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Reagentless Detection and Classification of Individual Bioaerosol Particles in Seconds

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The rapid chemical analysis of individual cells is an analytical capability that will profoundly impact many fields including bioaerosol detection for biodefense and cellular diagnostics for clinical medicine. This article describes a mass spectrometry-based analytical technique for the real-time and reagentless characterization of individual airborne cells without sample preparation. We characterize the mass spectral signature of individual *Bacillus* spores and demonstrate the ability to distinguish two *Bacillus* spore species, *B. thuringiensis* and *B. atrophaeus*, from one another very accurately and from the other biological and nonbiological background materials tested with no false positives at a sensitivity of 92%. This example demonstrates that the chemical differences between these two *Bacillus* spore species are consistently and easily detected within single cells in seconds.

The instantaneous physical and chemical characterization of many individual airborne cells in rapid succession is ideal for the detection of bioaerosols that have been intentionally released during a biological weapons attack. As an example, this article will focus on a rapid method of detecting individual airborne *Bacillus* cells in their spore form. Rapid detection of such attacks is inherently difficult due to the small sample sizes involved; for example, an aerosol particle containing a single *Bacillus anthracis* spore has a mass of $\sim 10^{-12}$ g.[1] The situation is further complicated by the fact that they would be widely dispersed among many other naturally occurring background particles that could be present at concentrations thousands of times their own.

While there are multiple standard microbiology- and molecular-based approaches for the detection of bioaerosols, current methods have shortcomings, particularly long analysis times, which range from hours to days when including sample collection, preparation, and actual analysis.[2] Various mass spectrometry techniques such as off-line matrix-assisted laser desorption/ionization (MALDI) and chemical ionization are effective at identifying certain microorganisms of interest but analyze samples that also contain large numbers of other atmospheric particles, potentially introducing interferences, or else require culturing prior to analysis, delaying identification.[3−10] An interesting permutation on MALDI whereby individual particles would be coated with a matrix and analyzed individually is currently in development.[11] Particle fluorescence sensors give real-time results but are susceptible to false positives and provide limited identification information.[12−17] We are currently developing a new technique, *bio*aerosol *m*ass spectrometry (BAMS), which has the potential to instantaneously detect species-level differences between single cells without the need for reagents, offering a high degree of detection specificity combined with both single-cell sensitivity and millisecond analysis times.

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Single-particle laser desorption/ionization mass spectrometry techniques[18] such as RSMS,[19] PALMS,[20] ATOFMS,[21] LAMPAS,[22] and others are methods of analyzing individual atmospheric particles in real time. Historically, nearly all single-particle mass spectrometric methods of analysis have been applied to bioaerosols at one point or another.[18,23−27] However, all previous attempts have been unable to distinguish individual organisms due to limits of instrumentation, including the use of mass spectrometric techniques incapable of collecting complete bipolar (simultaneous positive and negative ion) mass spectra for individual particles and the use of laser wavelengths and fluences that did not yield reproducible and identifiable spectra. As we will describe below, we have selected a laser operating at a wavelength of 266 nm and found it to yield reproducible, unique, identifiable mass spectra specific to *Bacillus* spores. The real-time aspect of single particle LDI-TOFMS techniques refers to the chemical analysis of individual particles within 1 ms of their removal from their natural environment. Here, we combine single-particle LDI-TOFMS with real-time pattern recognition to produce an instrument that is truly capable of performing real-time analysis of individual bioaerosol particles.

MATERIALS AND METHODS

Experimental Setup. Individual samples of powders and microorganisms enumerated below were aerosolized for analysis by BAMS. The powder samples were suspended by inserting an aliquot of the powder into a 1-L 0.22-μm filter/sterilizer (Corning, Inc., Corning, NY) which was then agitated. The smoke samples were collected by suspending a 5-gal plastic bottle over the source of combustion. Ambient particles were sampled directly through a hole in the laboratory wall. The microorganisms were washed and diluted in deionized water for suspension in a Collison nebulizer (TSI, Inc., Shoreview, MN). Upon suspension, the particles were conducted through a $^3/_8$-in. copper tube to a diffusion drier (TSI, Inc.), where they were dried. In all cases, the particles were finally conducted through a $^3/_8$-in. copper tube to an aerosol time-of-flight mass spectrometer[21] (TSI, Inc.), which was refitted as a bioaerosol mass spectrometer, as described in the text, for analysis. Mixtures of particles were generated by mixing the powders in a 5-gal plastic jug and agitating the jug. Nebulized microorganisms were dried and introduced through a hole in the jug for simultaneous analysis.

