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Rapid Identification of *Bacillus anthracis* Spores in Suspicious Powder Samples by Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS)

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Rapid and reliable identification of *Bacillus anthracis* spores in suspicious powders is important to mitigate the safety risks and economic burdens associated with such incidents. The aim of this study was to develop and validate a rapid and reliable laboratory-based matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis method for identifying *B. anthracis* spores in suspicious powder samples. A reference library containing 22 different *Bacillus* sp. strains or hoax materials was constructed and coupled with a novel classification algorithm and standardized processing protocol for various powder samples. The method's limit of *B. anthracis* detection was determined to be 2.5×10^6 spores, equivalent to a 55-μg sample size of the crudest *B. anthracis*-containing powder discovered during the 2001 Amerithrax incidents. The end-to-end analysis method was able to successfully discriminate among samples containing *B. anthracis* spores, closely related *Bacillus* sp. spores, and commonly encountered hoax materials. No false-positive or -negative classifications of *B. anthracis* spores were observed, even when the analysis method was challenged with a wide range of other bacterial agents. The robustness of the method was demonstrated by analyzing samples (i) at an external facility using a different MALDI-TOF MS instrument, (ii) using an untrained operator, and (iii) using mixtures of *Bacillus* sp. spores and hoax materials. Taken together, the observed performance of the analysis method developed demonstrates its potential applicability as a rapid, specific, sensitive, robust, and cost-effective laboratory-based analysis tool for resolving incidents involving suspicious powders in less than 30 min.

Bacillus anthracis is one of the most feared biological threat agents. This Gram-positive, spore-forming bacterium, designated a category A agent, is the causative agent of anthrax (1). The infective route of *B. anthracis* is inhalation, ingestion, or contact through skin lesions, leading to the development of inhalation, gastrointestinal, or cutaneous anthrax, respectively. *B. anthracis* can enter a resting stage by producing endospores that are highly resistant to environmental influences such as temperature, radiation, and humidity extremes, illustrated by the fact that *B. anthracis* spores have been shown to persist in various harsh environments for decades (2). Historically, *B. anthracis* has played a central role as a biological warfare agent, but in 2001, the mailing of letters containing powders of *B. anthracis* spores to news media and government offices in the United States (the Amerithrax incident) killed five people, sickened an additional 17, and resulted in economic disbursements of more than one billion U.S. dollars, demonstrating *B. anthracis* as a bioterrorism agent (3, 4). The majority of incidents involving suspicious powders eventually turn out to be hoaxes (i.e., the powder does not contain *B. anthracis* spores) (5). However, even hoax incidents have economic and psychological impacts, since they cannot easily be discriminated from bioterrorism incidents and must therefore be handled as real threats until the presence of *B. anthracis* spores or other hazards can be ruled out. In the United States alone, about 3,000 incidents involving suspicious powders occur each year (6). Rapid and reliable methods capable of ruling out hoax materials and confirming the presence of *B. anthracis* spores in powder samples are important to mitigate the safety risks and economic burdens associated with both bioterrorism and hoax incidents. Such methods assist in reducing the time needed to respond to possible and real bioterrorism events, as well as normalizing the situation after a hoax is encountered.

Several commercial methods are currently available for analyzing suspicious powder samples, ranging from basic powder screening kits that can provide first responders with an initial assessment of a powder's content to sophisticated laboratory techniques that are able to identify or rule out the presence of biological threat agents (7). The methods' sensitivity and specificity vary substantially, but they also differ in other analysis-related properties, such as speed, cost, and infrastructure and user experience level requirements. More than one method is generally needed when analyzing suspicious powder samples because the methods' analysis-related properties have a tendency to be inversely related (i.e., simple and rapid methods are often not sufficiently sensitive or specific, while sophisticated laboratory methods are often time-consuming and expensive and require expert users and a specialized infrastructure).

Recent advances in mass spectrometry (MS) and the introduction of soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) that allow MS analysis of intact organic macromolecules have led

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to an increased interest in MS-based microorganism identification methods, especially within infectious disease diagnostics and the biodefense community (8–14). MS is not restricted to predetermined targets, which stands in contrast to most other molecular detection techniques that generally rely on molecular recognition and selective binding (e.g., antibodies and nucleic acid probes and primers). In particular, MALDI-time of flight (TOF) MS has shown potential for rapid identification of microorganisms based on whole cells or whole-cell extracts (15), exemplified by the introduction of commercial analysis platforms (e.g., MALDI Biotyper from Bruker Daltonics and SARAMIS from AnagnosTec) that are now commonly used in hospital diagnostic laboratories (16–21).

MS-based methods (e.g., MALDI-TOF MS and liquid chromatography-tandem MS) involving several different analysis concepts such as mass spectrum fingerprinting and top-down or bottom-up proteomics have been successfully used to detect and characterize small acid-soluble proteins (SASPs) in *Bacillus* sp. spores (8–11, 22–39). SASPs, originally described in *B. subtilis* spores, have been shown to confer resistance to DNA damage and to function as a source of amino acids during spore germination (40). SASP family proteins are abundantly found in *Bacillus* sp. spores, and because of their basic nature, they can be selectively solubilized in acids and easily protonated to provide strong signals when ionized by MALDI or ESI (8). SASPs have been proposed as candidate biomarkers capable of discriminating between various *Bacillus* sp. spores, including those within the *Bacillus cereus sensu lato* group (*B. anthracis*, *B. cereus sensu stricto*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, and *Bacillus pseudomycoides*). The predominant SASPs found in *B. cereus sensu lato* group spores by MALDI-TOF MS are recognized as α-, β-, α/β-, and γ-SASPs (23).These SASPs are generally highly conserved within and between the various species of the *B. cereus sensu lato* group, and their true potential as unique biomarkers of *B. anthracis* spores has therefore been discussed and shown to depend on the analysis method used (24, 26, 27, 29, 32, 38, 39).

The aim of the present study was to develop a rapid, reliable, and cost-effective laboratory-based analysis method for identifying *B. anthracis* spores in suspicious powders. The development and validation of a MALDI-TOF MS-based classification method able to discriminate between powder samples consisting of *B. anthracis* spores, spores of closely related *Bacillus* spp., and commonly encountered hoax materials are described.

MATERIALS AND METHODS

Microorganisms and hoax materials. *Bacillus* sp. strains were obtained from the American Type Culture Collection; the Deutsche Sammlung von Mikroorganismen und Zellkulturen; the U.S. Department of Agriculture Agricultural Research Service culture collection; and Alvin Fox at the Department of Pathology, Microbiology, and Immunology, University of South Carolina, Columbia, SC. All of the *Bacillus* sp. strains used belong to the *B. cereus sensu lato* group (*B. anthracis*, *B. cereus sensu stricto*, *B. thuringiensis*, and *B. weihenstephanensis*) or the *B. subtilis* group (*B. subtilis* and *B. atrophaeus* [formerly known as *B. globigii*]). Emphasis was placed on obtaining several *B. cereus sensu stricto* and *B. thuringiensis* strains closely related to *B. anthracis*, based on the University of Oslo *B. cereus* group MultiLocus and MultiData Typing website (http://mlstoslo.uio.no) (41). The *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* strains were also selected to completely cover the previously described SASP diversity found within each of these species (8–11, 22–39). A dry powder formulation of *B. atrophaeus* spores was obtained from Dugway Proving Grounds (Dugway, UT), and a dry powder pesticide formulation (Turex WP 50) containing *B. thuringiensis* spores was obtained from Certis Europe (Utrecht, The Netherlands). The spores from these powders were subsequently recultured and isolated, and the isolates obtained were designated *B. thuringiensis* Kurstaki/Aizawai and *B. atrophaeus* Dugway, respectively. Commercially available powdered substances associated with powder letter hoaxes (42, 43) and referred to here as hoax materials were purchased from local supermarkets or obtained from Sigma-Aldrich (St. Louis, MO). Table 1 provides a complete list of the *Bacillus* sp. strains and hoax materials used in this study.

