

Rapid characterization of *Bacillus* spores targeting species-unique peptides produced with an atmospheric pressure matrix-assisted laser desorption/ionization source

Patrick A. Pribil,^{1*} Elizabeth Patton,¹ Gavin Black,² Vladimir Doroshenko² and Catherine Fenselau¹

¹ Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, USA

² MassTech, Inc., Columbia, Maryland 21046, USA

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New and improved strategies are eagerly sought for the rapid identification of microorganisms, particularly in mixtures. Mass spectrometry remains a powerful tool for this purpose. Small acid-soluble proteins (SASPs), which are relatively abundant in *Bacillus* spores, represent potential biomarkers for species characterization. Despite sharing extensive sequence homology, these proteins differ sufficiently in sequence for discrimination between species. This work focuses on the differences in sequence between SASPs from various *Bacillus* species. Compilation of SASP sequences from protein database searches, followed by *in silico* trypsin digestion and analysis of the resulting fragments, identified several species-specific peptides that could be targeted for analysis using mass spectrometry. This strategy was tested and found to be successful in the characterization of *Bacillus* spores both from individual species and in mixtures. Analysis was performed using an ion trap mass spectrometer with an atmospheric pressure MALDI source. This instrumentation offers the advantage of increased speed of analysis and accurate precursor ion selection for tandem mass spectrometric analysis compared with vacuum matrix-assisted laser desorption/ionization and time-of-flight instruments. The identification and targeting of species-specific peptides using this type of instrumentation offers a rapid, efficient strategy for the identification of *Bacillus* spores and can potentially be applied to different microorganisms. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: atmospheric pressure matrix-assisted laser desorption/ionization; *Bacillus* spores; bacteria; small, acid-soluble proteins; species-unique peptides

INTRODUCTION

In recent years, a great deal of emphasis has been placed on the development of instrumentation and protocols for the rapid identification of microorganisms, particularly in contaminated samples or unpurified mixtures. One of the more powerful tools for this purpose is matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).^{1,2} This technique is well suited to the analysis of protein and peptides, which are among the most useful biomarkers for species differentiation. MALDI ionization has distinct features that make it ideally applicable to the rapid analysis of organisms and their biomolecules; it generates primarily singly charged ions, and compared with electrospray ionization it has higher tolerances to impurities and requires less sample preparation.^{3,4} This

method of ionization can be coupled with different types of mass analyzers (e.g. time-of-flight (TOF), quadrupole ion trap (QIT)) to generate instruments with relatively high sensitivity, resolution and mass accuracy.

Among the more thoroughly studied microorganisms are bacterial spores of the genus *Bacillus*. Owing to the highly pathogenic nature of certain species and their potential use as bioweapons (e.g. *Bacillus anthracis*, the causative agent of anthrax), the analysis of *Bacillus* spores has elicited a great deal of medical and forensic interest.⁵ MALDI-MS analysis of bacterial proteins has figured prominently in the rapid characterization of *Bacillus* species, specifically in the ability to distinguish between pathogenic and non-pathogenic species⁶ and also in the discrimination between species strains.⁷ Strategies that have been employed to this end include spectral pattern analyses that make use of libraries of species-specific spectral profiles^{8–10} and the matching of suites of peaks to protein masses predicted from bacterial genome sequencing efforts.^{11–13} However, these strategies are hampered by the irreproducibility of

*Correspondence to: Patrick A. Pribil, Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, USA. E-mail: patrickpribil@hotmail.com

mass spectra and the complexity of signals in mixtures of microorganisms. An alternative approach involves the microsequencing of protein fragments using tandem mass spectrometry (MS/MS) and subsequent protein/organism identification by comparison of the peptide sequence with known protein sequences in protein/genome databases.^{14–16} In addition to this role, these databases can be exploited to achieve unambiguous species discrimination using a predictive approach; provided that these databases are sufficiently complete, it is possible to predict that certain protein and peptide fragments are unique for a given organism. Such unique peptide biomarkers can then be targeted specifically in the MS analysis, without the need for an initial survey scan.