Sample Preparation. *Bacillus atrophaeus*, formerly known as *Bacillus subtilis var. niger* or *Bacillus globigii* (ATCC No. 9372), *Bacillus thuringiensis* (ATCC No. 10792), and *Thelebolus stercoreus* (ATCC No. 16494) were obtained from the American Type Culture Collection (ATCC, http://www.atcc.org). All three organisms are risk group I organisms as defined by the ATCC and CDC. The species' identities were confirmed by the extramural testing of their 16S ribosomal DNA.

Following inoculation (1:25) with stationary-phase cultures, each species of *Bacillus* bacterial cells was grown in $^1/_4\times$ TY sporulation media and G media. In addition, *B. atrophaeus* cells were grown in Luria broth. *Bacillus* cells were grown at 32 °C with agitation for up to 10 days and harvested after ~90% were determined to be refractile. The percentage of sporulated cells was determined by a combination of spore stains, Gram stains, and phase contrast microscopy. Sporulated cells were harvested by centrifugation at 8000*g* for 12 min and washed in cold double-distilled water 3× before being reconstituted in 5 mL of double-distilled water. Approximately 10^5 cells were aliquoted into a Collison nebulizer for BAMS analysis.

Fungal spores of *T. stercoreus* (ATCC No. 16494) were analyzed both as received from the ATCC as a lyophilized spore preparation and as a prepared purified sample as part of a multicomponent experiment as presented in Figure 3. Fungal spores of *T. sterocoreus* preparations were cultured on potato dextrose agar (Difco, http://www.difco.com) at 25 °C. Fungal spores were isolated via filtration through sterile cheesecloth followed by centrifugation at 8000*g* and washed three times in double-distilled sterile water. The purity of the fungal spores was confirmed by phase contrast microscopy.

Knox Unflavored Gelatine, Gold Bond Medicated Powder, and Equal sweetener were procured from a local grocery store. Luria Broth Agar Powder was obtained from Sigma (Lot No. 80K1071). Smoke was generated by igniting a Marlboro cigarette and separately by igniting a wood chip found near our laboratory building. Environmentally sampled particulate standards for Urban Particulate Matter (SRM/RM 1648), San Joaquin Valley Soil (SRM/RM 2709), and Diesel Particulate Matter (Industrial Forklift, SRM/RM 2975) were ordered from the National Institute of Standards and Technology. A total of 1000 ambient particles were analyzed from outside of our laboratory building in Livermore, CA.

Aerosol Time-of-Flight Mass Spectrometry. Air and entrained aerosol particles are drawn directly from the environment through a converging nozzle into vacuum where each particle is accelerated to a terminal velocity as a function of its aerodynamic diameter. Each particle continues through three stages of differential pumping and passes through two continuous wave laser beams, scattering light from each in turn. The time between the scattering events indicates the velocity of the particle and, thus, its size. The measurement of the position and velocity of the particle is also used to determine its time of arrival in the center of the ionization region of a time-of-flight mass spectrometer. A pulsed laser operating at 266 nm is fired at this time and desorbs and ionizes material from the particle. Both positive and negative ion reflectron time-of-flight mass spectra for each particle are collected simultaneously in opposing directions by separate mass spectrometers.

Data Analysis. Briefly, each positive or negative mass spectrum is treated as a 350-element vector. The *n*th element in

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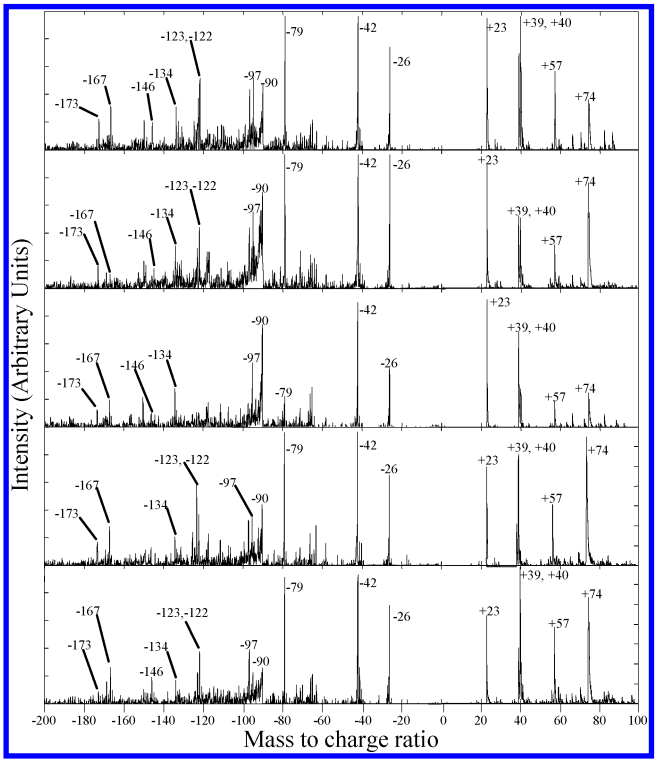

