthraxis)			9.5
<i>B. cereus</i> T (Cereus B)			8.3
<i>B. mycoides</i> ATCC10206 (Mycoides B)			7.8
<i>B. thuringiensis</i> 4Q281 (Thuringiensis B)			8.1
<i>B. subtilis</i> B-459			0.4

ps 25 ps 26

Fig. 6. Identification of microbial groups. Microarray containing probes ps25 and ps26 targeting 16S rRNA of the *B. cereus* group and the *B. subtilis* group, respectively, was hybridized with labeled bulk RNA of the reference microorganisms. Probe signal ratios are shown in the far right column. Images were captured with stationary microscope.

Fig. 7.

Two strategies for microbial identification. Hierarchically nested probe design and analysis strategy (C and D) and endpoint probe design and analysis strategy (A and B). The hierarchical strategy reduces false positives due to concordance of positive microarray signals (red dots) at predecessor nodes in the phylogenetic tree, resulting in a “branch” analysis (D) instead of an “endpoint” analysis (B) for data interpretation and reporting.

Degree of matching between oligonucleotide probes on microarray and the 16S rRNA sequences of reference microorganisms from the *B. cereus* group

Table 1

Probe's target	all other		Anthr.; Cer A, B		all other				Anthr.; Cer A; Myc B		all other		all other	
	Thur B		Thur A, B		Myc B								Myc A	
Probe's name	ps1	ps3	ps5	ps7	ps9	ps11	ps13	ps15	ps17	ps19	ps17	ps18	ps27	ps28
	ps2 (77)	ps4 (90,92)	ps6 (90,92)	ps8 (192)	ps10 (189,200)	ps12 (189,200,208)	ps14 (200,208)	ps16 (208)	ps18 (1015)	ps20 (1036,1045)				(133)
<i>B. anthracis</i> Ames (Anthr)	+/-	+/-2	+/-2	+/-	+/-2	+/-3	+/-2	+/-	+/-	+/-2	+/-	+/-	+/-	+/-
<i>B. anthracis</i> Sterne (Anthr)	+/-	+/-2	+/-2	+/-	+/-2	+/-3	+/-2	+/-	+/-	+/-2	+/-	+/-	+/-	+/-
<i>B. cereus</i> NCTC11143 (Cer A)	+/-	+/-2	+/-2	+/-	+/-2	+/-3	+/-2	+/-	+/-	+/-2	+/-	+/-	+/-	+/-
<i>B. thuringiensis</i> B8 (Cer A)	+/-	+/-2	+/-2	+/-	+/-2	+/-3	+/-2	+/-	+/-	+/-2	+/-	+/-	+/-	+/-
<i>B. cereus</i> T (Cer B)	+/-	+/-2	+/-2	+/-	+/-2	+/-3	+/-2	+/-	+/-	+/-2	+/-	+/-	+/-	+/-
<i>B. cereus</i> NCTC9620 (Cer B)	+/-	+/-2	+/-2	+/-	+/-2	+/-3	+/-2	+/-	+/-	+/-2	+/-	+/-	+/-	+/-
<i>B. cereus</i> ATCC43881 (Thur A)	+/-	+/-2	+/-2	+/-	+/-2	-4	+/-2	+/-	+/-	+/-2	+/-	+/-	+/-	+/-
<i>B. thuringiensis</i> 4Q281 (Thur B)	+/-	-2/+	-2/+	+/-	+/-2	-4	+/-2	+/-	+/-	+/-2	+/-	+/-	+/-	+/-
<i>B. mycoides</i> ATCC6462 (Myc A)	+/-	+/-2	+/-2	-3/-2	-3/-5	-2/-5	-3	+/-	+/-	+/-2	-2/-	-2/-	-2/-	-2/-
<i>B. mycoides</i> ATCC 10206 (Myc B)	+/-	+/-2	+/-2	-2/-3	-2/+	-3/+	-2/+	-/+	-/+	-2/+	+/-	+/-	+/-	+/-

“+”, denotes perfect matching; “-”, “-2”, and “-3”, denote one, two and three mismatches, respectively; p, designates polymorphism; Anthr, Cer A, Cer B, Thur A, Thur B, Myc A and Myc B, correspond to subgroups Anthracis, Cereus A, Cereus B, Thuringiensis A, Thuringiensis B, Mycoides A and Mycoides B; “all other”, depicts “all other subgroups”; numbers in parenthesis indicate positions of targeted subgroup-specific differences on 16S rRNA (Fig. 1A).

Table 2

Degree of matching between oligonucleotide probes on microarray and the 23S rRNA sequences of reference microorganisms from the *B. cereus* group

Probe's target	Anth; Thur A; Myc A, B	Anth; Cer B	Myc B	Thur B; Myc A
	Cer A, Cer B	Cer A; Thur A	Anth; Cer A, B; Thur B; Myc A	Anth; Cer A, B; Myc B
Probe's name	ps21	ps23	ps29	ps31
	ps22 (1559)	ps24 (insertion 1218-1219)	ps30 (346, 347)	ps32 (174)
<i>B. anthracis</i> Ames/Sterne (Anthr)	+/-	+/-	-2/+	-/+
<i>B. cereus</i> NCTC11143 (Cer A)	-/+	-/+	-2/+	-/+
<i>B. thuringiensis</i> B8 (Cer A)	-/+	-/+	-2/+	-/+
<i>B. cereus</i> NCTC9620 (Cer B)	-/+	+/-	-2/+	-/+
<i>B. thuringiensis</i> 4Q281 (Thur B)	N/A	N/A	-2/+	+/-
<i>B. mycoides</i> ATCC6462 (Myc A)	+/-	N/A	-2/+	+/-
<i>B. mycoides</i> ATCC10206 (Myc B)	+/-	N/A	+/-2	-/+

“+”, denotes perfect matching; “-”, “-2”, and “-3”, denote one, two and three mismatches, respectively; p, designates polymorphism; Anthr, Cer A, Cer B, Thur A, Thur B, Myc A and Myc B, correspond to subgroups Anthracis, Cereus A, Cereus B, Thuringiensis A, Thuringiensis B, Mycoides A and Mycoides B; numbers in parenthesis indicate positions of targeted subgroup-specific differences on 23S rRNA (Fig. 1B).