Owing to the large number of proteins present in *Bacillus* spores, strategies have been devised to solubilize selectively a limited set of proteins for rapid species differentiation.^{14–16} Among the most promising class of proteins for this purpose are the small acid-soluble proteins (SASPs), a suite of proteins that can be extracted (on the sample holder) from the interior of spores by treatment with acid.^{17,18} These proteins are relatively abundant within *Bacillus* spores and function in protecting bacterial DNA from physical and chemical damage, and also in spore germination processes.^{19–22} SASP sequences have been obtained from a wide variety of *Bacillus* species and, although SASPs from many species share a degree of homology, collectively these proteins differ enough in sequence to form a basis for species discrimination.²³

Recent studies of *Bacillus* SASPs using MALDI-MS have employed the strategy of *in situ* acid solubilization of SASPs from intact spores, followed by proteolytic digestion of these proteins, as a rapid procedure that can potentially be automated.^{15,16} In these studies, the resulting SASP peptides were characterized using MS/MS to obtain microsequence information, followed by database searches. Unfortunately, several of the identified peptides are common to more than one species of *Bacillus*, making the determination of the origin of these peptides difficult. This is true particularly among closely related members of the *cereus* group (*Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis*).^{5,24} In addition, it has recently been determined that the major SASPs of *Bacillus globigii* and *Bacillus stearothermophilus* are nearly identical.²⁵ In order to achieve more rapid, automatable species differentiation, it is advantageous to focus directly on species-unique peptide sequences. To expedite species characterization further, the overall analysis time could also be reduced by employing an instrument in which it is not necessary to bring the sample under vacuum. Such an instrument would also be favorable for automated sample preparation.

This paper describes the characterization of *Bacillus* species via directed scanning of species-unique SASP tryptic peptide markers using an atmospheric pressure MALDI source on an ion trap analyzer.²⁶

EXPERIMENTAL

Materials and *Bacillus* spores

Trifluoroacetic acid (TFA) and α -cyanohydroxycinnamic acid (CHCA) were purchased from Sigma (St. Louis, MO, USA).

Trypsin immobilized to agarose beads was purchased from Pierce Biotechnology (Rockford, IL, USA). *Bacillus anthracis* str. S-Sterne, *Bacillus cereus* str. T, *Bacillus globigii*, *Bacillus subtilis* str. 168 and *Bacillus thuringiensis* str. Kurstaki were grown on new sporulation medium and harvested in-house using previously reported protocols.^{27,28} Dried spores ($\sim 5 \times 10^7$ spores mg^{-1} dry mass) were stored at -20°C until use.

Generation of species-unique SASP peptide lists

Sequences were obtained for SASP- α , - β , -A, -B, -1, -2, and - γ proteins from all *Bacillus* species available from the NCBI protein database (<http://www.ncbi.nlm.nih.gov>). SASP sequences for *Bacillus globigii* were sequenced in our laboratory.²⁵ *In silico* trypsin digestions were performed using PeptideCutter (<http://us.expasy.org>). Peptides that were found uniquely in a given *Bacillus* species were tabulated, and the species uniqueness of each was tested by performing a MASCOT sequence query (<http://www.matrixscience.com>) (see below for details of the search parameters).

Sample preparation

Suspensions of spores were prepared in water (concentration 2.5 mg ml^{-1}) immediately prior to their analysis. These solutions were then mixed in 1:1 or 1:10 volume ratios to generate various mixtures. Aliquots ($1 \mu\text{l}$) of spore suspensions were applied to a gold-coated stainless-steel 96-well MALDI plate, to which $1 \mu\text{l}$ of 10% aqueous TFA was added to solubilize the SASPs. After drying the spot, $1 \mu\text{l}$ of immobilized trypsin suspension was added (trypsin was washed with $25 \text{ mM NH}_4\text{HCO}_3$ prior to use), and the digestions were allowed to proceed for 5 min at ambient room temperature in a humidification chamber to prevent drying. Digestions were terminated by the addition of $1 \mu\text{l}$ of 0.1% aqueous TFA. After the spots had dried, $1 \mu\text{l}$ of CHCA matrix solution (5 mg ml^{-1} in 70% acetonitrile–0.1% aqueous TFA) was applied.

