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Stable Isotope Labeling of Entire *Bacillus atrophaeus* Spores and Vegetative Cells Using Bioaerosol Mass Spectrometry

Gregg A. Czerwieniec,† Scott C. Russell,† Herbert J. Tobias,‡ Maurice E. Pitesky,§ David P. Fergenson,‡ Paul Steele,‡ Abneesh Srivastava,‡ Joanne M. Horn,‡ Matthias Frank,‡ Eric E. Gard,‡ and Carlito B. Lebrilla*,†

Department of Chemistry, University of California, Davis, California 95616, Lawrence Livermore National Laboratory, Livermore, California 94550, and School of Veterinary Medicine, University of California, Davis, California 95616

Single vegetative cells and spores of *Bacillus atrophaeus*, formerly *Bacillus subtilis var. niger*, were analyzed using bioaerosol mass spectrometry. Key biomarkers were identified from organisms grown in ^{13}C and ^{15}N isotopically enriched media. Spore spectra contain peaks from dicipolinate and amino acids. The results indicate that compounds observed in the spectra correspond to material from the spore's core and not the exosporium. Standard compounds and mixtures were analyzed for comparison. The biomarkers for vegetative cells were clearly different from those of the spores, consisting mainly of phosphate clusters and amino acid fragments.

The ability to perform real-time detection of airborne pathogens such as *Bacillus anthracis* is a necessary safeguard against biological attacks. Traditional bioaerosol detection and identification involves collection and culturing of aerosol particles for susceptibility tests to a number of phages and antibiotics.[1] Alternatively, PCR amplification of cellular extract from bioaerosols has been used to detect pathogens.[2,3] The drawback of these techniques is the considerable time required for analysis.

Mass spectrometry provides a more rapid and sensitive means of analysis, making it highly suitable for a rapid bioaerosol detection system. Early work to characterize microorganisms with mass spectrometry (MS) employed fast atom bombardment,[4,5] plasma desorption,[5−7] laser desorption/ionization,[5,8] and pyrolysis MS,[9] to identify lipids and fatty acids from whole bacterial cells or cell lysates. The advent of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) made possible the detection of large biopolymers of proteins and oligonucleotides. ESI-MS of complex environmental and tissue samples has detected specific lipid and protein biomarkers, allowing identification of vegetative cells and bacterial spores.[10−14] MALDI-MS of vegetative bacterial cells or extracts of their protein constituents has provided a means of species and strain level identification using protein biomarkers.[11,15−18] Pyrolysis MS of lysed vegetative cells with electron impact (EI) has yielded biomarkers consisting of fatty acids, glycerides, lipids, and nucleic acids.[19−23]

Various MS techniques have also been applied to the analysis of bacterial spores. MALDI-MS has been used for the analysis of the small protein extract from spores[24−26] and has differentiated

* To whom correspondence should be addressed. E-mail: cblebrilla@ucdavis.edu.

† Department of Chemistry, University of California, Davis.

‡ Lawrence Livermore National Laboratory.

§ School of Veterinary Medicine, University of California, Davis.

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Bacillus spores of species *atrophaeus*, *cereus*, *thuringiensis*, and *anthracis*.[26] MALDI MS of intact spores has been used to identify spores of *Bacillus atrophaeus* and *Bacillus cereus*.[17,27,28] Pyrolysis MS with EI and chemical ionization (CI) has also been used to identify spores based on small-molecule biomarkers such as dipicolinic acid (DPA), a chemical found primarily in *Bacillus* and *Clostridia* spores.[21,29−32] Mass spectrometric analysis employing IR and UV desorption/ionization has revealed characteristic differences between several *Bacillus* species based on small protein biomarkers detected from collections of intact spores.[33] An ion trap MS instrument with a 308-nm desorption laser has been developed for single-cell bioaerosol analysis.[34] The mass fingerprints in the former differ considerably from the spectra obtained in this study on the TOF mass spectrometer using a 266-nm laser.

Microorganism detection using MS, whether spores or vegetative cells, depends on identification of taxonomically important biomarkers. Bioaerosol mass spectrometry (BAMS) has been used to analyze various species of bacterial spores.[35] The algorithm currently being developed in our laboratory for biowarfare detection involves obtaining mass spectra from a single airborne particle. Each spectrum is then compared with fingerprints from a mass spectral database of possible threat agents. Discrimination based on peak occurrence and intensity then identifies similar mass spectral patterns. Using this technique, single *B. atrophaeus* spores aerosolized from a solution of cultured cells were correctly identified with 93% success rate by comparison to reference mass fingerprints.[35] Also, no spectra obtained when sampling spores of *Bacillus thuringiensis* were incorrectly identified as *B. atrophaeus*. In the field, BAMS can function autonomously and sample directly from ambient air. The major advantage of BAMS is the ability to directly sample single particles from ambient air for real-time detection with no sample workup or preconcentration.