Figure 1. Dual-polarity mass spectra selected at random from five individual *B. atrophaeus* spores. This figure exhibits the degree of reproducibility between the spectra of individual cells. The real-time data analysis algorithm was designed to recognize the origin of the particles despite these variations.

each vector represents the total area of all peaks within 0.5 Da of *n*. Similar spectra produce similar vectors, and the angle between the vectors is small. Standard spectra collected from particles of known compositions can therefore be compared easily with analyte spectra. If the analyte's positive and negative vectors are both within a certain minimum angle of a standard's positive and negative values, in our case 46°, it is considered a match. In the event that multiple standards match, the analyte is identified as the standard with the most similar negative spectrum. If there is no matching standard, the spectrum is considered "other." Species of *Bacillus* may be further resolved from each other by the rules-based method described in the text.

RESULTS

Analysis of *Bacillus* Endospores. To evaluate our instrument's application to the detection of *B. anthracis*, we selected surrogates from within the genus *Bacillus*. *B. atrophaeus* is commonly used as a surrogate for *B. anthacis* in the biodefense community, while *B. thuringiensis* is a commonly used pesticide that differs from *B. anthracis* primarily by the latter's two virulence plasmids.[1,28] Figure 1 shows five individual mass spectra of *B. atrophaeus* selected randomly. There was some variability among spectra from the same sample, and the quality and reproducibility of the data from each material depend heavily on the desorption/ionization laser wavelength and fluence. The wavelength was selected to be absorbed well by dipicolinic acid (DPA) that comprises between 5 and 15% of the dry weight of a *Bacillus* spore,[1] and the power was optimized empirically to provide the most information about the spores in each spectrum while minimizing spectrum-to-spectrum variability. A Nd:YAG laser operating at a wavelength of 266 nm with a 7-ns pulse width and a fluence of ~0.2 J/cm^2 was used in this study. Because the rapidly moving microorganism may encounter the desorption/ionization laser beam at any point in the beam field, laser beam inhomogeneity would introduce variability in the fragmentation/ion generation and degrade the rate of recognition. A study on the influence of laser power on *Bacillus* spore mass spectra has recently been published.[29]

Average mass spectra from *B. atrophaeus* and *B. thuringiensis* spores are shown in Figure 2 to illustrate their unique signatures. Results from two different growth media are included. Spectra A−C of Figure 2 were generated from 1000 individual spectra while (D) was generated from 608. The BAMS instrument[21] was adapted from a system that we originally designed for environmental analysis and thus currently has a limited mass range and resolution. Despite these limitations, however, many peaks were seen in both species' spectra that are consistent with what is known about the microbiology of bacterial endospores. To help identify the ions observed, we analyzed standards of DPA, DNA, and various amino acids as well as microorganisms grown in C^{13} and N^{15} isotopically labeled growth media. Detailed reports of both of these efforts are forthcoming.[30]

DPA molecular ion and its fragment peaks were observed at $m/z = -167$, -166, and -122. A metabolic precursor of DPA, 2,3 dihydrodipicolinate, was tentatively assigned to a peak observed at $m/z = -169$. DPA is found in a complex polymer with calcium in endospore cores, and peaks at $m/z = +40$, $+57$, $+66$, and $+82$ may result from Ca^+, $CaOH^+$, $CaCN^+$, and $CaCNO^+$, respectively. Glutamic acid is also commonly found in *Bacillus* spores and is consistent with the peak at $m/z = -146$. The peak at $m/z = -134$ has been tentatively assigned to aspartic acid. Peaks at $m/z = -79$ and -97 have been tentatively attributed to PO_3^- and $H_2PO_4^-$ from phosphates, which are found in endospore nucleic acids, in adenosine di- and triphosphates, and in cell membranes. Subtle differences among the spectra are apparent upon close inspection. In particular, a peak at $m/z = -173$, ascribed to arginine, and an unidentified peak at $m/z = +74$ are present in both growths of *B. atrophaeus* and absent from *B. thuringiensis*. (B) and (D) of Figure 2 show average mass spectra of spores of *B. atrophaeus* and *B. thuringiensis*, respectively, grown under nonnutritive conditions.[31] While a more extensive study of growth conditions versus mass spectra is in progress, the samples presented here are similar, with the only major difference between the spectra being the ratios of sodium and potassium.