Mass spectrometric analysis

Spectra were obtained on an LCQ-Deca XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an atmospheric pressure MALDI source (MassTech, Columbia, MD, USA) with a 337 nm nitrogen laser. The laser power was optimized between 70 and $120 \mu\text{J}$, with a laser firing rate of 10 Hz. Laser shots were rastered manually across the sample. Spectra were accumulated for 1 min intervals and averaged.

Database searches

Spectra were exported as text files and submitted to MASCOT MS/MS Ion Search. Manual sequencing was performed in some cases. Searches were performed against the NCBI nr database, with the taxonomy restricted to eubacteria. Peptide and MS/MS fragment mass tolerances were set to ± 1.0 and $\pm 1.5 \text{ Da}$, respectively. One missed cleavage was allowed.

RESULTS AND DISCUSSION

Identification of species-unique peptides

Complete genome sequence information is available for several *Bacillus* species at <http://www.tigr.org>; *Bacillus anthracis* (strains Ames and A2012),²⁹ *Bacillus cereus* (strains 10 987 and ATCC 14 579),^{30–32} *Bacillus halodurans*³³ and *Bacillus subtilis* str. 168,³⁴ providing access to a comprehensive list of annotated SASPs. More limited SASP sequence information is also available for several species whose genomes have not yet been completely sequenced, including *Bacillus globigii*, *Bacillus mycoides*, *Bacillus megaterium*, *Bacillus stearothermophilus* and *Bacillus thuringiensis*. Using information available from public protein databases and recent protein sequencing efforts, sequences of known major SASPs (including SASP-1, -2, -A, -B, - α , - β and - γ) for the above organisms are compiled in Table 1.

As mentioned above, SASPs are highly abundant in spores and are readily solubilized by treatment of the intact spores with acid. Although significant sequence homology exists between many members, SASPs collectively differ sufficiently in sequence between organisms to allow for species discrimination. The objective of this work was to focus solely on these sequence differences in SASPs from various species and to determine whether species-specific peptides could be identified and targeted for automated analysis using MS. To accomplish this, the SASP sequences from Table 1 were digested *in silico* with trypsin. The lists of peptide products were compared and those that were found exclusively in a given species were further tested by performing MASCOT searches for each peptide against prokaryotic protein sequence databases. The list of species-unique SASP peptide ions in the practical range for MS and MS/MS analysis and peptide identification (peptides composed of ≥ 6 amino acid residues, with m/z values ranging from 600 to 3000) is shown in Table 2.

It is evident from Table 2 that the number of peptides unique to *Bacillus anthracis* and *Bacillus cereus* is limited, and that no unique *Bacillus thuringiensis* SASP peptides could be identified from the incomplete list of SASPs in the database. Recognition of the close relation of these three species has caused them to be classified as *cereus* group *Bacilli*, along with *Bacillus mycoides*.^{5,24} To overcome the lack of species-unique peptides among members of this group, the similarities in their protein sequences were exploited and focus was placed on peptides that are common within the *cereus* group and not found elsewhere. A list of such *cereus* group-specific peptides is shown in Table 3 (peptides that are present in a given species are labeled X). Comparison of *Bacillus anthracis* and *Bacillus cereus*, whose genomes have been completely sequenced, shows that most SASP peptides are shared. Nevertheless, there are some SASP peptides that are absent in one species or the other (for example, peptides having m/z 2712.8 and 2728.8 are present in *Bacillus cereus* but not in *Bacillus anthracis*). The genomes of *Bacillus mycoides* and *Bacillus thuringiensis* have not been completely sequenced and far fewer *cereus* group-specific peptides were predicted for these species in the present strategy. Some entries for *Bacillus thuringiensis* have been recently derived experimentally (labeled Y in Table 3).

Owing to the high degree of similarity between sequenced SASPs of *Bacillus globigii* and *Bacillus stearothermophilus*,²⁵ only a limited number of species-unique peptides were identified for each of these (Table 2). For *Bacillus subtilis*, whose genome is completely sequenced and whose SASPs differ in sequence to a larger extent from those of other species, numerous specific SASP peptides were identified for subsequent targeting.

As more protein or genome information becomes available for different *Bacillus* species, these peptide lists will be amended. Nevertheless, the strategy discussed here has the potential to discriminate between species by focusing directly on species-unique or group-specific SASP peptides and was implemented by analyzing acid-treated *Bacilli*, both individually and in mixtures.