Small-molecule biomarkers for *B. atrophaeus*, a surrogate for *B. anthracis*, were determined by isotope incorporation and BAMS. The mass fingerprint is composed of ubiquitous ionic species such as sodium or potassium and small organic compounds. Elucidating the identity of the MS peaks and understanding how they relate to the metabolism of the cell will allow the development of more robust biomarkers. The algorithm used for comparing fingerprints could then be adjusted to concentrate on more significant biomarker peaks relative to other peaks. Determining the origin and relationship of the observed components will also give further insight into cellular processes of single microorganisms. Vegetative cells were also studied and were easily differentiated from spores by their mass spectra.

METHODS

Microbiology. *B. atrophaeus* (ATTC 9372, Dugway Proving Ground, Dugway, UT) cells were grown and sporulated in LB media in a shaker incubator at 35 °C and 170 rpm. Nutrient depletion yielded 90% sporulation. Spores were harvested by centrifugation at 8000*g* for 15 min and washed with double-distilled water, treated with TRIS buffer to lyse any remaining vegetative cells, and washed three times with distilled water. Visual inspection of spores under a microscope using a combination of phase contrast microscopy, Gram stains, and Shaeffer−Fulton stains confirmed sporulation. Isotopically labeled spores were initially grown until midlog phase in nonlabeled media, transferred at a 1:200 dilution into the isotopically enriched media, and grown until starvation in the same manner as the nonlabeled spores. The labeled medium was the LB-type broth Bio-express-1000 from Cambridge Isotopes (Andover MA). The ^{15}N medium is 96−99% pure, and the ^{13}C medium is 97−98% pure. Vegetative cells were grown in the same manner but removed during the culture's midgrowth phase, washed, and resuspended in deionized water.

Aerosol Generation. Aerosols of spores and vegetative cells were generated from a suspension in deionized water using a TSI Collison nebulizer. Standards Glu, Arg, DPA, and $Ca(OH)_2$ (Sigma-Aldrich Inc., St. Louis, MO) were nebulized from 0.001 M solutions in deionized water. The aerosolized particles pass through a silica drying column and are sampled into the mass spectrometer.

Mass Spectrometry. A dual-polarity time-of-flight mass spectrometer, previously discussed, was used for analysis.[36] Air was sampled into the mass spectrometer through a nozzle at a rate of 1 L/min and pulled through a series of particle focusing lenses by differential pumping. Particles of 1 μm are preferentially focused; however, particles ranging from 0.5 to 2 μm are detected. Two scattering lasers tracked the incoming particles and provided the necessary timing for the 266-nm Nd:YAG desorption/ionization laser. Both positive and negative ions were extracted into separate TOF regions and detected simultaneously.

RESULTS AND DISCUSSION

An average mass spectrum (1000 shots averaged) of *B. atrophaeus* is presented in Figure 1. Positive and negative spectra were obtained individually and then combined into a single mass spectrum with m/q plotted on the *x*-axis. A number of the peaks have been tentatively assigned. The signal at m/q −167 is due to the electron capture species of DPA (MW = 167.12). It is accompanied by the loss of CO_2 from the quasimolecular ion to yield the signal at m/q −123. Deprotonated DPA at m/q −166 is also often present in spore spectra, accompanied by a loss of CO_2 to yield m/q −122. DPA is a major component of bacillus spores, typically about 5−15% by dry weight.[37]

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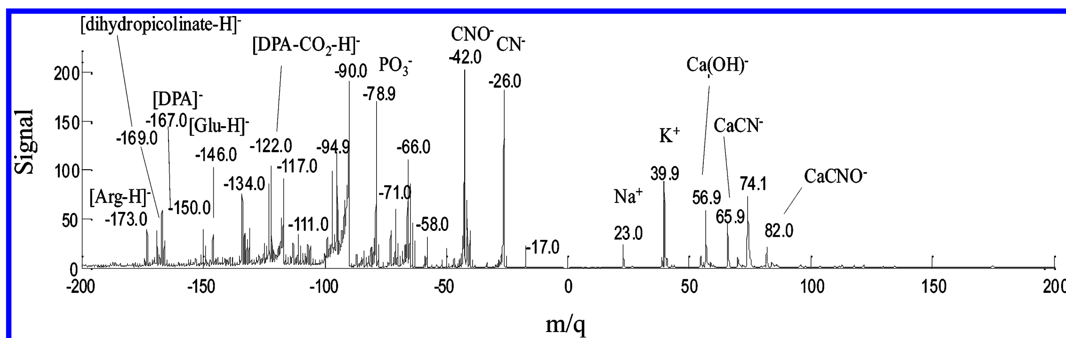

Figure 1. Average spectra (1000) of *B. atrophaeus* spores.