The particles were categorized in real time by a data analysis algorithm that is novel to this study, and its details will be published shortly. The method functions in two stages. First, a prescreening stage is used to identify all nonmicrobial particles according to their origin and to select the spectra of bacterial endospores for further analysis. This method is similar to the ART-

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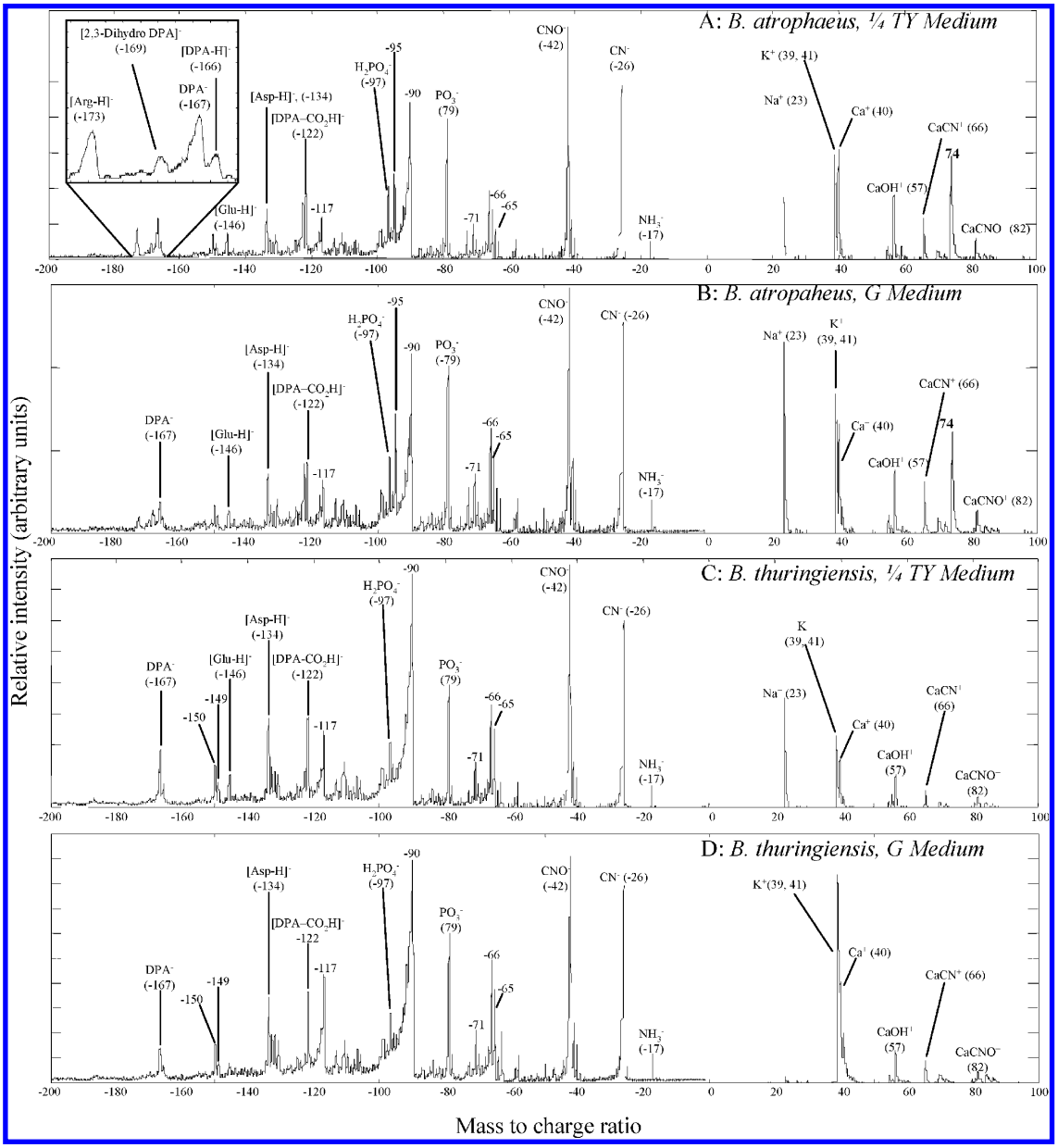

