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Discrimination of *Bacillus anthracis* Spores by Direct *in-situ* Analysis of Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry

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The rapid and accurate identification of biological agents is a critical step in the case of bio-terror and biological warfare attacks. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry has been widely used for the identification of microorganisms. In this study, we describe a method for the rapid and accurate discrimination of *Bacillus anthracis* spores using MALDI-TOF MS. Our direct *in-situ* analysis of MALDI-TOF MS does not involve subsequent high-resolution mass analyses and sample preparation steps. This method allowed the detection of species-specific biomarkers from each *Bacillus* spores. Especially, *B. anthracis* spores had specific biomarker peaks at 2503, 3089, 3376, 6684, 6698, 6753, and 6840 *m/z*. Cluster and PCA analyses of the mass spectra of *Bacillus* spores revealed distinctively separated clusters and within-groups similarity. Therefore, we believe that this method is effective in the real-time identification of biological warfare agents such as *B. anthracis* as well as other microorganisms in the field.

Key Words : Spore, *Bacillus*, Anthrax, MALDI-TOF MS, Direct *in-situ* analysis

Introduction

Bacillus species are rod-shaped bacteria exhibiting catalase and endospore-forming abilities; they are facultative aerobes. The genus *Bacillus* includes 2 kinds of species: *B. subtilis* and *B. cereus*. The former group is a well-characterized model organism that is used for genetic research. Species of the *B. cereus* type such as *B. cereus*, *Bacillus thuringiensis*, *Bacillus atrophaeus*, and *Bacillus amyloliquefaciens* have a high degree of phenotypic similarity, and their phylogenetic relationships are not easily distinguishable.[1] *Bacillus anthracis* (genus *Bacillus*; family Bacillaceae) is a spore-forming gram-positive bacterium. The spores of *B. anthracis* are used in biological warfare and bio-terror activities because they are the causative agents of anthrax. For example, the mailing of *B. anthracis* spores in the US caused anthrax in 18 patients and an additional 4 were suspected to have cutaneous anthrax.[2]

This suggests that *B. anthracis* spores used as biological warfare agents (BWAs) might become a possible threat from terrorist groups.[3] Biochemical, chemotaxonomic, physiological, serological, and genomic methods are typically used for the identification of microorganisms.[4] Nevertheless, novel, accurate, and rapid methods for the identification of bacteria as well as detection of BWAs are of great significance. A matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has been introduced in bacterial taxonomy and successfully applied to many taxa in a high-throughput manner.[5-11] In some cases microorganism identification by MALDI-TOF MS, direct whole cell mass spectrometry was applied. This method was simple and could be successfully used for analyzing vegetative cells or colonies. However, purification steps were required to identify spores.[8] Some researchers reported on the analysis of intact spores by MALDI-TOF. However their results showed that insufficient detection level to identify biomarkers within species level.[16,17] In other studies, high-resolution mass analyses such as MALDI-TOF/TOF MS were used to identify the spores of *Bacillus* species.[12] Therefore, achieving rapid detection and identification of *B. anthracis* spores by using low-resolution mass analysis methods such as MALDI-TOF MS seems challenging. In this study, we describe the use of MALDI-TOF MS as an accurate and high-throughput identification tool for detection of *Bacillus* spp. spores without involving any purification step (Figure 1).

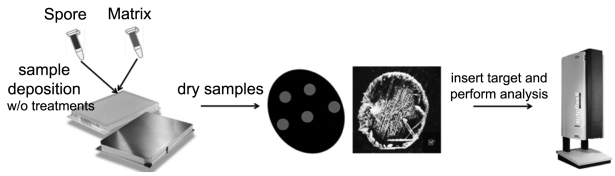

Figure 1. Schematic diagram of the direct spotting method for the analysis of mass spectra of *Bacillus* species spores.

Experimental

The following strains were used in this study: *B. anthracis* Sterne, *B. cereus*, *B. globigii*, *B. subtilis*, *B. thuringiensis*. They were provided by the Korea Center for Disease Control and Prevention, Osong, Republic of Korea. The procedure for the preparation of spores was as reported previously.[13] Briefly, a single colony of the strains was inoculated into nutrient broth sporulation medium for sporulation, and incubated in a shaking incubator for 2-4 days at 32 °C. The

strains were cultured until they showed > 99% spore formation as revealed by optical microscopy. Cultivated spores were collected by centrifugation for removal of remnant vegetative cells and cellular debris, as previously reported.[4] Purified spores were diluted to 1×10^9 colony forming unit (CFU) mL^{-1} by suspending in sterile water and stored at 4 °C until used. Then *B. anthracis* spores were autoclaved during 20 min at 212 °C, 15 lb/in^2 for researcher safety before storing them.[14] The concentration and an inactivation of *B. anthracis* spores were confirmed by plate count in triplicate.