Spore production. The *Bacillus* sp. strains were recovered from frozen stocks, maintained on blood agar plates, and clonally seeded into cultivation flasks containing 2×SG modified Schaeffer sporulation broth (44, 45). The cultivation flasks were incubated (30°C, 250 rpm) until more than 90% phase-bright free spores were observed by phase-contrast microscopy. The cultures were centrifuged (4,500 × *g*, 30 min), and the supernatants were discarded. The spores were washed (10,000 × *g*, 5 min) three times with cold (4°C) MilliQ water (Millipore, Billerica, MA). Between the first and second washing steps, the spore solutions were freeze-thawed (−20°C, 18 h) and incubated (room temperature, 2 h) to induce autolysis of residual endospore-containing vegetative cells. The spores were further purified by a density gradient centrifugation procedure (46). Briefly, the spores were resuspended in 20% (wt/vol) Nycodenz (Axis-Shield, Oslo, Norway) in MilliQ water and overlaid onto 50% (wt/vol) Nycodenz in MilliQ water in a centrifuge tube (2 ml). The supernatant was discarded after centrifugation (13,000 × *g*, 45 min), and the spore pellets were washed (10,000 × *g*, 5 min) three times with cold (4°C) MilliQ water to remove residual Nycodenz. The final spore preparations were quantified with a counting chamber and a phase-contrast microscope (Olympus BX41; Olympus Nederland, Zoeterwoude, The Netherlands) and confirmed to contain more than 99% phase-bright free spores. Stock solutions were made by diluting the spores to a final concentration of 1.0×10^9/ml in MilliQ water. The spore stocks were stored at 4°C for the duration of the study.

Acid-assisted protein extraction procedure. An acid-soluble protein extraction protocol suitable for powder samples containing *Bacillus* sp. spores or hoax materials was developed by modifying a previously described formic acid (FA)-based method (47). The entire protocol was completed in less than 10 min. The standard input amount for samples included in this study was 1 mg powder (Fig. 1) or $\sim 10^7$ spores (when starting directly with liquid spore stocks). The modified protein extraction protocol was as follows. MilliQ water (1.0 ml) was added to a centrifuge tube (2 ml) containing the sample and mixed by pipetting up and down. The sample tube was centrifuged (13,000 × *g*, 2 min), and the supernatant was aspirated from the center of the tube and discarded without disturbing the pellet. The pellet was resuspended in 1.0 ml MilliQ water, mixed by pipetting up and down, and centrifuged (13,000 × *g*, 2 min). The supernatant was removed, the tube was recentrifuged (13,000 × *g*, 1 min), and any residual liquid was removed. The pellet was resuspended in 15.0 μl 70% FA and mixed by pipetting up and down without introducing air bubbles. After incubation (2 min, room temperature), 15.0 μl acetonitrile (ACN; Sigma-Aldrich) was added and the solution was mixed as described above. The tube was centrifuged (13,000 × *g*, 30 s), and the supernatant was transferred without disturbing the pellet to the filter membrane of a luer-lock syringe filter (0.22-μm-pore-size, 4-mm, Millex-GV_4 hydrophilic Durapore polyvinylidene difluoride [PVDF] filters; Millipore). The liquid was filtered with a luer-lock syringe (1 ml), and the filtrate was captured in a new tube.

MALDI-TOF MS. The protein extracts were spotted (1.0 μl) onto MTP AnchorChip 600/384 T F targets (Bruker Daltonics), air dried, and overlaid with 0.5 μl matrix solution containing 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) in 50% ACN (Sigma-Aldrich) and 2.5% trifluoroacetic acid (Sigma-Aldrich). Mass spectra were acquired with FlexControl 3.0 software (Bruker Daltonics) and an AutoFlex III Smartbeam mass spectrometer (Bruker Daltonics) in positive linear

TABLE 1 *Bacillus* sp. strains and hoax materials included in this study and results concerning the MALDI-TOF MS-based classification method's specificity and robustness

Bacillus sp. strain or hoax material	Included in library?	SASGCL (classification library)	Similarity group	Evaluation of specificity and robustness [no. of correct classifications (total)]			Performance measurements for *B. anthracis* [no. of classifications (total)]	
				Specificity (blinded samples)	Robustness (operator dependence)	Robustness (instrument dependence)	False positive	False negative
B. cereus sensu lato group								
B. anthracis								
Ames(pXO1^{+}/pXO2^{+})	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. anthracis*	4 (4)	1 (1)	1 (1)	NA[a]	0 (6)
Vollum(pXO1^{+}/pXO2^{+})	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. anthracis*	4 (4)	1 (1)	1 (1)	NA	0 (6)
Sterne(pXO1^{+}/pXO2^{-})	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. anthracis*	4 (4)	1 (1)	1 (1)	NA	0 (6)
Farmer cute(pXO1^{+}/PXO2^{+})	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. anthracis*	4 (4)	1 (1)	1 (1)	NA	0 (6)
B. cereus sensu stricto								
DSM 336	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
NVH0597-99	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 31 (ATCC 14579)	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 9378 (ATCC 10876)	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 3648 (ATCC 11950, W)	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	4 (4)	1 (1)	1 (1)	0 (6)	NA
R3	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 8438	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
B. thuringiensis								
CEB97/27	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
BGSC 4AJ1	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 6102 (ATCC 33679, HD-1)	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
BGSC 4CC1	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	4 (4)	1 (1)	1 (1)	0 (6)	NA
Kurstaki/Aizawai[b]	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
B. weihenstephanensis DSM 11821	Yes	*B. weihenstephanensis*/*B. subtilis*/*B. atrophaeus*	*B. weihenstephanensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
B. subtilis group								
B. subtilis DSM 10 (ATCC 6051)	Yes	*B. weihenstephanensis*/*B. subtilis*/*B. atrophaeus*	*B. subtilis*	5 (5)	1 (1)	1 (1)	0 (7)	NA
B. atrophaeus Dugway[c]	Yes	*B. weihenstephanensis*/*B. subtilis*/*B. atrophaeus*	*B. atrophaeus*	3 (3)	1 (1)	1 (1)	0 (5)	NA
Hoax materials								
Wheat flour	Yes	Hoax materials	Wheat flour	4 (4)	1 (1)	1 (1)	0 (6)	NA
Rye flour	Yes	Hoax materials	Rye flour	4 (4)	1 (1)	1 (1)	0 (6)	NA
Dry milk	Yes	Hoax materials	Dry milk	4 (4)	1 (1)	1 (1)	0 (6)	NA
Coffee creamer				NA[f,g]	ND[i]	ND	0 (3)	NA
Washing powder				NA[f,g]	ND	ND	0 (3)	NA
Talcum powder				NA[f,g]	ND	ND	0 (3)	NA
Powdered sugar				NA[f,g]	ND	ND	0 (3)	NA
Nutrient broth				NA[f,g]	ND	ND	0 (3)	NA
Yeast extract				NA[f,g]	ND	ND	0 (3)	NA

(Continued on following page)

TABLE 1 (Continued)