Figure 2. Positive and negative polarity average mass spectra of *B. atrophaeus* (A, B, 1000 spectra each) grown in $^1/_4\times$ TY growth medium and G medium, respectively, and *B. thuringiensis* (C, 1000 spectra; D, 608 spectra) grown in the same. Note that both growths of each species are nearly identical, with the exception of the sodium and potassium peaks.

2a clustering algorithm that has been previously applied to single-particle LDI-TOFMS data.[32,33] Next, the endospores are classified by species according to a series of rules that had been induced from previously acquired data. In this study, spectra with peaks at $m/z = +74$ making up greater than 0.0169 of all peak areas were considered potential *B. atrophaeus* and those without were considered potential *B. thuringiensis.* The presence or absence of a peak at $m/z = +74$ influences the threshold of the area of a peak at $m/z = -173$ required to confirm the identity of one species or the other. This is an example of how interpreting the presence or absence of one marker peak in the context of another can enhance the sensitivity of a rules-based analysis. The identities of the suspected *B. atrophaeus* were confirmed by the presence of peaks at $m/z = -173$ in relative abundances 0.001 18 and greater, while those of suspected *B. thuringiensis* were confirmed by the absence of peaks at $m/z = -173$ in relative abundances 0.001 93 or greater. Spectra whose identities were not confirmed according to $m/z = -173$ were considered unidentified *Bacillus* spores. These rules were induced from one culture of spores grown under nutritive conditions and applied to another in order to determine their effectiveness. In this manner, we were able to identify *B. atrophaeus* spores 93.2% of the time with absolute specificity versus *B. thuringiensis.* The same analysis was reproduced for the identification of the nonnutritively grown endospores, resulting in recognition rates of 80.1% for *B. atrophaeus* with one spore of *B. thuringiensis* misassigned as *B. atrophaeus.* The rate of identification for *B. atrophaeus* grown in Luria broth

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(data not shown) was 73.4%, but specificity could not be determined as the experiment was not run with *B. thuringiensis*. Given the fact that the determination of species is currently based upon the intensities of only two peaks at relatively low m/z and that they were optimized for one set of growth conditions while another produced minor spectral differences, it is unsurprising that the rates of recognition were not identical. Indeed, we are still experimenting with different growth conditions to ensure that these marker peaks are present consistently and are currently experimenting with alternative desorption/ionization laser schemes and mass spectrometer designs to observe higher m/z biological markers, such as those from endospore coat proteins, to make our detection even more robust.

Although the *Bacillus* spores analyzed in these experiments were *B. atrophaeus* and *B. thuringiensis*, separate field tests run at a biosafety level 3 facility, operated by the Bureau of Laboratories—Miami, FL, and affiliated with the Centers for Disease Control and Prevention, have demonstrated the ability of BAMS to detect *B. anthracis* spores versus nonmicrobial background particles. These preliminary experiments with *B. anthracis* were conducted prior to several instrumental and experimental changes that may allow species-level identification. We are currently working toward developing a unique signature for the species-level identification of *B. anthracis*, which will require additional live agent testing soon. The larger objective of the deployment was to test the efficacy of the BAMS system for screening the overwhelming number of suspicious powders sent to the Florida Department of Health shortly after the anthrax attacks of 2001.

Real-Time Recognition of Endospores versus Background. To demonstrate the ability of the technique to distinguish *Bacillus* spores from a variety of background aerosols, we designed our proof-of-principle experiments to demonstrate the detection of *Bacillus* spores within a background of both biological and nonbiological materials. Some of these challenge aerosols were widely available "white powders", intended to simulate those found in the large numbers of hoax letters mailed during the postal anthrax attacks of Autumn 2001. Cells of *Clostridium acetobutylicum* and spores of *T. stercoreus* were used to demonstrate the technique's robustness in the presence of a bioaerosol background. We analyzed Luria broth, a microbial growth medium, as well.[31] Other simple organic molecules were represented by aspartame in Equal sweetener; collagen from Knox Unflavored Gelatine represented purified biological extracts; and Gold Bond Medicated Powder represented mineral aerosols. Average mass spectra of each of the confounders are found in Figure 3.