MALDI analysis of tryptic SASP peptides

It was of initial interest to determine which species-specific (and *cereus* group-specific) SASP peptides would be observed in the spectra of individual spore extracts. *Bacillus anthracis* is considered to be an extremely monomorphic species of bacterium, i.e. having low genetic variability between strains.^{35,36} Therefore, it was expected that the species-specific SASP peptides identified would apply to the non-pathogenic strain S-Sterne studied here (lacking the plasmid encoding the anthrax toxin). *Bacillus anthracis* str. S-Sterne, along with *Bacillus cereus* str. T, *Bacillus globigii*, *Bacillus subtilis* str. 168 and *Bacillus thuringiensis* str. Kurstaki, were individually treated with acid to solubilize their SASPs and were subsequently digested with trypsin and analyzed by MS to determine which predicted species-unique or *cereus* group-unique peptides would be observed. Since it is likely that different SASP proteins are expressed at different levels in spores and that some peptides ionize more efficiently than others using MALDI, it was expected that some SASP peptides would be more prevalent than others in the spectra. The overall speed of the analysis was increased by performing the SASP solubilization and trypsin digestion directly on the MALDI plate, by ionizing the sample in the atmosphere, and, where applicable, by directly selecting peptides of interest for MS/MS verification instead of first generating a survey scan. The results of the analysis of individual *Bacilli* species are shown in Fig. 1. The observed species-specific peptides particular to the given organism and the *cereus* group-specific peptides are indicated in the spectra.

Analysis of *Bacillus anthracis* str. S-Sterne (Fig. 1(a)) shows that the species-specific peaks observed are those with m/z 1490.1 and 1528.1, corresponding to a major SASP- α/β protein (see Tables 1 and 2). The *cereus* group-specific markers that are observed are those with m/z 1139.5, 1429.8, 1488.3, 1517.9, 1594.0, 1885.3, 1940.3, 1956.3, 1971.0, 2257.8 and 2835.1. The identities of these peaks were confirmed using MS/MS analysis (data not shown). The peak at m/z 1189.1 corresponds to the SASP peptide ANGSVGGGEITKR, which is found in the SASPs of numerous *Bacilli* including *Bacillus anthracis*, *cereus*, *halodurans*, *megaterium* and *subtilis* and it was therefore not targeted for further analysis here. The species observed at m/z 2274.8 corresponds to