Bacillus sp. strain or hoax material	Included in library?	SASGCL (classification library)	Similarity group	Evaluation of specificity and robustness [no. of correct classifications (total)]			Performance measurements for *B. anthracis* [no. of classifications (total)]	
				Specificity (blinded samples)	Robustness (operator dependence)	Robustness (instrument dependence)	False positive	False negative
Spore-containing powders								
Turex WP 50[d]				3 (3)	1 (1)	1 (1)	0 (5)	NA
Dugway *B. atrophaeus*[e]				3 (3)	1 (1)	1 (1)	0 (5)	NA
Other bacterial agents (vegetative)[h]				NA[g]	ND	ND	0 (39)	NA
Total				83 (83)	24 (24)	24 (24)	0 (164)	0 (24)

[a] NA, not applicable.
[b] Isolated from Turex WP 50.
[c] Isolated from Dugway *B. atrophaeus*.
[d] Commercial pesticide formulation containing *B. thuringiensis* Kurstaki/Aizawai spores (Certis Europe, Utrecht, The Netherlands).
[e] Freeze-dried *B. atrophaeus* Dugway spores, lot 19076-03267 (Dugway Proving Grounds, Dugway, UT).
[f] Flat-lined mass spectrum, no peaks found.
[g] No false-positive classifications observed.
[h] Vegetative cells of other bacterial agents, including various strains of *Escherichia coli* ($n = 5$), *B. anthracis* ($n = 5$), *B. cereus sensu stricto* ($n = 4$), *B. subtilis* ($n = 1$), *B. atrophaeus* ($n = 1$), *Francisella tularensis* ($n = 2$), *Clostridium botulinum* ($n = 2$), *Vibrio cholerae* ($n = 2$), *Burkholderia mallei* ($n = 2$), *Burkholderia pseudomallei* ($n = 1$), *Yersinia pestis* ($n = 2$), *Acinetobacter baumannii* ($n = 1$), *Coxiella burnetii* ($n = 1$), *Enterococcus faecalis* ($n = 1$), *Staphylococcus aureus* ($n = 1$), *Staphylococcus epidermis* ($n = 1$), *Proteus mirabilis* ($n = 1$), *Pseudomonas aeruginosa* ($n = 1$), *Serratia marcescens* ($n = 1$), *Brucella ceti* ($n = 1$), *Brucella melitensis* ($n = 1$), *Shigella flexneri* ($n = 1$), and *Shigella sonnei* ($n = 1$).
[i] ND, not determined.

mode between 2 and 20 kDa. The pulsed ion extraction time was 350 ns, the acceleration voltages were 20 kV (source 1) and 18.7 kV (source 2), the lens voltage was 8 kV, and the linear detector voltage was 1.522 kV. Each sample spot was measured by using a hexagon acquisition pattern, and the mass spectra were recorded as the sum of 2,000 laser shots with the Smartbeam Nd:YAG (355 nm) laser at 200 Hz. The instrument was externally calibrated with the Bacterial Test Standard (255343; Bruker Daltonics).

Mass spectrum data processing. The mass spectra acquired were converted into MZXML format by using a script file from Bruker Daltonics (CompassXport.exe). Matlab R2012b (MathWorks, Natick, MA) and the Bioinformatics toolbox (version 3.0) were used for data processing. Data processing involved import (mzxmlread.m), resampling (msresample.m; mass range, 3,000 to 10,000 m/z; 31,500 data points), smoothing (mslowess.m; Lowess smoothing; order, 2; span, 40), baseline subtraction (msbackadj.m; window and step size, 5.0 and $0.005 \times m/z$), normalization (msnorm.m; single peak max intensity, 300), and peak selection (mspeaks.m; minimum intensity threshold, 10.0 between 3,000 and 8,000 m/z and 3.0 between 8,000 to 10,000 m/z). An additional filtering step was used after peak selection to remove peak shoulders initially identified as peaks by the intensity-based algorithm (mspeaks.m). By comparing the intensities of the peaks to the intensities of the neighboring valleys (identified by using the first-, and second-order derivatives), peak shoulders were removed by applying a bidirectional peak-to-valley minimum intensity ratio of 2.0.

Reference library construction. Protein extracts were prepared by the modified FA-based method of protein extraction from samples consisting of the *Bacillus* sp. spores or hoax materials selected for library construction (Table 1). Three extractions of each sample were done on separate days, and each protein extract was measured eight times. The peak lists obtained were used to generate one consensus peak list for each *Bacillus* sp. strain or hoax material. The inclusion criteria used when binning the individual peak lists into a consensus peak list required that the peak be present in >75% (18/24) of the individual peak lists within ±300 ppm of the peak's m/z average. A preliminary reference library was created by binning the consensus peak lists into common library peaks by using a bin size of ±500 ppm of the peak's m/z average and associating each library peak with present and absent designations for each entry of *Bacillus* sp. spores or hoax materials. Three stand-alone, similarity-grouped reference

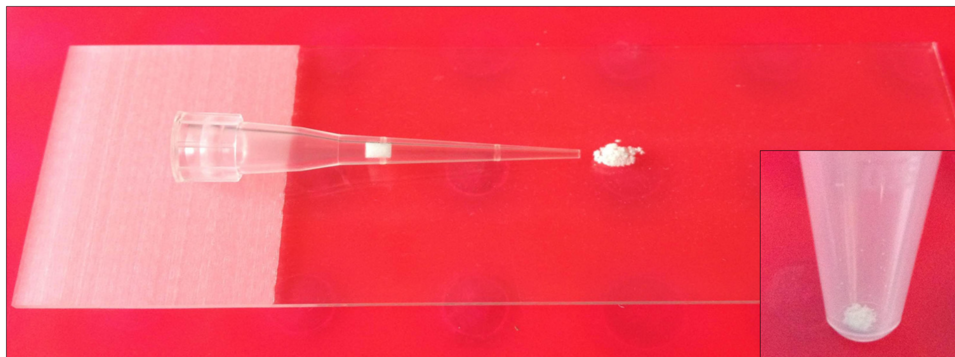

FIG 1 Powder sample (1 mg) on a microscopy slide. The inset shows the same amount of powder in a 1.5-ml centrifuge tube. A pipette tip (10 μl) is shown as a scale reference. The end facing the powder is 1 mm in diameter.