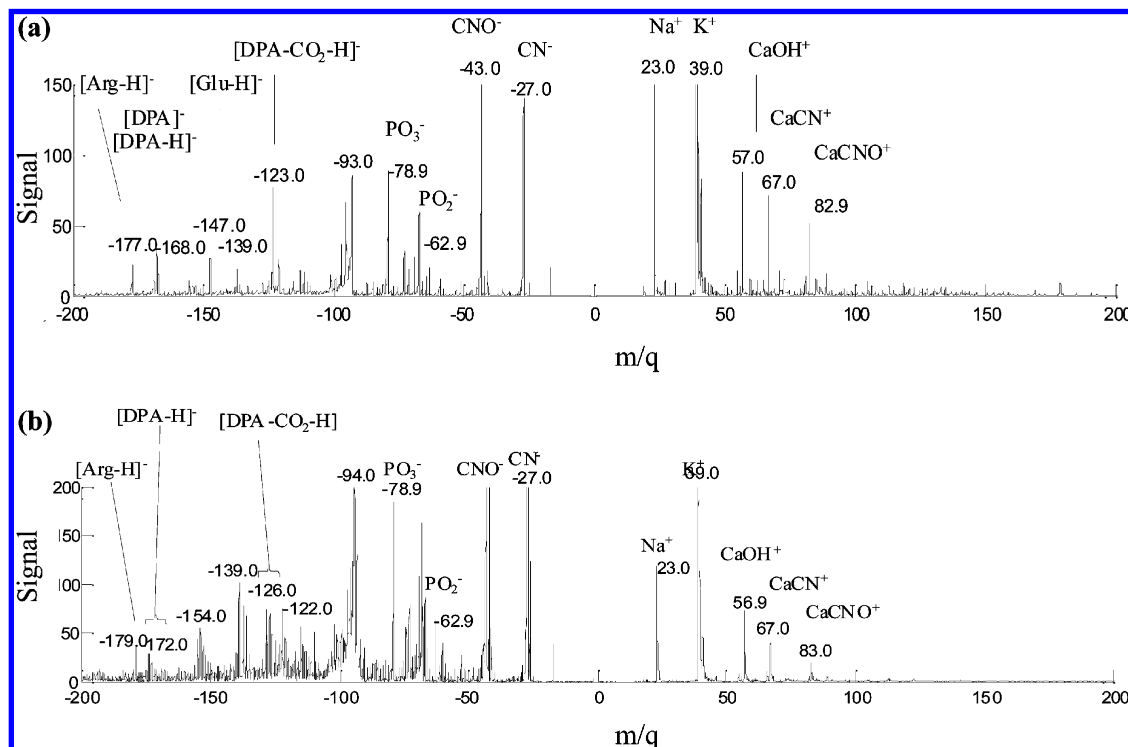

Figure 2. (a) Average spectra (94) of ^{15}N-labeled *B. atrophaeus* spores and (b) average spectra (28) of ^{13}C-labeled spores.

The *B. atrophaeus* mass spectra obtained using BAMS contrast those from other existing MS techniques. For instance, pyrolysis MS detected the electron capture species of DPA. However, no other similarities were observed in the spectra, which displayed peaks consisting generally of fatty acids, DNA bases, and glycerides.[30] Also, differences in desorption/ionization laser wavelength produce different biomarkers from single cells.[38] This is evidenced by the single-cell laser ablation studies at 308 nm[38] performed by Ramsey et al. Their findings contrast findings in the present study obtained using 266 nm. No DPA peaks were reported at 308 nm, presumably due to the difference in the compound's absorption at this wavelength.[38] Similar peaks belonging to phosphates were observed in the negative spectra for the two different techniques.[38] No other similarities between the two methods are apparent.

Isotopically Labeled Spores. To further aid peak identification, spores and vegetative cells of *B. atrophaeus* were grown in both ^{15}N- and ^{13}C-enriched medium. Growing the organisms in isotopically enriched media allows the nitrogen and carbon content of peaks to be determined. The spectra of spores grown in ^{15}N- and ^{13}C-enriched media are shown in panels a and b of Figure 2, respectively. A number of peaks did not shift in either medium. For example, those corresponding to Na^+(m/q 23), K^+(m/q 39), PO^{3-}(m/q −79), and $CaOH^+$(m/q 57) did not shift. Calcium is an important component of the spore and is well represented in the mass spectra.[37,39] A number of peaks moved by only 1 mass unit in the ^{15}N media (Figure 2a). These ions corresponded to species containing only one N atom. The putative signals for DPA, m/q −167.0 and −166.0, both shifted by a single mass unit to m/q −168 and −167, respectively. Similarly, the signal corresponding to the loss of CO_2 from DPA, m/q −122, shifted by only 1 mass unit. The peak tentatively assigned as glutamic acid (m/q −146) similarly shifted by 1 mass unit (m/q −147). A number of small ions also contained one N atom. For example, m/q −42.0 shifted to m/q −43.0 in the ^{15}N media. A similar shift was obtained