To make direct *in-situ* analysis of spores by using MALDI-TOF MS, about 1×10^6 CFU of purified spores of *Bacillus* species were directly spotted onto a single well of a MTP 384 target ground steel T F (Bruker Daltonics, Germany) without any treatments such as acidic purification and allowed to evaporate for 5 min at room temperature. Subsequently, 1 μL of 12-mg mL^{-1} α-cyano-4-hydroxycinnamic acid (HCCA) solution prepared in TA_2, 2:1 (vol/vol) mixture of 100% acetonitrile (ACN), and 0.3% trifluoroacetic acid (TFA) was applied to each dried spore spot on the MALDI target plate and then allowed to dry for 5 min at room temperature. After the spotted samples were dried, the target plate was swiftly transferred to the vacuum area of the MALDI-TOF system, and mass spectra were analyzed. Three kinds of matrices –HCCA, 2,5-dihydroxybenzoic acid (DHB), and sinapinic acid (SA; Bruker Daltonics)– were tested for identifying suitable matrices for mass analysis of our samples. HCCA was the only matrix that yielded the most reliable mass spectral data. Therefore, HCCA was thereafter used as the matrix for the analysis. To compare other sample preparation methods for MALDI-TOF MS, we assessed an inactivation method and a modified method combined with bead beating and trifluoroacetic acid (TFA) extraction. The former method was carried out using the modified TFA inactivation method as described by a previous work.[8] In the latter protocol, first, equal volume of absolute ethanol (Merck, Germany) was mixed with purified spore samples by vortexing for 5 min, and then, the mixed samples were centrifuged at 13,000 rpm for 3 min. The supernatants were removed, and the samples were dried for 2 min at room temperature. Next, 7 μL of ACN and beads were introduced in the precipitated spore samples, and the samples were vortexed for 5 min to ensure effective purification by mechanical shear force. Finally, 7 μL 70% formic acid was added to the samples, and the samples were vortexed for 10 min. Each sample was spotted at least in triplicate.

Mass spectra of spores were measured using a Bruker autoflex speed™ LRF MALDI-TOF. The instrument was equipped with an Nd/YAG laser operating at 355 nm with pulse rates of up to 1 kHz. The pulse ion extraction time was 200 ns. Measurements of spectra were carried out in the linear mode of a MBT_FC parameter by using acceleration voltages of 19.51 kV and 18.26 kV at ion sources 1 and 2, respectively. A laser power in the MBT_FC parameter was equipped to 77%. Reliable mass spectra were not observed when the laser power was below 50% (data not shown). The lens voltage was 7.00 kV. The mass spectra were analyzed in the intermediate mass rage between 2 and 20 kDa. *Escherichia coli* DH5α (Bruker Daltonics, Germany) was used as a reference strain for mass calibration, with a peak assignment tolerance of about 1000 ppm. At least 200 individual laser shots were co-added for each spectrum.

The mass spectra of spores were processed by smoothing, baseline subtraction, and intensity normalization using Flex Analysis 3.3 software (Bruker Daltonics, Germany). The smoothing and baseline subtraction were done by using Savitzky Golay algorithm[15] (10 cycles with 2 *m/z* width) and TopHat algorithm, respectively. Then peak picking was performed by centroid algorithm with a signal to noise threshold of 2, a peak width of 2 *m/z*, and 90% height. For more visualized pattern of spore biomarkers, a gel view was used with the processed mass spectra. The gel view displayed peak intensities to gray-scaled with abscissa values as mass to charge ratios (*m/z*) and spectral numbers as the ordinates. Cluster analysis and principle component analysis (PCA) were performed by using Bruker BioTyper 3.0 software. Mass spectrum data of each spore were obtained in 5 different runs of experiments to confirm the reproducibility of peak patterns.