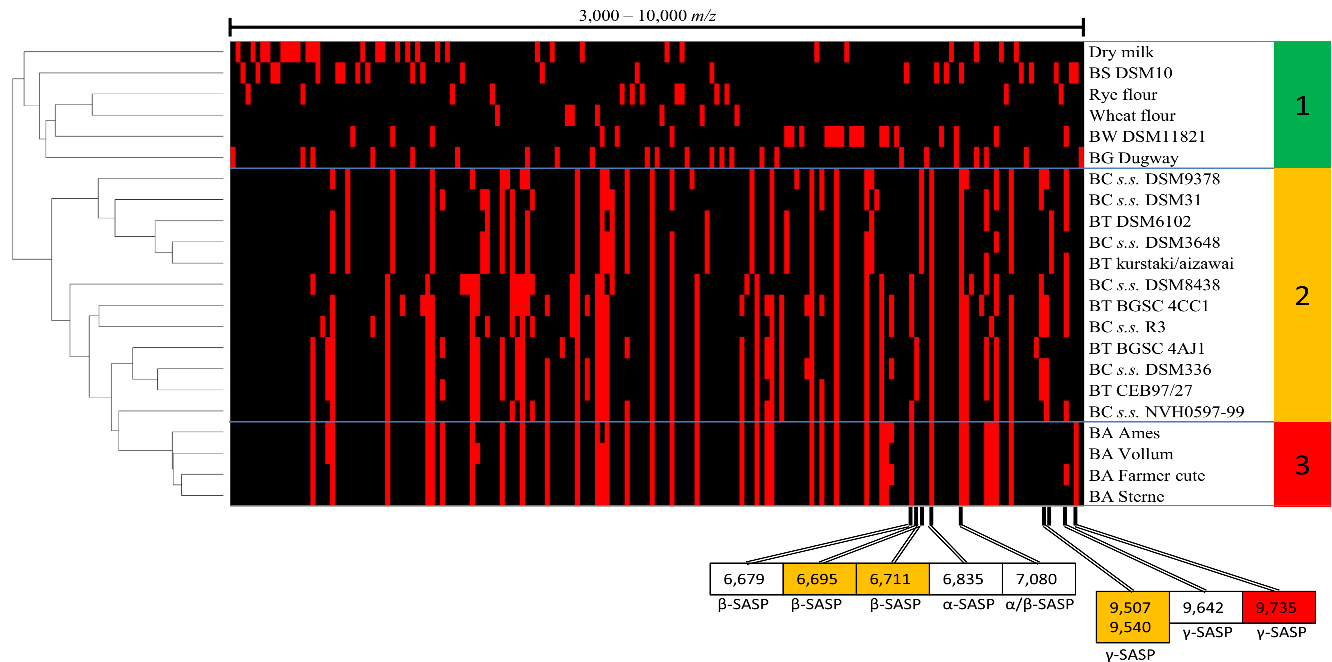

FIG 2 Dendrogram from average linkage hierarchical clustering of the preliminary reference library, including spores of 19 different *Bacillus* sp. strains and three hoax materials. The green region (region 1) corresponds to the *B. weihenstephanensis* (BW), *B. subtilis* (BS), *B. atrophaeus* (BG), dry milk, wheat flour, and rye flour entries. The orange region (region 2) corresponds to the *B. cereus sensu stricto* (BC *s.s.*) and *B. thuringiensis* (BT) entries. The red region (region 3) corresponds to the *B. anthracis* (BA) entries. The α-, β-, α/β-, and γ-SASPs observed in the *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* entries are presented below the dendrogram. SASPs found exclusively in *B. cereus sensu stricto*-*B. thuringiensis* entries (and not in *B. anthracis*) are orange, while SASPs found exclusively in *B. anthracis* entries (and not in *B. cereus sensu stricto*-*B. thuringiensis*) are red.

libraries (SASGRLs) were further constructed on the basis of the results of a hierarchical cluster analysis (average linkage) of the preliminary reference library (Fig. 2). The SASGRLs contained (i) *B. anthracis* and *B. cereus sensu stricto*-*B. thuringiensis* (merged into a composite similarity group) entries (the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library); (ii) *B. weihenstephanensis*, *B. subtilis*, and *B. atrophaeus* entries (the *B. weihenstephanensis*-*B. subtilis*-*B. atrophaeus* library); and (iii) dry milk, wheat flour, and rye flour entries (the hoax material library). In the SASGRLs, a single library entry was created to represent each similarity group by merging together all of the individual entries that were assigned to the similarity group. The peak present and absent designations in the SASGRLs were reported as percent present values (PPVs; range, 0 to 100) calculated from the number of entries used to construct the similarity group that had the peak divided by the total number of entries used to construct the similarity group. From each SASGRL, a final stand-alone similarity-grouped classification library (SASGCL) was created. Each similarity group's PPV was transformed into a PPV ratio (the similarity group's PPV divided by the sum of the PPVs of all of the similarity groups in the SASGRL) and further into an odds ratio (PPV ratio/[1 − PPV ratio]). Each SASGCL contained the same library peaks as the SASGRL it was generated from, in addition to each peak's odds ratio for each similarity group in the library. The SASGCLs also contained information about whether the library peaks could be linked to observed and/or predicted masses of SASP family proteins previously described in the literature (8, 9, 22–34, 37–39) or identified through a protein search of GenBank (http://www.ncbi.nlm.nih.gov) for SASPs. A 5× weighting correction was added to the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library to adjust the PPVs (adjusted PPV range, 0 to 500) of all of the SASP-associated library peaks observed exclusively in a single similarity group (i.e., unique similarity group SASP markers).

Sample measurement. All of the samples analyzed in this study were processed according to the procedures described for acid-assisted protein extraction, MALDI-TOF MS measurement, mass spectrum data processing, and library-based classification, unless otherwise stated. The protein extracts were measured as five spots on the MALDI target, thus generating a total of five mass spectra per analyzed sample. A consensus peak list was generated for each sample by binning peaks observed in >60% (3/5) of the individual peak lists within ±300 ppm of the peak's *m/z* average.

Classification algorithm. A custom classification algorithm was developed that matched the consensus peak list of a measured sample to library peaks (±500 ppm of the peak's *m/z* average) in each of the three SASGCLs. The classification algorithm summed the odds ratios for each similarity group in each SASGCL using matched peaks only and outputted summed size-sorted odds ratios together with the ratio of the highest to the second highest summed odds ratio. The classification algorithm also extracted the number of matched peaks in a SASGCL that could be mapped to SASP family proteins. The confidence threshold for a sample classification result was based on three different criteria that all had to be met. The first criterion was used to obtain a group classification and select one of the SASGCLs for further classification. When all of the common SASPs from the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library, α-SASP (~6,835 Da), β-SASP (~6,679, ~6,695, or ~6,711 Da), and α/β-SASP (~7,080 Da), were observed in a sample's consensus peak list, the classification algorithm used the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library for further classification and reported *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* as a confident group classification. When the common SASPs of the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library were absent, the classification algorithm selected the *B. weihenstephanensis*-*B. subtilis*-*B. atrophaeus* library when two or more peaks could be mapped to SASP family proteins found in this library and reported *B. weihenstephanensis*-*B. subtilis*-*B. atrophaeus* as a confident group classification. If fewer than two SASPs could be mapped to the *B. weihenstephanensis*-*B. subtilis*-*B. atrophaeus* library, the classification algorithm selected the hoax material library for further classification but did not report a confident group classification. For the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* or *B. weihenstephanensis*-*B. subtilis*-*B. atro-*

phaeus library, the second and third confidence criteria necessary to report a confident similarity group classification were a minimum summed odds ratio of 500 and a minimum ratio of the highest to the second highest summed odds ratio of 5, respectively. For the hoax material library, the second and third confidence criteria were more stringent to reduce the likelihood of assigning false-positive classifications to hoax materials because of random (by chance) matching of only a limited number of sample consensus peaks to the hoax material library. Specifically, a minimum summed odds ratio of 1,000 and a minimum ratio of the highest to the second highest summed odds ratio of 500 were necessary to assign a confident similarity group classification in the hoax material library. When none of the classification criteria described were met, the classification algorithm reported "no reliable classification."

LOC. The limit of classification (LOC) by the end-to-end classification method, defined here as the smallest amount of spores that allowed consistent, confident, and correct classification of a sample, was determined on 2 separate days by using spores of *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0597-99, and *B. atrophaeus* Dugway. Two-fold serial dilutions of the original spore stocks were made fresh each day with MilliQ water to generate samples ranging in concentration from 4.0×10^7 to 1.25×10^6 spores ml^{-1}, as determined by phase-contrast microscopy. Initial testing was done with 10-fold serial dilutions between 1.0×10^{10} and 1.0×10^5 spores ml^{-1} to establish an appropriate concentration window for LOC testing. Each dilution was extracted in duplicate and measured as five spots on the MALDI target. The analysis was scored as successful when the sample was correctly classified. The overall mass spectrum quality (i.e., peak resolution, peak signal-to-noise, and peak-to-peak intensity ratio) was also assessed, and the mass spectra were assigned a quality indicator (excellent, good, medium, or poor) in reference to the mass spectra generated during reference library construction. These quality indicators were used to complement the classification results when determining the method's LOC.