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in the ^{13}C media yielding the formula CNO^-. The other ions that shifted by 1 mass unit were assigned as CN^- (m/q −26.0), $CaNO^+$ (m/q 66), and $CaCNO^+$ (m/q 82.0).

The peak assigned as deprotonated Arg (m/q −173) shifted by 4 mass units in the ^{15}N-labeled medium. This species represented the biggest mass shift that could be identified in the ^{15}N-labeled mass spectra. The mass shift is consistent with the presence of four nitrogen atoms in the molecule. An important feature of the mass spectrum of the ^{15}N-labeled spore is the completeness of the incorporation. For example, Arg, DPA, and Glu shift by precisely the correct number of nitrogens with no noticeable unlabeled component in the mass spectrum. Complete incorporation of the ^{15}N isotope contrasts the ^{13}C isotope labeling (see below), where incorporation was less complete and complicated some peak assignments.

The spores grown in ^{13}C-labeled medium (Figure 2b) exhibited an isotopic distribution that was not apparent in the ^{15}N-labeled spores. This is evidenced by envelopes of peaks representing varying degrees of shifting due to incomplete ^{13}C incorporation instead of a single shifted peak. For example, ionic species that contain only one carbon partially shift by 1 mass unit. CNO^- and CN^- shifted by 1 mass unit (to m/q −43 and −27, respectively) but were both accompanied by a large unshifted component with 1 mass unit less. The peak for DPA shows a multiplet of signals centered around m/q −172. An expansion of the spectrum around this peak shows an incorporation of up to seven carbon atoms (not shown). A similar multiplet of peaks is observed around m/q −126 for the fragment corresponding to CO_2. Another group of peaks centered around m/q −149 corresponds to Glu. The only exception is Arg, where the peak shifted by precisely 6 mass units as expected from the amount of carbon. This behavior is unique as compared to the other ionic species and could suggest a faster metabolic turnover rate for Arg relative to the other components represented in the spectra.

DPA is found only in the spore core.[40] Glutamic acid and arginine are also found in the core and are used as a nitrogen reservoir for the spore.[40] The prominence of these species in the spectra suggest the signals obtained from the spore originate primarily from the core. There is additional evidence for the core as the source of the signals. In the sporulation process, DPA forms long polymeric chains with Ca^{2+}.[40,41] Indeed, several Ca^{2+} species are observed in the mass spectra.

Standard Compounds. To obtain further evidence that the mass spectra consist primarily of core constituents, particles composed of DPA, pure amino acids, and their mixtures were examined. The spectra of pure DPA (Figure 3a) showed the dominant $[M - H]^-$ peak at m/q −166 along with the distinctive loss of one and two CO_2 groups, giving rise to peaks m/q −122 and −78. Also present was the protonated species at m/q 168, with its corresponding loss of one and two CO_2 groups to yield m/q 124 and 80, respectively. DPA is notably absent in the positive ion mass spectra of the spore. In the spore, DPA is strongly bound to Ca^{2+} in long linear chains.[39] The binding of calcium to doubly deprotonated DPA, $[DPA - 2H]^{2-}$, would result in a neutral species that would not be detected using MS. The number of calcium species in the mass spectra prompted an investigation of DPA and Ca^{2+} mixtures (from solutions of $Ca(OH)_2$). An average of spectra from the mixture is shown in Figure 4 and differed from the pure DPA spectra (Figure 3a). However, there were important similarities. In the pure DPA and spore samples, fragmentation of deprotonated DPA is observed, corresponding to the loss of one and two CO_2 groups from the aromatic ring (m/q −122 and −78). There are other similarities between the spore and the DPA/Ca^{2+} mixture. Prominent peaks at m/q −117 and −134 were found in the spectra from both the *B. atrophaeus* spores and the DPA/Ca^{2+} mixture. These peaks were not observed in the isotopically labeled studies or in the spectra of pure DPA. The compositions of these peaks have not yet been determined.