Results and Discussion

The *Bacillus* spores were analyzed with MALDI-TOF MS by using a direct spotting method (direct *in-situ* analysis)

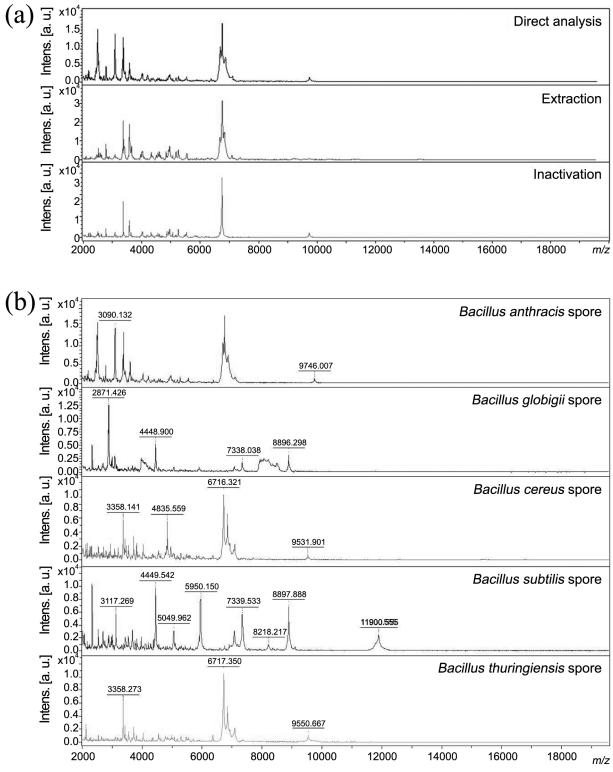

Figure 2. Mass spectra of *Bacillus* spores. (a) Comparison of *B. anthracis* spore mass spectra by using 3 different sample preparation methods. (b) Mass spectra of 5 spores of *Bacillus* family. The X- and Y-axes represent *m/z* and mass intensity, respectively.

that did not involve biomarker extraction and inactivation or mechanical shear force application. In this method, the spores are spotted on a MALDI target plate without any treatments (Figure 1).[8] The results of this analysis were compared with those obtained using previously known inactivation and extraction methods. The direct spotting method yielded more distinctive biomarker peaks when the MALDI-TOF mass analysis was performed using the MBT_FC parameter with a laser power of over 50% (Figure 2(A)). In general, the MBT_FC parameter set in Bruker Flex Analysis software requires a laser power of 32% for analyzing microorganisms; however, this laser power was not sufficient to obtain biomarker peaks of *Bacillus* spores while using the direct spotting method. Indeed, higher laser power was needed to break down spores to increase the ionization efficiency of the internal contents of the spores. The lower and upper bound of detection level were 1×10^5 CFU/well and 2×10^7 CFU/well, respectively. The optimal condition to achieve the best signal-to-noise ratio in mass spectra was when 1×10^6 CFU/well was used. The spectrum for *B. anthracis* spores yielded the following main mass peaks: 2503, 3089, 3376, 6684, 6698, 6753, and 6840 *m/z*. Further, peaks at 6684, 6698, 6753, and 6840 *m/z* showed significantly discriminative pattern for identification of *B. anthracis* (Table 1, Figure 2(a)). However, some peaks such as those at 3089, 6684, 6698, and 6840 *m/z* were not observed when the inactivation and extraction methods were applied. The peak at 2503 *m/z* was not observed when the extraction method was used. The reliability of the mass peak data of *B. anthracis* spores obtained using the direct spotting method was confirmed by conducting several runs of mass spectrum measurements on a given *B. anthracis* spore sample under the previously suggested analysis conditions of MALDI-TOF MS. The main peaks of *B. anthracis* spores were thus found to be reproducible in terms of *m/z* values and intensities (data not shown). Since the mass peaks of *B. anthracis* spores were very distinct from those of other *Bacillus* family spores, they could be used as putative biomarkers for the identification of *B. anthracis*. Therefore, the direct spotting method can be used as a simple and high-throughput method for the identification of spores by using MALDI-TOF MS.

The putative biomarker peaks of *B. anthracis* were conveniently detected using the direct *in-situ* analysis involving MALDI-TOF MS (Figure 2(b), Table 1). Few studies have focused on the identification of spores from *Bacillus* species, including *B. anthracis* spores, a putative BWA. The distinctive mass spectra of 5 kinds of *Bacillus* spores were successfully obtained in the mass range of 2,000 to 20,000 Da by using the direct *in-situ* analysis method. In the *B. cereus* group, the spectra of *B. cereus* and *B. thuringiensis* were very similar, and *B. anthracis* spores also yielded similar spectra. The spectra of the spores of these species showed specific mass patterns in the mass range of 6.5 to 7.1 kDa, although the markers for the spores of each species had very similar mass in this region (Table 1). The spores of *B. globigii* and *B. subtilis* yielded distinct spectra and were different from the mass spectra of *B. cereus* group. Interestingly, *B. globigii* spores had a mass range of 7,800 to 8,200 kDa, with a very broad mass pattern (Figure 2(b), Table 1). Specifically, the mass spectra of *B. anthracis* spores were clearly distinguishable from those of other *Bacillus* spores (Figure 2(b), Table 1).