Validation of the classification method with blinded samples. Eighty-three samples consisting of *Bacillus* sp. spores or hoax materials were created by random selection from the available *Bacillus* sp. strains and hoax materials (Table 1) and analyzed according to the classification method described. Each sample was number coded, and the operator performing the analysis was blinded to the sample content until the analysis was completed. Additionally, 18 samples containing hoax materials not included in the reference libraries (Table 1) and 39 samples containing vegetative cells of various bacterial species (Table 1) were also analyzed to investigate the method's potential for obtaining false-positive classification results.

Evaluation of the classification method's robustness. All of the *Bacillus* sp. spores or hoax materials (Table 1) were analyzed by an untrained operator (i.e., no previous experience with the method). The analysis results from these 24 samples were used to evaluate the operator-dependent robustness of the classification method. A similar set of the same 24 samples were independently processed and express shipped on dry ice to the Norwegian Defense Research Establishment FFI (Kjeller, Norway) for MS measurement on a MicroFlex LT MALDI-TOF MS instrument (Bruker Daltonics) to evaluate the instrument-dependent robustness of the classification method. To evaluate the classification method's potential for analyzing samples containing *Bacillus* sp. spores mixed with hoax materials, spores of *B. anthracis* Ames, *B. cereus sensu stricto* NVH0595-97, or *B. atrophaeus* Dugway were mixed with various hoax materials and analyzed by the method described. Each sample was analyzed on 2 separate days, and the hoax materials used were dry milk, wheat flour, rye flour, coffee creamer, talcum powder, and washing powder, for a total of seven samples (one control and six mixtures) for each *Bacillus* sp. strain per analysis round. The mass spectra generated were assigned quality indicators in accordance with the procedure described for the LOC experiments. These were used to complement the classification results when evaluating the impact of sample mixtures on the method's classification potential.

RESULTS

Spore production. High-purity spore preparations (>99% phase-bright free spores) were successfully produced for all of the 19 *Bacillus* sp. strains selected for reference library construction (Table 1). In an attempt to include at least one representative species of each member of the *B. cereus sensu lato* group, production of *B. mycoides* and *B. pseudomycoides* spores were initially attempted by using the standard sporulation protocol, but these efforts failed because of low sporulation efficiencies (<1%).

Acid-assisted protein extraction protocol. A modified FA-based protein extraction procedure was developed that includes a pre-extraction washing step to retain a high level of *Bacillus* sp. spore recovery while removing water-soluble substances (data not shown). The added sterile filtration step was shown to consistently exclude spores from the final protein extract following repeated testing with up to 1.0×10^{10} spores of *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0597-99, and *B. atrophaeus* Dugway. The filter step did not have an impact on the mass spectra obtained from *Bacillus* sp. spores, consistent with observations made by Lasch et al. (48).

Reference library construction and classification algorithm. The scope of the present work was to develop a rapid method able to confidently discriminate *B. anthracis* spores from other *Bacillus* sp. spores and hoax materials in powder samples. High-quality mass spectra with consistently observed mass peaks could be generated from all of the 19 *Bacillus* sp. spore preparations produced. Of the nine hoax materials selected for the library construction (Table 1), only three (dry milk, wheat flour, and rye flour) generated mass spectra with consistent mass peaks while the rest failed to generate mass spectra with peak information (flat-lined mass spectra). Reference library construction was therefore performed with a total of 22 entries, 19 *Bacillus* sp. strains (4 *B. anthracis*, 7 *B. cereus sensu stricto*, 5 *B. thuringiensis*, 1 *B. weihenstephanensis*, 1 *B. subtilis*, and 1 *B. atrophaeus*) and three hoax materials (dry milk, wheat flour, and rye flour). A hierarchical cluster analysis (average linkage) was performed on the preliminary reference library containing all of the *Bacillus* sp. strains and hoax materials as independent library entries in order to guide the binning of the individual entries into similarity-grouped entries. The dendrogram from the cluster analysis correlated well with the known phylogenetic grouping of *Bacillus* spp. (Fig. 2). The interdispersed clustering of *B. cereus sensu stricto* and *B. thuringiensis* strains and the positioning of the *B. anthracis* strains in a homogeneous and separable cluster inside the *B. cereus sensu stricto*-*B. thuringiensis* branch also corresponded well to results of previous phylogenetic analyses of the *B. cereus sensu lato* group (41). The dendrogram also revealed that the *B. weihenstephanensis*, *B. subtilis*, *B. atrophaeus*, and hoax material entries constituted a heterogeneous cluster well separated from the *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* entries. Since the main objective of the classification method was to identify *B. anthracis* spores and because the *B. cereus sensu stricto* and *B. thuringiensis* strains were interdispersed with each other in the dendrogram, the *B. cereus sensu stricto* and *B. thuringiensis* entries were merged into a single similarity group while the *B. anthracis* entries were kept as a similarity group separate from the composite *B. cereus sensu stricto*-*B. thuringiensis* group. The preliminary similarity-grouped reference library therefore consisted of the following entries after the merging all of the individual entries into a single entry per similarity group: *B.*

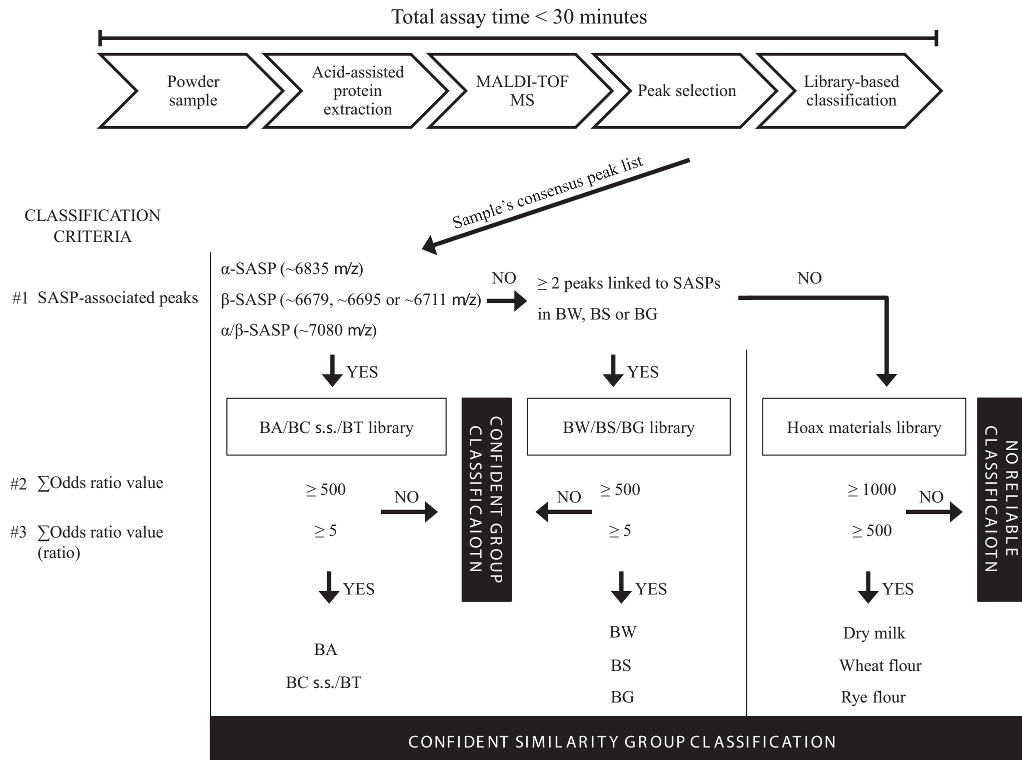

FIG 3 Flow chart summary of the proposed MALDI-TOF MS-based classification method. The library-based classification algorithm consisted of three classification criteria applied sequentially: number 1, selection of one of the three stand-alone similarity-grouped classification libraries using SASP-associated peaks in the sample's consensus peak list; number 2, similarity group summed odds ratio above a predetermined threshold; number 3, ratio of the highest to the second highest summed odds ratio above a predetermined threshold. A confident similarity group classification (*B. anthracis* [BA], *B. cereus sensu stricto-B. thuringiensis* [BC *s.s.*/BT], *B. weihenstephanensis* [BW], *B. subtilis* [BS], *B. atrophaeus* [BG], dry milk, wheat flour, or rye flour) was assigned when all of the classification criteria were met (numbers 1 to 3 for the *B. anthracis-B. cereus sensu stricto-B. thuringiensis* and *B. weihenstephanensis-B. subtilis-B. atrophaeus* libraries and numbers 2 and 3 for the hoax material library). A confident group classification (*B. anthracis-B. cereus sensu stricto-B. thuringiensis* or *B. weihenstephanensis-B. subtilis-B. atrophaeus*) was assigned when only the first classification criterion was met (not applicable to the hoax materials). "No reliable classification" was reported when the second or third classification criteria were not met for the hoax material library.