Aerosols of amino acids Arg and Glu were examined by employing the same ionization conditions used for the spores. Pure Arg and Glu did not ionize well in the instrument. Arg did not readily produce spectra. However, when obtained, a distinctive peak at m/q −173 was present (Figure 3b) corresponding to deprotonated Arg. The Arg standard sample, however, also yielded protonated arginine at m/q 175, which was not observed in the spectra of the spore. Glutamic acid behaved in the same manner as Arg (Figure 3c). A deprotonated parent was observed in the negative spectra, and a protonated species was observed in the positive spectra. These amino acids did not ionize as readily as DPA, yet were commonly present in spore spectra. However, adding DPA to the mixture of amino acids increased their ionization probability by at least an order of magnitude and yielded predominantly deprotonated species of both Arg and Glu,[42] similar to the peaks in the spore mass spectra. It has been previously shown that the aromatic ring in DPA is a convenient chromophore and provides high ionization efficiency for compounds that do not strongly absorb at the D/I laser wavelength.[42] Nonaromatic amino acids absent an aromatic ring generally ionize poorly in laser desorption/ionization using a 266 nm laser pulse, although mixing the amino acid with DPA increases the ionization of the amino acid to as high as DPA.[42] We suspect the high concentration of DPA in the spore behaves as the spore chromophore, allowing the ionization of Arg and Glu in the spore even though these amino acids generally ionize poorly in pure forms.

Vegetative Cells. The mass spectra of the vegetative *B. atrophaeus* cells were easily differentiated from the spores (Figure 5). The optimal laser power found for the vegetative cells was greater than that of the spore, 6.7 versus 2.1 mJ/pulse, corresponding to fluences of ~1.7 and ~0.55 J/cm^2, respectively. This reflects differences in the composition of a spore and a vegetative cell. Spores absorb strongly at 266 nm due to the absorbance of DPA at this wavelength. It can be speculated that the absence of DPA in the vegetative cells necessitates a higher laser power for efficient ionization.

The vegetative cell spectra contain significantly more peaks in the positive region than the spore. Absent in the vegetative cells are signals corresponding to DPA, Arg, and Glu. As with the spore, isotopic labeling using ^{15}N- and ^{13}C-enriched media allowed the identification of many of the peaks. The negative

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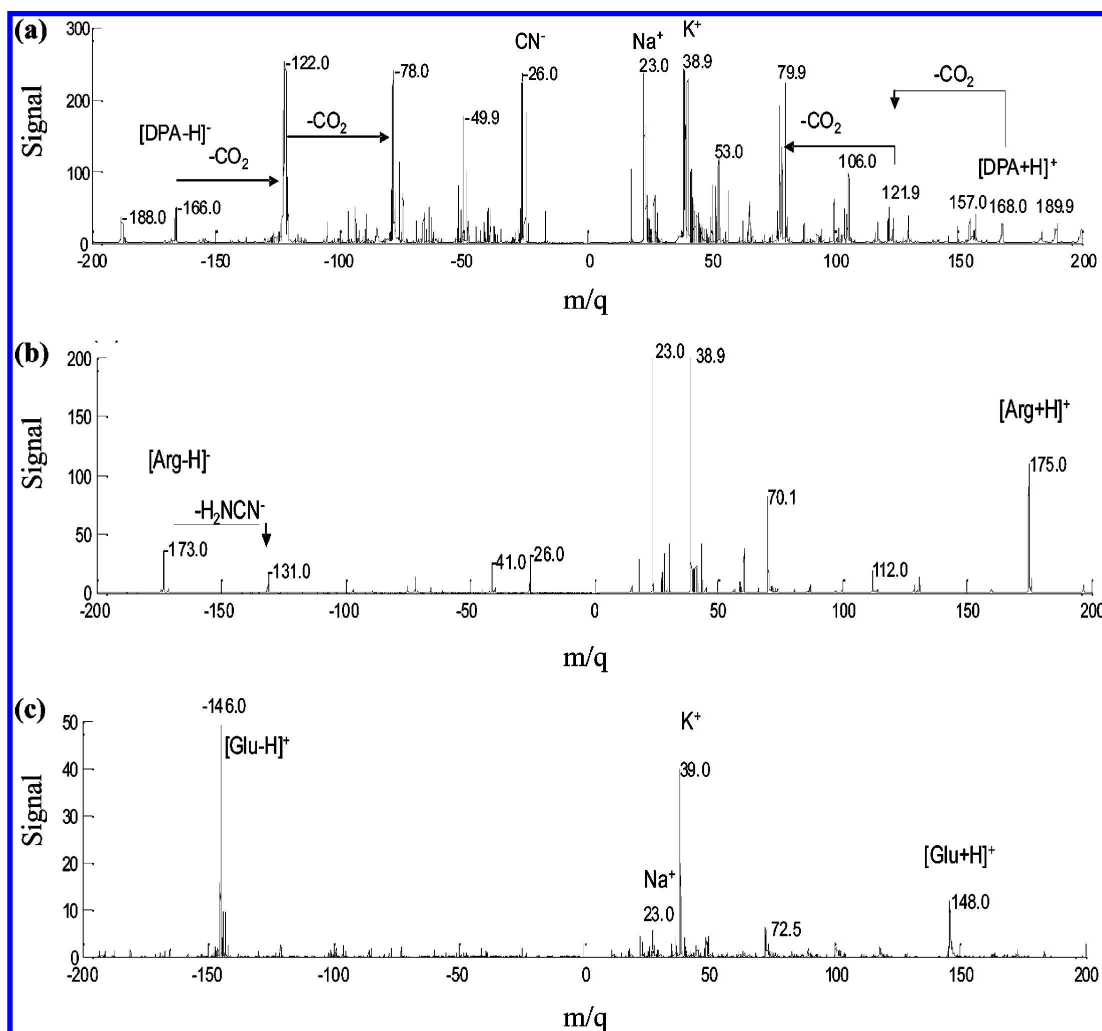