Figure 3(a) shows the gel view of 25 spectra from 5 *Bacillus* spores in the mass range of 2 to 15 kDa. This gel view analysis suggested that the mass spectra of *Bacillus* spores were reproducibly and distinguishably detected by the direct *in-situ* analysis method with MALDI-TOF and indicated a relatively high similarity between the spectra of spores of the *B. cereus* group as well as heterogeneity for spectra between species. Cluster analysis using mass spectra of 5 *Bacillus* spores (Figure 3(b) and PCA analysis by mass spectra of 7 *Bacillus* spores and vegetative cells (Figure 3(c)) were carried out. We noticed that the mass spectral of spectra of *Bacillus* spores and vegetative cells were distinct each other. However, precisely classifying the spores of *B. cereus* and *B. thuringiensis* was difficult because they showed high similarity patterns in the mass spectra (Figure 2, Figure 3, and Table 1). Nonetheless, the *B. anthracis* spores could be easily distinguished from the other spores of the *B. cereus* family. The spectra of *Bacillus* spores used in this research were not detected by Bruker BioTyper 3.0 software and library version 3.3.1.0 (4,613 entries) which includes extensive library of spectrum database of microorganisms. This further confirms the efficacy of the direct *in-situ* analysis method in detecting and identifying *Bacillus* spores.

Moreover, species-specific putative biomarkers were identified for spores of each *Bacillus* species by 20 times of repeated experiments (Table 1). The peaks of *B. cereus* and *B. thuringiensis* mostly overlapped and therefore were difficult to differentiate. On the other hand, other spores included various putative biomarkers that were distinguishable in mass. The *B. anthracis* spores had specific and unique putative biomarkers (6684, 6698, 6753, and 6840 *m/z*) that were similar with the marker peaks of the *B. cereus* group but were distinguishable. These biomarkers of *Bacillus* spores can be used as references for their detection and identification in the environment.

Conclusion

Nowadays, the incidences of bio-terror threats are increasing and necessitate appropriate action to be prepared for such incidences. In this regard, rapid detection and identification of BWAs are crucial for minimizing the number of casualties. MALDI-TOF MS has been investigated as a method for the rapid and accurate identification of BWAs. In this study, direct *in-situ* analysis of mass spectrometry data of spores from *Bacillus* species was conducted with low-resolution MALDI-TOF MS and was found to be a fast and accurate identification tool for BWAs in near real-time.

In conclusion, the *in-situ* analysis by direct spotting method allowed the discrimination of *B. anthracis* spores from the spores of other *Bacillus* species. The mass spectra pattern of *B. anthracis* spores was reproducible and showed distinct

Table 1. Putative biomarkers for the *Bacillus* spores analyzed by direct *in-situ* analysis with MALDI-TOF MS