Table 1. Small, acid-soluble proteins (SASPs) from various *Bacillus* species

Species	SASP	Accession No.	Protein sequence
<i>B. anthracis</i>	SASP-1	gi 134222	MGKNSGRNEVLVRGAEALDQMKYEIAQEFVQLGADTTARNSGSGGEITKRLVAMAEQQLGGRANR
	SASP-2	gi 134231	MSRSTNKLA VPGAESALDQMKYEIAQEFVQLGADATARANGSVGGEITKRLVSLAEQQLGGYQK
	SASP- α / β	gi 30263073	MSNNSSGNQLVRGAEALDQMKYEIAQEFVQLGADATARANGSVGGEITKRLVSLAEQQLGGVYTR
	SASP- α / β	gi 30262016	MARNRNSQLASHGAQAALDQMKYEIAQEFVQLGADTSSRANGSVGGEITKRLVAMAEQQLGGGYTR
	SASP- α / β	gi 30261413	MVKTNKL VPGAQAELQFKYEIAQEFVSLGSNTASRNSGSGGEITKRLVALAQQLRG
	SASP- α / β	gi 21402693	MARSTNKLA VPGAESALDQMKYEIAQEFVQLGADATARANGSVGGEITKRLVSLAEQQLGGFQK
	SASP- α / β	gi 21401007	MANNNSGRNELLVRGAEALDQMKYEIAQEFVQLGADTTARNSGSGGEITKRLVAMAEQQLGGRANR
	SASP- α / β	gi 21398813	MANQNSSNL VPGAATAIDQMKYEIAQEFVQLGADSTARANGSVGGEITKRLVAMAEQSLGGFHK
	SASP- α / β	gi 21398007	MSRRRGVMSNQFKEELA KELGFYDVVQKEGWWGIRAKDAGNMVKRAIEIAEQQLMKQNQ
	SASP- γ	gi 30260683	MSKKQQGYNKATSGASIQSTNASYGTETETDVAVKQANAQSEAKKAQASGASIQSTNASYGTETETDVHA VKQNAQSAAKQSQSSSNQ
<i>B. cereus</i>	SASP- α / β	gi 42781133	MARNRNSQLASHGAQAALDQMKYEIAQEFVQLGADTSSRANGSVGGEITKRLVAMAEQQLGGGYTR
	SASP- α / β	gi 42780498	MCIIAMKTNKLLVPGAELDQFKYEIAQEFVSLGSNTASRNSGSGGEITKRLVSLAEQQLRG
	SASP-B	gi 42739759	MSRSTNKLA VPGAESALDQMKYEIAQEFVQLGADATARANGSVGGEITKRLVSLAEQQLGGFQK
	SASP-B	gi 80084	MSKKQQGYNKATSGASIQSTNASYGTETETDVAVKQANAQSEAKKAQASGASIQSTNASYGTETETDVHVS KKQNAKSAAKQSQSSSNQ
	SASP- γ	gi 42779659	MSKKQQGYNKATSGASIQSTNASYGTETETDVAVKQANAQSEAKKAQASGASIQSTNASYGTETETDVHAV KKQNAQSAAKQSQSSSNQ
	SASP- γ	gi 30018712	MIVEVRKMSKKQQGYNKATSGASIQSTNASYGTETETDVAVKQANAQSEAKKAQASGASIQSTNASYGTETEFATE TDVHAVKKQNAKSAAKQSQSSSNQ
<i>B. globigii</i>	SASP-1	— ^a	MPNQSGSNSSNQLL VPGAQAIDQMKFEIASEFVNLGAFTTSRANGSVGGEITKRLVSAFAQQQMGGGVQ
	SASP-2	— ^a	MAQNSQNGNSSNQLL VPGAQAIDQMKFEIASEFVNLGAFTTSRANGSVGGEITKRLVSAFAQQNMSGQQF
	SASP- γ	— ^a	MANSNKNTNAQQVQRKQNSASGQQGFTETAFSETNVQVQRKQNSAAGQQGFTETAFSETDAQVRRQQ NQSAEQNKQNS
<i>B. halodurans</i>	SASP- α	gi 25299033	MANNNSQLL VPGVQALDQMKTEIAQEFVQLGADTTSRANGSVGGEITKRLVAMAEQQLGGGFQK
	SASP- α / β	gi 15615764	MARSNSNL VPGVQALDQMKTEIAQEFVQLGADTTSRANGSVGGEITKRLVAMAEQQLGGFQQQ
	SASP- α / β	gi 15613701	MARSNKL VPGVEQALNEMKYEIAQEFVRLGSDTTSRANGSVGGEITKRLVQQSEREF
	SASP- α / β	gi 15612623	MSRRRIMSRLKEEIAKELGFYDVVQKEGWWGIRARDAGNMVKRAIEIAEQQLAERESSR
	SASP- γ	gi 15613502	MNNKRRQQPQASKTNAQEVKRNQASEQNAQFATEFASSETNAQEVKRNQQAQAKKQQQAQQNRQQNQ
<i>B. megaterium</i>	SASP-A	gi 134242	MANTNKL VPGSAADQMKYEIASEFVNLGPEATARANGSVGGEITKRLVQMAEQQLGGK
	SASP- γ	gi 134245	MAKQTKTASGTSTQHVKKQ
			NAQASKNNFGTEFCSETNVQEVKQNAQAAANKSQNAQASKNNFGTEFASQEVRRQNAQAQAKKNQNSGKYQQ