Figure 3. Average of (a) 117 DPA spectra, (b) 134 Arg spectra, and (c) 56 Glu spectra.

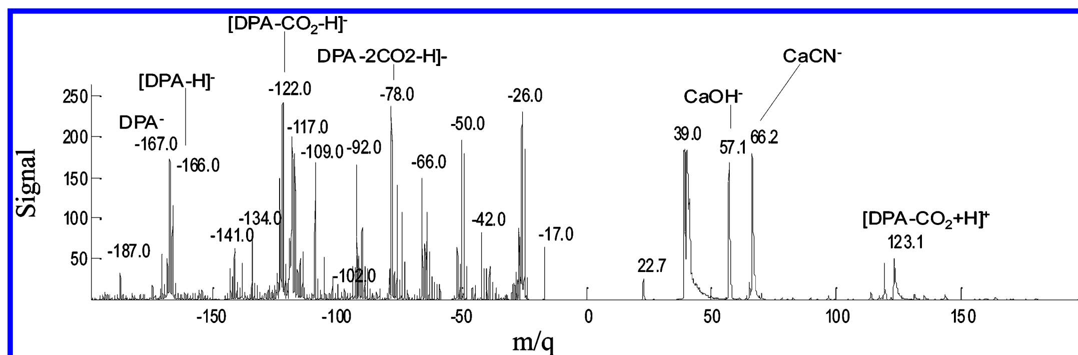

Figure 4. Average (200) of 1:2 mixture of $Ca(OH)_2$.

spectra were dominated by peaks that did not shift when the organisms were grown in either ^{15}N- or ^{13}C-labeled media, indicating an absence of nitrogen and carbon (Figure 6). These same peaks were observed in standards containing phosphates (spectra not shown). Their masses were assigned to phosphate clusters and are listed in Table 1.

The positive peaks belong predominantly to fragments of amino acids and are tentatively assigned in Table 2. The assignments were made according to the mass shifts from the ^{15}N- and ^{13}C-labeling experiments. Peaks m/q +23, +39, and +40 belong to Na, K, and Ca, respectively. Peaks m/q +28 and +30, shifted 1 mass unit for both ^{15}N- and ^{13}C-labeled species and were determined to be CNH_2^+ and $H_2CNH_2^+$. These fragments coincide with a loss of CO_2H_3 and CO_2H from the amino acid glycine, respectively. Several positive peaks from the vegetative cell spectra can also be explained as amino acids with a loss of 45 mass units from the compound's full molecular weight. These fragments correspond to losses of CO_2H from the amino acids' molecular formula. Corresponding mass shifts in the isotopic labeling experiments support these assignments. For example, peak m/q 70 shifted 4 mass units in the ^{13}C medium and 1 mass unit in the ^{15}N medium. This ionic species corresponds to $HNCH(CH_2)_3^+$, which is equivalent to a proline minus CO_2H. In the same manner, the following assignments were made: m/q +72 from valine (H_2-