Table 3

Probe pairs	<i>B.thuringiensis</i> B8 (Cereus A)	<i>B.thuringiensis</i> 4Q281 (<i>Thuringiensis</i> B)	<i>B.thuringiensis</i> B8 : <i>B.thuringiensis</i> 4Q281 1 : 2		<i>B.thuringiensis</i> B8 : <i>B.thuringiensis</i> 4Q281 1 : 5			
			Experimental	Theoretical	Exp/Theoret	Experimental	Theoretical	Exp/Theoret
ps3/ps4	4.245	0.653	0.905	1.216	0.744	0.863	0.941	0.917
ps5/ps6	1.860	0.182	0.426	0.412	1.033	0.306	0.298	1.027
ps7/ps8	2.810	0.270	0.442	0.470	0.941	0.351	0.368	0.954
ps17/ps18	6.697	0.256	0.621	0.611	1.016	0.414	0.427	0.970

Table 4
Probe signal ratio for *B. mycoides* ATCC10206 sample hybridization with the microarray

Probe pairs	Degree of probe matching	Hybridization time					
		Amount of labeled sample					
		2.5 µg	5 µg	2.5 µg	5 µg	1 µg	
ps3/ps4	+/-2	1.53	2.94	2.77	4.62	13.40	
ps5/ps6	+/-2	1.37	1.82	1.61	1.96	3.98	
ps14/ps13	+/-2	2.80	3.66	3.95	5.07	15.40	
ps17/ps18	+/-	1.52	2.06	1.97	2.51	10.00	
ps27/ps28	+/-	1.80	2.16	2.27	2.68	4.09	
ps31/ps32	+/-	1.49	1.70	1.43	1.62	2.54	

“+”, denotes perfect matching; “-”, or “-2”, denote one or two mismatches. Images were captured with stationary microscope.

Table 5

Influence of environmental admixtures on probe signal ratio

Probe pairs	K1 Pure Cells* (p/mm)	K2 Environmental Samples (p/mm)	K1/K2
ps3/ps4	3.100	2.302	1.347
ps5/ps6	2.719	1.960	1.387
ps14/ps13	7.404	6.424	1.153
ps17/ps18	3.462	1.925	1.798
ps27/ps28	4.088	3.111	1.314
ps31/ps32	1.974	1.862	1.060

* - logarithmic *B. mycoides* ATCC10206 cells were used for these experiments. Images were captured with portable reader after 1 hour hybridization of 3 micrograms labeled sample with microarray.

Table 6Subgroup affiliation^a of type strains and reference microorganisms that are common in the cited papers

Subgroups	Strains
Anthraxis	All <i>B. anthracis</i> strains
Anthraxis-Cereus A	<i>B. cereus</i> G9241 ^b
Cereus A	<i>B. cereus</i> ATCC10987 ^c ; <i>B. cereus</i> WSBC10030; <i>B. thuringiensis</i> 97-27 ^b ; <i>B. cereus</i> E33L ^b ; <i>B. cereus</i> NCTC11143 ^c ; <i>B. cereus</i> IMSNU 12078
Cereus B	<i>B. cereus</i> ATCC14579 ^c (DSM31 ^T) ^d ; <i>B. cereus</i> ATCC11778 ^c (ATCC9634; DMS345); <i>B. thuringiensis</i> HD1 ^e
Thuringiensis A	<i>B. thuringiensis</i> ATCC33679 ^f ; <i>B. cereus</i> ACTC43881
Thuringiensis B	<i>B. thuringiensis</i> ATCC10792 ^c (IMSNU 1208; DSM2046 ^T); <i>B. cereus</i> AH527; <i>B. thuringiensis</i> KCTC 1509 ^c
Mycoides A	All <i>B. weihenstephanensis</i> strains; <i>B. cereus</i> AH521 ^c ; <i>B. cereus</i> WSBC10204 ^T ; <i>B. mycoides</i> ATCC6462 ^c (DSM2048T)
Mycoides B	All <i>B. pseudomycoides</i> strains

^a For more detailed information about these strains see references [85,112].

^b Affiliation of these isolates to correspondent subgroups were obtained from analysis of rRNA and *gyrB* gene sequences extracted from whole genome sequences of these bacteria (AAEK00000000; AE017355; NC_006274). *B. cereus* G9241 represents intermediate form between subgroups Anthracis and Cereus A (S. Bavykin, personal communication).

^c Sequences of these isolates perfectly match with sequences of their subgroups.

^d Synonyms of strain title are shown in parenthesis.

^e Relation of the isolate was established in accordance with its *gyrB* gene sequence only, GeneBank accession number AF136390.

^f Isolate contains substitution C/Y(192) in its 16S rRNA sequence that makes it to be intermediate form between Thuringiensis A and Cereus B [85]. Finally this dilemma may be solved after sequencing of their 23S rRNA and *gyrB* genes.