anthracis, 4 merged entries; *B. cereus sensu stricto-B. thuringiensis* composite similarity group, 12 merged entries; *B. weihenstephanensis*, 1 entry; *B. subtilis*, 1 entry; *B. atrophaeus*, 1 entry; milk powder, 1 entry; wheat flour, 1 entry; rye flour, 1 entry. The hierarchical clustering and the preliminary similarity-grouped reference library revealed that a core set of SASPs, α (~6,835 *m/z*), β (~6,679, ~6,695, or ~6,711 *m/z*), and α/β (~7,080 *m/z*), were observed in all of the *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* entries and could be used to separate them from all of the other library entries (Fig. 2). This led to the separation of these entries from the other *Bacillus* spp. and hoax materials by creating a SASGRL including only the *B. anthracis* and *B. cereus sensu stricto-B. thuringiensis* similarity groups (*B. anthracis-B. cereus sensu stricto-B. thuringiensis* library). *B. weihenstephanensis* did not have the common core set of SASP peaks found in all of the other *B. cereus sensu lato* group entries. Thus, *B. weihenstephanensis* was not included in the *B. anthracis-B. cereus sensu stricto-B. thuringiensis* library, even though it is phylogenetically recognized as a member of the *B. cereus sensu lato* group. It was found that two or more peaks corresponding to SASP family proteins were consistently observed in all of the *B. weihenstephanensis*, *B. subtilis*, and *B. atrophaeus* entries. Subsequently, this was used to separate these entries from the hoax materials by creating two SASGRLs containing (i) the *B. weihenstephanensis*, *B. subtilis*, and *B. atrophaeus* similarity groups (*B. weihenstephanensis-B. subtilis-B. atrophaeus* library) and (ii) the milk powder, wheat flour, and rye flour similarity groups (hoax material library). Figure 3 provides a flow chart summary of the proposed classification method, including the classification algorithm and final SASGCLs. Figure 4 presents representative MALDI-TOF MS spectra obtained by the analysis method described, highlighting the predominant SASPs observed in *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* spores.

LOC. The LOC experiments revealed clear differences between the *B. cereus sensu lato* group spores tested (*B. anthracis* Sterne and *B. cereus sensu stricto* NVH0595-97) and the *B. atrophaeus* Dugway spores, with a 4-fold higher LOC observed for the *B. atrophaeus* Dugway spores than for the *B. cereus sensu lato* group spores (Table 2). By employing stringent criteria for the LOC (4/4 correct classifications and good-quality mass spectra), the LOCs for *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0595-97, and *B. atrophaeus* Dugway spores were 2.5×10^6, 2.5×10^6, and 1.0×10^7 spores per sample, respectively (Table 2). Nearly all of the samples containing spores corresponding to half the LOC were correctly classified but did not fulfill all of the defined quality criteria (Table 2).

Method validation and robustness evaluation. One hundred percent (83/83) of the blinded samples consisting of *Bacillus* sp. spores or hoax materials were correctly classified by the proposed

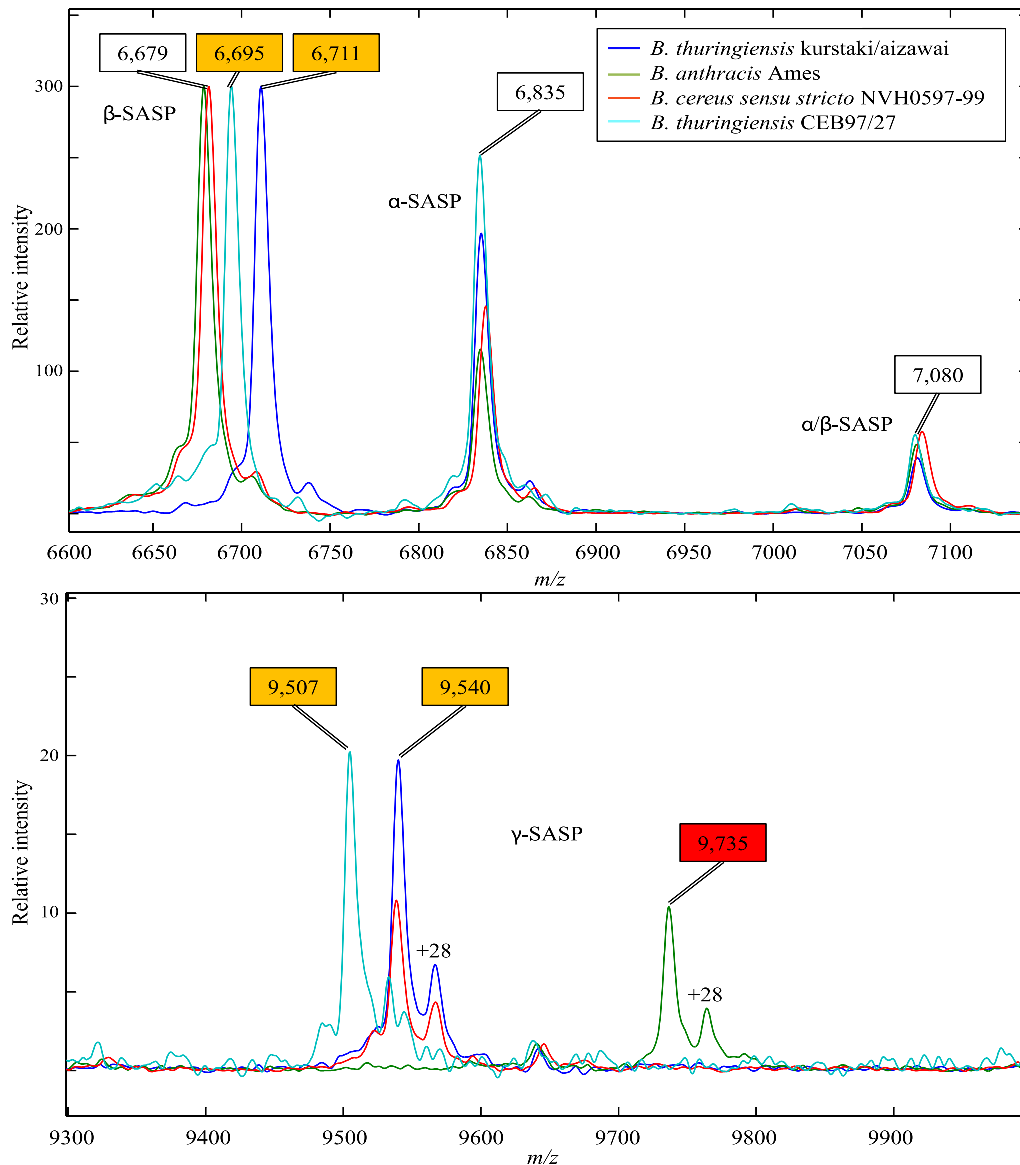

FIG 4 Representative mass spectra obtained by the MALDI-TOF MS-based analysis method described for *B. thuringiensis* Kurstaki/Aizawai, *B. anthracis* Ames, *B. cereus sensu stricto* NVH0597-99, and *B. thuringiensis* CEB97/27 spores. The upper plot presents the α-, β-, and α/β-SASPs, and the bottom plot presents the γ-SASPs. SASP-associated peaks found exclusively in *B. cereus sensu stricto* and/or *B. thuringiensis* and not *B. anthracis* are in orange, while SASP-associated peaks found exclusively in *B. anthracis* and not *B. cereus sensu stricto* and/or *B. thuringiensis* are in red. The γ-SASP peaks were commonly associated with low-intensity shoulder peaks (+28 *m/z*), probably because of FA-induced protein formylation.