The BAMS real-time recognition algorithm was programmed to detect individual particles of all seven analytes described above, and in real-time recognition tests, the system detected each with absolute specificity, illustrating the uniqueness of the spectral signatures. The sensitivity varied by sample. *Bacillus* spores were recognized 92% of the time; Gold Bond powder, 91%; growth medium, 86%; Equal sweetener, 78%; fungal spores, 56%; and Knox Gelatine, 46%. Spectra that were unrecognized were classified as "other". Higher recognition rates are possible for the fungal spores and the other materials, but the laser power was optimized to identify *Bacillus* spores.

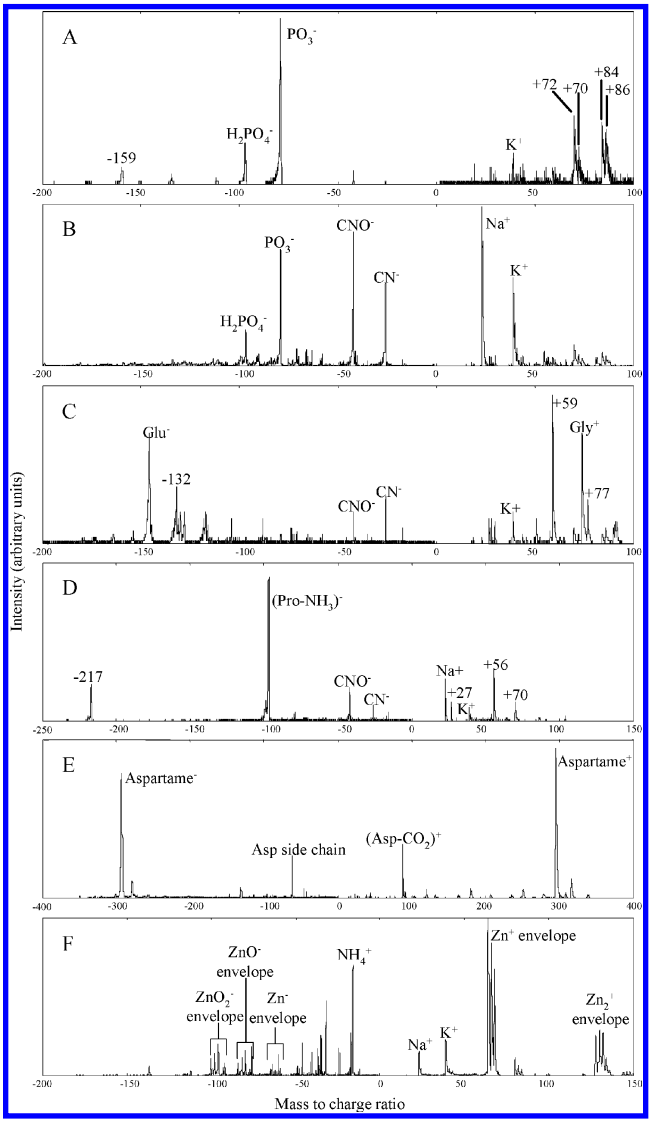

Figure 3. Average mass spectra from 1000 particles each of six different samples. *C. acetobutylicum* (A); a combination of fungal spores and fungal heife from *T. sterecoreus* (B); Luria broth powder (C), a bacterial growth medium; Knox Unflavored Gelatine (D); Equal brand artificial sweetener (E); and Gold Bond Medicated Powder (F). Identified peaks are labeled. All spectra appear distinguishable from one another by the naked eye.