	B. anthracis				*B. cereus*				*B. thuringiensis*				*B. globigii*				*B. subrilis*			
	Biomark-ers	STDEV	Relative intensity	STDEV	Biomark-ers	STDEV	Relative intensity	STDEV	Biomark-ers	STDEV	Relative intensity	STDEV	Biomark-ers	STDEV	Relative intensity	STDEV	Biomark-ers	STDEV	Relative intensity	STDEV
	2080.66	±0.41	0.11	±0.05																
	2096.97	±0.47	0.11	±0.04																
					2108.78	±0.49	0.19	±0.14	2108.93	±0.65	0.12	±0.03								
					2123.44	±3.58	0.19	±0.16	2126.59	±0.57	0.30	±0.19								
	2196.16	±0.47	0.19	±0.10																
									2266.09	±0.59	0.07	±0.03								
													2324.17	±0.40	0.52	±0.26	2324.43	±0.53	0.88	±0.14
									2379.60	±0.66	0.12	±0.08								
	2446.05	±0.70	0.12	±0.05																
	2473.07	±0.52	0.30	±0.08																
	2503.17	±0.52	0.65	±0.24																
	2517.88	±0.57	0.35	±0.12																
	2523.19	±0.66	0.33	±0.11					2528.89	±0.59	0.22	±0.16								
	2579.21	±0.49	0.16	±0.05													2720.02	±1.03	0.23	±0.10
	2786.00	±0.54	0.32	±0.08									2870.15	±0.55	0.88	±0.18	2870.75	±0.79	0.73	±0.13
													2886.32	±0.46	0.80	±0.16	2886.92	±0.68	0.90	±0.14
													2918.40	±0.45	0.26	±0.07	2918.93	±0.72	0.30	±0.09
													2934.46	±0.53	0.24	±0.07	2935.14	±0.71	0.50	±0.11
													2992.31	±0.77	0.25	±0.15	2991.48	±0.61	0.26	±0.04
	3075.22	±0.58	0.26	±0.06	3079.19	±0.66	0.04	±0.01												
	3089.28	±0.58	0.44	±0.11					3095.14	±0.63	0.22	±0.17								
													3122.69	±0.47	0.49	±0.27	3116.29	±0.64	0.50	±0.10
	3150.47	±0.59	0.10	±0.03																
					3193.42	±0.78	0.03	±0.01												
	3341.10	±0.64	0.22	±0.09																
					3356.79	±0.56	0.24	±0.05	3356.72	±0.64	0.31	±0.05								
	3375.69	±0.66	0.28	±0.08																
					3418.83	±0.57	0.13	±0.03	3418.82	±0.67	0.11	±0.03								
													3430.19	±0.61	0.16	±0.07				
																	3528.22	±0.74	0.21	±0.05
					3542.03	±0.66	0.10	±0.03	3541.98	±0.67	0.08	±0.02								
	3576.24	±0.72	0.26	±0.09																
	3593.73	±0.71	0.52	±0.17																
m/z	3653.23	±0.79	0.12	±0.04																
					3708.67	±0.71	0.14	±0.05	3708.64	±0.65	0.18	±0.07								
													3759.76	±0.56	0.27	±0.09				
					3807.23	±0.72	0.09	±0.08												
	4030.99	±0.89	0.16	±0.05	4031.21	±0.92	0.06	±0.01	4031.11	±0.70	0.06	±0.01								
	4195.93	±0.75	0.13	±0.02																
	4327.88	±0.86	0.18	±0.08																
					4335.03	±0.71	0.09	±0.02	4334.98	±0.71	0.07	±0.02								
	4382.51	±0.93	0.12	±0.03																
					4424.50	±0.57	0.15	±0.04	4424.56	±0.67	0.09	±0.02	4418.42	±0.56	0.23	±0.11				
													4447.29	±0.61	0.60	±0.32	4447.75	±0.87	0.15	±0.06
	4553.50	±1.01	0.09	±0.02									4681.63	±0.75	0.14	±0.06				
					4836.27	±0.62	0.15	±0.03	4837.39	±0.74	0.08	±0.02								
	4956.00	±0.98	0.18	±0.03	4953.16	±0.68	0.11	±0.03	4971.42	±0.75	0.08	±0.02								
													5047.38	±0.81	0.22	±0.09	5048.26	±0.88	0.14	±0.05
					5173.10	±0.70	0.07	±0.02	5172.84	±0.66	0.05	±0.01								
	5263.00	±1.00	0.15	±0.03													5299.15	±0.74	0.19	±0.06
	5540.56	±1.13	0.09	±0.01													5948.39	±1.04	0.26	±0.09
									6352.44	±0.74	0.05	±0.00								
	6683.73	±1.27	0.98	±0.04																
	6698.86	±2.22	0.60	±0.06																
					6714.36	±0.91	1.00	±0.00	6714.57	±0.86	1.00	±0.00								
	6753.46	±1.36	0.94	±0.07																
	6839.77	±1.50	0.83	±0.06	6839.01	±1.07	0.70	±0.03	6840.21	±1.08	0.54	±0.04								
					7085.05	±0.16	0.26	±0.04	7086.28	±1.01	0.21	±0.02	7071.86	±0.96	0.16	±0.07	7073.19	±1.13	0.15	±0.06
													7336.33	±1.03	0.22	±0.10	7337.78	±1.16	0.27	±0.10
													7906.83	±1.32	0.20	±0.11				
													8053.02	±1.36	0.23	±0.13				
													8199.33	±1.28	0.20	±0.11	8201.02	±1.44	0.36	±0.11
													8345.36	±1.46	0.11	±0.06	8347.19	±1.52	0.23	±0.07
													8492.05	±1.32	0.21	±0.11	8493.71	±1.52	0.18	±0.07
													8895.01	±1.55	0.59	±0.30	8896.30	±1.60	0.16	±0.06
	9745.74	±2.13	0.06	±0.01													11055.56	±2.79	0.04	±0.01
																	11898.19	±2.17	0.15	±0.03