classification method (Table 1). Additionally, analysis of 18 samples containing hoax materials not included in the classification library and 39 samples containing vegetative cells of various bacterial species did not lead to any (0/57) false-positive classifications (Table 1). Analysis of 24 different samples representing each of the *Bacillus* sp. strains and hoax materials by an untrained operator led to 100% (24/24) correct classification (Table 1), indicating that the performance of the classification method was not influenced by operator-dependent factors. A similar set of the 24 samples was analyzed at an external research facility (FFI, Kjeller, Norway), revealing that all of the samples (24/24) were correctly classified (Table 1). This indicated that the classification method's performance was not impacted by instrument-dependent factors (i.e., not influenced by the transport of protein extracts on dry ice or the use of a MicroFlex LT MALDI-TOF MS instrument at the external facility). In summary, the method's specificity and robustness were proven by classifying a total of 131 samples with 100% accuracy (Table 1). The performance criteria related solely to the successful discrimination of *B. anthracis* spores were demonstrated by obtaining 0% (0/164) false-positive and 0% (0/24) false-negative classification results after subjecting the classification method to samples containing a wide range of *Bacillus* sp. spores (including closely related *B. cereus sensu lato* group strains), hoax materials, and vegetative cells of other bacterial species (Table 1).

Classification potential for sample mixtures. The analysis of 42 samples containing *B. anthracis* Ames, *B. cereus sensu stricto* NVH0595-97, or *B. atrophaeus* Dugway spores mixed with hoax

TABLE 2 End-to-end detection limit (i.e., sensitivity) of the MALDI-TOF MS-based classification method

Strain and no. of spores/sample	Result	No. correct (total)	Spectrum quality
B. anthracis Sterne			
4.0×10^7	*B. anthracis*	4 (4)	Excellent
2.0×10^7	*B. anthracis*	4 (4)	Excellent
1.0×10^7	*B. anthracis*	4 (4)	Excellent
5.0×10^6	*B. anthracis*	4 (4)	Excellent
2.5×10^6	*B. anthracis*	4 (4)	Good
1.25×10^6	*B. anthracis*[b]	3 (4)	Medium
B. cereus sensu stricto NVH0595-97			
4.0×10^7	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Excellent
2.0×10^7	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Excellent
1.0×10^7	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Excellent
5.0×10^6	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Excellent
2.5×10^6	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Good
1.25×10^6	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Medium
B. atrophaeus Dugway			
4.0×10^7	*B. atrophaeus*	4 (4)	Excellent
2.0×10^7	*B. atrophaeus*	4 (4)	Good
1.0×10^7	*B. atrophaeus*	4 (4)	Good
5.0×10^6	*B. atrophaeus*[b]	3 (4)	Poor
2.5×10^6	No reliable classification[c]	0 (4)	NA[a,d]
1.25×10^6	No reliable classification[c]	0 (4)	NA[d]

[a] NA, not applicable.
[b] A single sample (1/4) led to "No reliable classification."
[c] All samples (4/4) led to "No reliable classification."
[d] Flat-lined mass spectrum, no peaks found.

materials revealed that all three types of *Bacillus* sp. spores could be successfully classified in a mixture with dry milk, wheat flour, rye flour, coffee creamer, talcum powder, or washing powder. Generally, the mass spectra obtained from *Bacillus* sp. spores mixed with hoax materials differed marginally from those of the control samples containing *Bacillus* sp. spores only. Also, all of the acquired mass spectra were of high spectrum quality. When spores were mixed with hoax materials present in the classification library, mass peaks from both the spores and the hoax materials were consistently observed without major changes in the overall mass spectrum quality or the normalized intensities of the individual mass peaks. However, one exception was seen when spores were mixed with wheat flour (see Fig. S1 in the supplemental material), since when measured alone, wheat flour generated mass spectra with two high-intensity mass peaks (~4,820 and ~4,920 *m*/*z*). These were the only hoax material-derived peaks that displayed higher intensities than the predominant SASPs from *Bacillus* sp. spores. Still, their intensities were only marginally stronger than those of the SASP peaks and therefore did not influence the peak selection process. However, because the first criteria of the classification algorithm (detection of two or more SASP-associated mass peaks) were achieved, only the *Bacillus* sp. spores were classified and no attempt was made to identify the hoax material by the proposed method when the sample contained *Bacillus* sp. spores.

DISCUSSION

Powder letters containing *B. anthracis* spores can represent a real threat to human health and to our society, as revealed by the 2001 Amerithrax incidents in the United States (1, 3, 4, 49). Besides the actual acts of bioterrorism, letters containing harmless powders are commonly encountered. A major challenge with these hoax incidents is that they cause anxiety, consume resources, and must be handled as a real threat until the presence of *B. anthracis* spores and/or other hazardous substances can be confidently ruled out, even if they *per se* do not represent a direct health hazard.

The present work has demonstrated the successful development of a MALDI-TOF MS-based rapid classification method for powder samples suspected of containing *B. anthracis* spores. Taken together, the observed performance of the classification method demonstrates its applicability as a rapid, reliable, and cost-effective laboratory-based analysis tool for powder samples. The end-to-end classification method achieved the sensitivity and specificity levels needed to resolve incidents involving suspicious powders and can be completed in less than 30 min.

The presence of *B. anthracis* spores is generally the first suspected hazard when encountering suspicious powders, but other threat agents could potentially be considered as well (50). The MALDI-TOF MS-based classification method developed in this work was originally aimed at suspicious powder threats involving *B. anthracis* spores but could be extended to include additional threat agents, which could increase the applicability of the proposed assay in a broader sense. This is supported by the method's observed performance, and there is reason to believe that it could be adapted and validated for other sample types (e.g., swabs and air samples), although this was outside the scope of the present study.