A series of experiments was performed in which a complex background of aerosolized materials, including Equal sweetener, Gold Bond Medicated Powder, Knox Unflavored Gelatine, and growth medium was analyzed to show how each material can be identified within the mixture (Figure 4a). The large "other" category in Figure 4a is a result of gelatin in the mixture, which has only a 50% recognition rate. The next experiment used a new mixture of these same background materials with the addition of aerosolized *Bacillus* spores and two samples that did not appear in the database, baking soda and powdered sugar. As Figure 4b shows, the spores are easily identified within this complex aerosol background, as are the other components. Note that a large fraction of the particles are called "other" in response to the addition of the unknown samples. Furthermore, other common background aerosols, including cigarette and wood smoke, particles sampled directly from the environment, and NIST standards of common environmental confounders including ve-

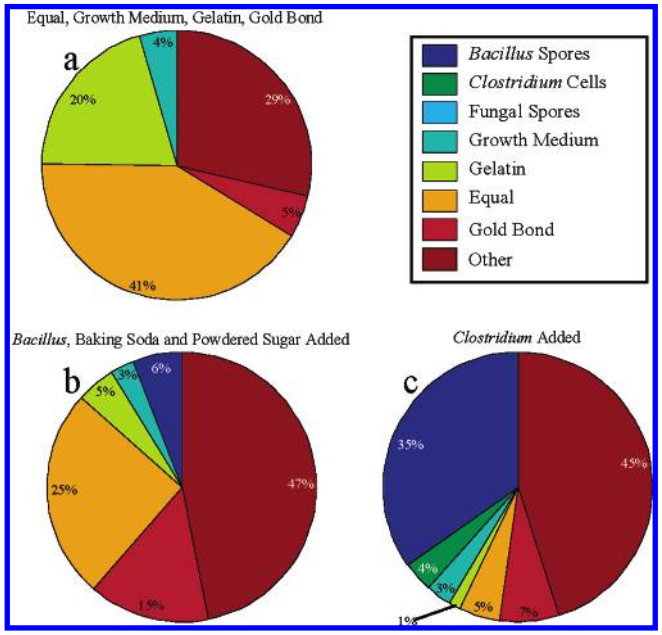

Figure 4. Pie charts showing real-time classifications of mixed aerosols. It is worth noting that fungal spores were not present in any of the mixtures and so were not detected.

hicular exhaust, ambient particles, and soil dust have been tested against the recognition algorithm and are not falsely detected as a known particle type. Figure 4c shows the recognition of *Clostridium* cells that had been added. It is worth noting that fungal spores were not present in either of the mixtures and so were not detected by the BAMS system.

DISCUSSION

A biological agent sensor's limit of detection is typically given in terms of agent-containing particles per liter of air (ACPLA). At present, due to instrumental limitations, the BAMS system can process ~2 particles/s in typical urban background conditions[34] and has a draw of ~1 L of air/min. This rate of acquisition implies that, statistically, the instrument could detect the presence of biological agents present at concentrations of 10^4 ACPLA in 1 min. We are currently developing a new instrument to be 4−5 orders of magnitude more sensitive by accelerating its rate of acquisition. This requires an entirely new form of single-particle LDI-TOFMS, especially when operating within very high background environments. Our ultimate performance goal is to be able to detect on the order of 1 ACPLA in 1 min in a particle background typical of an urban environment. As a proof-of-principle experiment, however, the system shows promise and, even in its current form, there are circumstances in which high concentrations of biological agents are present and need to be identified very rapidly.

In addition to increasing the speed and sensitivity of the technique, it is also important to investigate sources of variability in the organism signatures. These include the growth conditions, growth media, harvesting techniques, storage conditions, and weaponization procedures. This work begins to address variation introduced by growth conditions by showing consistency in the signature even though the organisms are grown in widely differing media. Although promising, these results do not represent a complete understanding of this issue, and fully resolving it will require additional work. We are also investigating the other possible confounding factors.

Another challenge in detecting an atmospheric release is the possibility that heterogeneous atmospheric chemical reactions as well as biological particles coagulating with other background particles may alter the signature of *Bacillus* spores, preventing their recognition. Fortunately, heterogeneous gas-phase reactions and aerosol coagulation processes typically occur on the order of hours or days, even in a polluted environment such as the Los Angeles basin. The time scales of these processes make them unimportant for many applications where rapid detection of a new release is of primary concern.[35,36] When BAMS is used in applications where regional-scale networks are implemented, these atmospheric processes will become relevant. However, as we have shown in previous work,[37] it is possible to follow the atmospheric reactions that occur on aerosol particles. By tracking the extent of reaction on bioaerosol particles, it might also become possible to determine how long they have been aloft in the atmosphere. We have an ongoing research effort assessing the utility of this approach to aid in locating a regional-scale biological agent release in space and time. Finally, although the applications presented in this study deal with the detection of biological attack, with future improvements as a single-cell analysis technique, BAMS may have far-reaching contributions in the fields of oncology, microbiology, and public health.

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