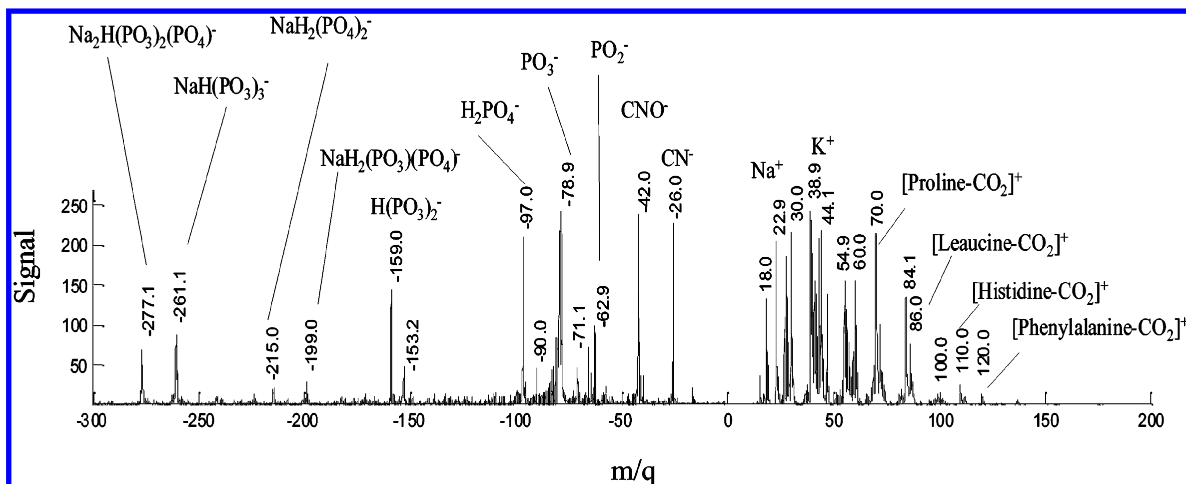

Figure 5. Average of 1000 vegetative *B. atrophaeus*.

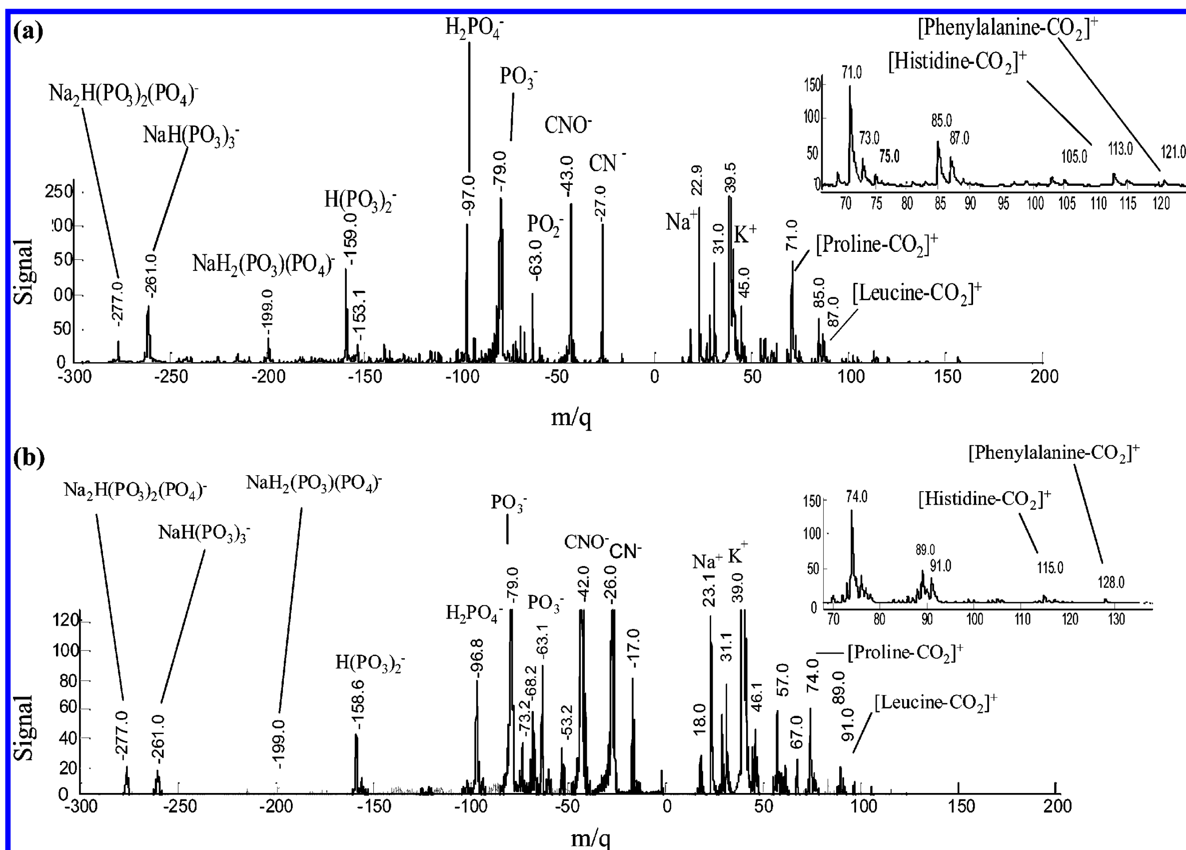

Figure 6. Average spectra of (a) 196 ^{15}N-labeled vegetative *B. atrophaeus* cells and (b) 21 ^{13}C-labeled vegetative *B. atrophaeus* cells.