The mass spectra obtained from *Bacillus* sp. spores in this study were consistently dominated by mass peaks that could be linked to SASPs. Previous MS-based investigations have demonstrated the use of SASPs as discriminatory biomarkers to separate various *Bacillus* sp. spores from each other (8–11, 22–39). The challenges

associated with SASP-based discrimination increase significantly when attempting intragroup discrimination inside the *B. cereus sensu lato* group, which is not surprising considering their close genetic relationship (41). Recent studies, have pointed out that *B. weihenstephanensis*, *B. mycoides*, and *B. pseudomycoides* can be discriminated from the rest of the members of the *B. cereus sensu lato* group on the basis of their α-, and β-SASPs, except for *B. mycoides* group 1 strains, which have the same α-SASP as *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* strains and the same β-SASP as some *B. cereus sensu stricto* and *B. thuringiensis* strains (22–25). It has been proposed that the mass (~6,679 Da) of the β-SASP from *B. anthracis* can be used to discriminate *B. anthracis* from *B. cereus sensu stricto* and *B. thuringiensis* on the basis of the consistent observation of one or two amino acid substitutions in the β-SASP (~6,695 or ~6,711 Da, respectively) from *B. cereus sensu stricto* and *B. thuringiensis* compared to that from *B. anthracis* (24–27, 34, 38). Recent work has revealed that some *B. cereus sensu stricto* and *B. thuringiensis* strains, although presumably rare, also have this previously assumed *B. anthracis*-specific β-SASP (22, 23, 36). To ensure the robustness of the proposed classification method when encountering such *B. cereus sensu stricto* and *B. thuringiensis* strains, several of these strains were included in the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library. Three of seven *B. cereus sensu stricto* strains (NVH0597-99, R3, and DSM8438) and one of five *B. thuringiensis* strains (BGSC 4CC1) in the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library (Table 1) have the same β-SASP (~6,679 Da) as *B. anthracis*. The γ-SASP has recently been highlighted as a potential biomarker for the discrimination of *B. anthracis* from *B. cereus sensu stricto* and *B. thuringiensis* (32). We consistently observed the γ-SASP (~9,735 Da) in all of the *B. anthracis* spores in this study. None of the *B. cereus sensu stricto* or *B. thuringiensis* strains had the same γ-SASP as *B. anthracis* but had other γ-SASP masses instead (e.g., ~9,642, ~9,540, and ~9,507 Da). By combining the collective discriminatory power of the α-, β-, α/β-, and γ-SASPs and several other non-SASP-associated peaks, we were able to develop a MALDI-TOF MS library-based classification method that could confidently discriminate *B. anthracis* spores from all other *Bacillus* sp. spores, even when challenged with closely related *B. cereus sensu stricto* and *B. thuringiensis* strains that contained the same α-, β-, and α/β-SASP (~6,835, ~6,679, and ~7,080 Da, respectively) as *B. anthracis*.

Generally, most of the MALDI-TOF MS methods in use for microorganism identification rely on cultivation before MS analysis. Thus, previous studies have not focused on the determination of exact detection limits. The proposed end-to-end classification method was found to have sensitivity comparable to that of other MALDI-TOF MS-based analysis methods for microorganism identification (51–53). Previously described MALDI-TOF MS-based methods for the direct identification and discrimination of *Bacillus* sp. spores have focused primarily on elucidating the specificity of various SASPs as biomarkers (22–25, 30, 31, 33, 34, 37, 38). To our knowledge, limited information is available on end-to-end functional assays and on sensitivity measurements (i.e., detection limits). Our proposed end-to-end classification method was shown to have stringently set LOCs for *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0595-97, and *B. atrophaeus* Dugway of 2.5×10^6, 2.5×10^6, and 1.0×10^7 spores, respectively. However, it should be noted that our modified protein extraction method led to the use of constant volumes of FA and ACN (15 μl each), independently of the observed size of the spore pellet after the pre-extraction washing step, while only 1 μl of the total protein extract (30 μl) was analyzed per MALDI spot. The LOC could therefore probably be further improved by adjusting the FA and ACN volumes to the size of the pellet or, alternatively, by analyzing a larger fraction of the available protein extract. Compared to those of other sensitive detection technologies, such as PCR-based methods, the reported LOC of the proposed classification method is orders of magnitude higher. However, for applications involving the screening of suspicious powders, it can be assumed that the powder must be present in an amount discernible by the naked eye for it to be recognized as a suspicious powder threat. On the basis of the physical size of a 1-mg powder sample (Fig. 1), it seems to be unlikely that amounts far below 1 mg would warrant analysis by such methods. The powder letters involved in the 2001 Amerithrax incident in the United States contained gram quantities of *B. anthracis* spores at concentrations ranging from 2.1×10^{12} to 4.6×10^{10} spores/g (54), which would translate into 1-mg samples that contain 4.6×10^7 spores for the crudest spore preparation involved. This corresponds to more than 18 times the LOC described for *B. anthracis* spores (2.5×10^6 spores) in our study, indicating that for even the most impure powder involved in the Amerithrax incident, a sample amount as small as 55 μg would be sufficient for successful classification by the method described. In this study, the proposed classification method was successfully tested with up to 1.0×10^{10} *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0595-97, and *B. atrophaeus* Dugway spores, indicating that analysis of a larger amount of powder would be possible. Still, increasing the amount of powder could compromise the robustness of the method if the powder does not primarily contain spores. A sound approach could be to analyze different amounts of the same powder samples. The results obtained in the LOC experiments showed that the LOC for *B. atrophaeus* spores was four times higher than that for *B. anthracis* and *B. cereus sensu stricto* spores. This could possibly be attributed to physical differences between *B. cereus sensu lato* and *B. subtilis* group spores, including the presence or absence of exosporium, respectively, and spore coat and cortex compositional differences. This is supported by previous reports showing lower SASP yields from *B. subtilis* group spores than from *B. cereus sensu lato* spores following extraction with ACN–5% TFA (70:30, vol/vol) (34, 55).

The results obtained from the analysis of sample mixtures demonstrated the robustness of the proposed classification method, since all of the samples containing both *Bacillus* sp. spores and hoax materials could be consistently and correctly classified. This observation also suggested that the acid-assisted protein extraction was not sensitive to the presence of additional materials in the samples, possibly helped by the pre-extraction washing step that was included in the standardized protocol to remove water-soluble substances. The proposed method classified only the spore content and not the hoax material when the sample contained a mixture of both. Since the method was developed primarily to identify *B. anthracis* spores, no attempt was made to implement hoax material classifications when *Bacillus* sp. spores were present in the sample. The classification method described was also challenged with samples consisting of *Bacillus* sp. spores generated by production methods that differed from the standard protocol described, including several solid agar production methods (e.g., Columbia blood agar, Trypticase soy agar, and nutrient agar). The classification method was also challenged with crude

spore preparations (i.e., without postproduction purification) and spore preparations that were intentionally harvested before complete sporulation (i.e., <90% phase-bright free spores). All of the samples containing *Bacillus* sp. spores produced with the alternative production protocols were correctly classified, and no discernible changes in the mass spectra obtained were seen (data not shown), which is in agreement with previous observations (31, 33, 37). The analysis of samples containing crude spore preparations generated mass spectra with several additional peaks compared to pure spores. The additional peaks were most likely derived from vegetative cells and/or medium residues, but the expected spore-associated peaks were still consistently observed. Since the additional peaks were not present in the classification library and the SASP-associated peaks were present in the mass spectra irrespective of the additional peaks, no effect on the final classification results was seen and all of the samples were correctly classified (data not shown). Taken together, these observations support the perceived robustness of the proposed classification method.

The analysis method described here is a supplement to other laboratory methodologies (e.g., cultivation-, PCR-, and sequencing-based methods). The main focus of the proposed method is to obtain a reduction of the time between the initial discovery of a suspicious powder and the first confirmed laboratory-based answer about whether it contains *B. anthracis* spores or not, in order to decide if the initiation of other countermeasures is needed. Finally, the MALDI-TOF MS method developed is a cost-effective and potentially valuable assay that can easily be deployed in response laboratories and will help to elucidate suspicious powders and substantially reduce the burden of handling them.

In conclusion, a MALDI-TOF MS-based classification method for the rapid and reliable screening of powder samples and identification of *B. anthracis* spores was developed. The results showed that the assay is specific enough to discriminate *B. anthracis* spores from genetically highly similar *B. cereus sensu lato* strains and that the method is sensitive enough to allow the successful analysis of barely visible powder samples in less than 30 min.

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