[†]n = 20. [‡]Bold numbers: main mass peaks, gray and narrow numbers: relatively minor mass peaks

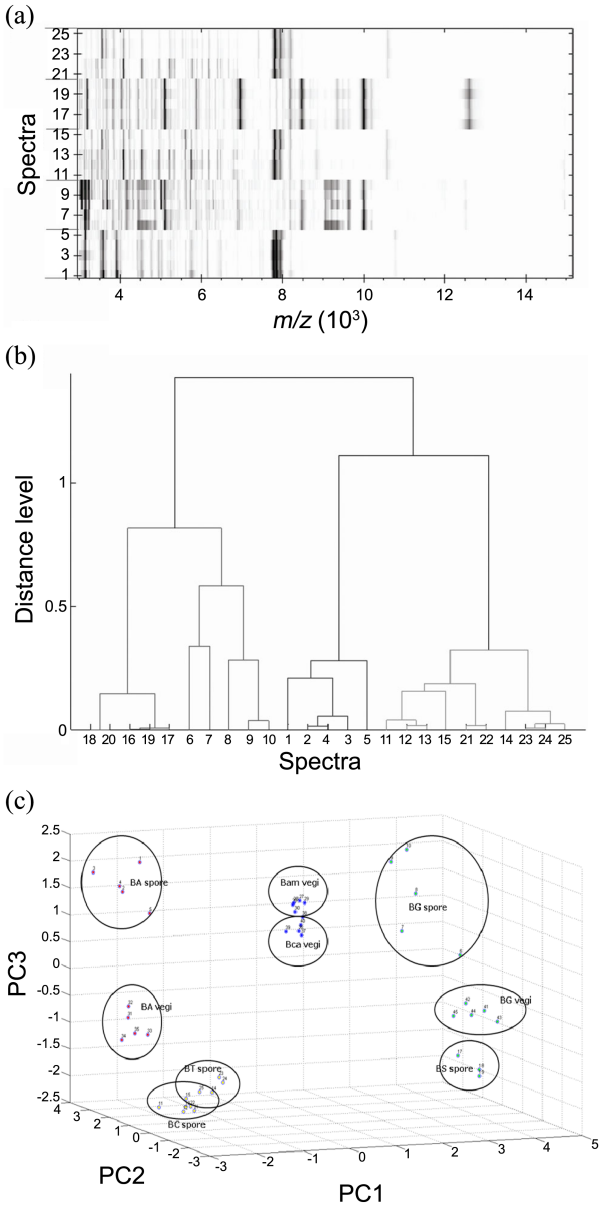

Figure 3. Analyses of the spectra for *Bacillus* spores. (a) Gel view representation of mass spectra for *Bacillus* spores in the mass range of 2 to 15 kDa. The X- and Y-axes represent *m/z* and analyzed sample number, respectively. The intensities of MALDI-TOF MS spectra were gray-scaled and plotted as a function of *m/z*. The spectra were baseline subtracted, smoothed, and vector normalized. Vertical lines indicate reproducible mass peaks. In the Y-axis, 1-5, *B. anthracis*; 6-10, *B. globigii*; 11-15, *B. cereus*; 16-20, *B. subtilis*; 21-25, *B. thuringiensis*. (b) Cluster analysis of mass spectra. The X-axis displays the spectrum numbers of each spore: 1-5, *B. anthracis*; 6-10: *B. globigii*; 11-15, *B. cereus*; 16-20, *B. subtilis*; 21-25, *B. thuringiensis*. (c) PCA analysis of the mass spectra of *Bacillus* spores and vegetative cells.

biomarker peaks (2503, 3089, 3376, 6684, 6698, 6753, and 6840 *m/z*) from those of other *Bacillus* family spores. The mass spectrum of the spores of each *Bacillus* species was well separated and clustered with those of other family members. Moreover, the biomarkers and specific mass patterns for 5 *Bacillus* spores were distinguishably detected. Therefore, this method allows real-time identification of spores and other microorganisms at the species level without requiring tandem MS and extraction methods and can be used efficiently for the detection of BWAs as well as other microorganisms.

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