$NCHCH(CH_2)_2)^+$, m/q +74 from threonine $(H_2NCHCHOHCH_3)^+$, m/q +86 from either leucine $(H_2NCHCH_2CH(CH_3)_2)^+$ or isoluecine $(H_2NCH(CH_3)CH_2CH_3{}^+$, m/q +110 from histidine $(H_2NCHCH_2(C_3H_3N_2))$, and m/q +120 from phenylalanine $(HNCHCH_2(C_6H_6)$. Other peaks, m/q +59, +84, and +104, were determined to m/q shift 2, 5, and 2 from ^{13}C and 2, 1, and 1 from ^{15}N labeling, respectively, but are not assigned at this time. The nature of these species is not known, but the fragmentation of the aforementioned free amino acids is typical of the high laser power needed for efficient ionization. The sources of the amino acid fragments are not yet fully understood. Vegetative *Bacillus* cells are ~5% free amino acid by dry weight and are the primary metabolites for the formation of proteins.[37] Alternatively, aminoacyl-tRNA is roughly 7% of the dry weight of the cell, and as much as 50−60% of the dry weight of *B. atrophaeus* vegetative cells is protein mass.[37] However, no signals representing larger fragments of proteins

Table 1. Negative Peak Assignments for Vegetative *B. atrophaeus*

formula	m/q	formula	m/q
63	PO_2^-	181	$Na(PO_3)_2^-$
79	PO_3^-	199	$NaH_2(PO_3)(PO_4)^-$
97	$H_2PO_4^-$	215	$NaH_2(PO_4)_2^-$
159	$H(PO_3)_2^-$	261	$NaH(PO_3)_3^-$
177	$H_3(PO_3)(PO_4)^-$	277	$NaH(PO_3)_2(PO_4)^-$

Table 2. Positive Peak Assignments for Vegetative *B. atrophaeus*

m/q	no.of C	no. of N	formula	source
28	1	1	$HCNH^+$	
30	1	1	$H_2NCH_2^+$	glycine − CO_2H
39	0	0	K^+	potassium
40	0	0	Ca^+	calcium
59	2	0	$H_2NCHCH_2NH_2^+$	
70	4	1	$HNCH(CH_2)_3^+$	proline − CO_2H
72	4	1	$H_2NCHCH(CH)_2^+$	valine − CO_2H
74		1	$H_2NCHCH(OH)CH_3^+$	threonine − CO_2H
84	5	1		
86	5	1	$H_2NCHCH_2CH(CH_3)_2^+$	leucine − CO_2H
			$H_2NCHCH)CH_3)CH_2CH_3^+$	isoleucine − CO_2H
104		1		
110	5	3	$H_2NCHCH_2C_3N_2H_3)^+$	histidine − CO_2H
120	8	1	$HNCHCH_2(C_6H_6)^+$	phenylalanine − CO_2H

such as dipeptides or tripeptides have been observed, suggesting that the amino acids do not originate from proteins.

CONCLUSION

Growing the organisms in isotopically enriched media is an effective method for determining the number of carbon and nitrogen atoms in the signals. The majority of the peaks in the BAMS spectra of the spore of *B. atrophaeus* have been assigned. These signals correspond to compounds generally found in the core of the spore. Prominent peaks in the mass spectra correspond to dipicolinic acid, a major component of the spore. Other important peaks correspond to arginine and glutamic acid. Both of these compounds are important nitrogen reservoirs for the organism. In the ionization of the spore, DPA acts as a chromophore for the absorption of the 266-nm radiation. This role for DPA may be the reason that the compounds observed originate primarily in the core.

Identification of peaks in spore and vegetative cell samples is complicated by cell-to-cell differences. For example, differences in mass fingerprints are seen between cells grown in different types of media. Cells grown in isotopically labeled media did not display all the peaks seen in the unlabeled media, and therefore, some peak identifications were hindered. For example, m/q −117, −134, and 74 are prominent peaks of *B. atrophaeus* spectra when grown in LB broth media, but their presence was diminished when other growth conditions were used. Development of a bioaerosol detection system will require an understanding of this range of variability in the target threat agents.

The BAMS spectra of the vegetative cell are dominated by other amino acids and phosphates. As phosphates are generally important in essentially all metabolic processes, the presence of more and higher order forms of phosphates in a living cell is expected. The presence of decarboxylated amino acids is consistent with free amino acids in the vegetative cells. These compounds most likely do not originate from peptides or proteins as at least di- or tripeptide fragments should be observed.

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