<i>B. mycoides</i>	SASP- γ	gi 29 378 307	MNKKQQYNKATSGASIQSTNASYGTEFATETNVQAVQKQNAQSEAKKAQASGAQSANASYGTEFATETDVQAVKKQ NAQSAANKSQSSSSNQ
	SASP- γ	gi 29 378 305	MSKKQQYNKATSGASIQSTNASYGTEFATETNVQA VKQANAQSEAQAQAASAAQSANASYGTEFATETDVHAV KKQNAQSAATQSQSSSSNE
	SASP- γ	gi 29 378 301	MSKKQQYNKATSGASIQSTNASYGTEFATETNVQA VKQANAQSEAQAQAASAAQSANASYGTEFATETDVHAV KKQNAQSAKQSQSSSSNE
	SASP- γ	gi 29 378 297	MSKKQQYNKATSGASIQSTNASYGTEFATETNVQA VKQANAQSEAKKAQTSGAQSANASYGTEFATETD VHAVKKQNAKSAKQSQSSSSNE
<i>B. stearother- mophilus</i>	SASP-1	gi 80 225	MPNQSGSSSNQLLVPGAQVIDQMKFEIASEFGVNLGAETTSRANGSVGGEITKRLVSFAQQQMGGGVQ
	SASP- γ	gi 134 246	MANSNKNTNAQQVRKQNQOSAAGQGFTEFASSETNVQQVRKQNQOSAAGQGFTEFASSETDAQQVRQONQ SAEQNKQONS
<i>B. subtilis</i>	SASP- α / β	gi 16 080 009	MANNSGNSNLLVPGAQAIDQMKLEIASEFGVNLGADTTSRANGSVGGEITKRLVSFAQQNMGGGQF
	SASP- α / β	gi 16 079 053	MAQQSRSRNNDLIPQAASAIQMKLEIASEFGVNLGADTTSRANGSVGGEITKRLVRLAQQNMGGGQFH
	SASP- α / β	gi 16 078 411	MASRNKLVVPGVEQALDQFKLEV AQEFGVNLGSDTVARANGSVGGEITKRLVQQAQSQNLNGTTK
	SASP- α / β	gi 16 077 113	MGRRRGVMDEFKYEAKDLGFYDTVKNCGWGEIRARDAGNMVKRAIEAEQQMAQNQNNR
	SASP- β	gi 16 078 040	MANQNSSNDLLVPGAQAIDQMKLEIASEFGVNLGADTTSRANGSVGGEITKRLVSFAQQQMGGRVQ
	SASP- γ	gi 134 247	MANSNFSKNTNAQQVRKQNQOSAAGQGFTEFASSETNAQQVRKQNQOSAAGQGFTEFASSETDAQQVRQONQSAE QNKQONS
	SASP- γ	gi 13 676 638	MANSNFSKNTNAQQVRKQNQOSAAGQGFTEFASSETNAQQVRKQNQOSAAGQGFTEFASSETDAQQVRQONQSAE QNKQONS
<i>B. thuringiensis</i> Kurskati	SASP- γ	gi 13 676 636	MANSNFSKNTNAQQVRKQNQOSAAGQGFTEFASSETNAQQVRKQNQOSAAGQGFTEFASSETDVQQVRQONQSAE QNKQONS
	SASP- γ	gi 42 781 133	MSKKQQYNKATSGASIQSTNASYGTEFATETNVQA VKQANAQSEAKKAQASGAQSANASYGTEFATETDVHAVKQKQ AKSAKQSQSSSSNE
	SASP- γ	gi 42 780 498	MSKKQQYNKATSGASIQSTNASYGTEFSTETDVQAVKQANAQSEAKKAQASGAQSANASYGTEFATETDVHVSVKKQNAKS AAKQSQSSSSNQ

^a See Ref. 25.

Table 2. Species-unique tryptic SASP markers

Species	SASP	Accession No.	Sequence	Span	[M + H] _{calc} ^a
<i>B. anthracis</i>	SASP- α/β	gi 21 401 007	ANNNSGSR ^b	2–9	819.8
	SASP- α/β	gi 30 263 073	SNNNSGSSNQLLVR ^b	2–15	1490.6
	SASP- α/β	gi 30 263 073	LVSLAEQQLGGGVTR	56–70	1528.7
<i>B. cereus</i>	SASP- α/β	gi 42 780 498	CIIMAK ^b	2–7	677.9
	SASP- α/β	gi 42 780 498	LVSLAQQQLR	55–65	1156.4
	SASP- α/β	gi 42 780 498	LLVPGAEQALDQFK	11–24	1529.8
<i>B. globigii</i>	SASP-2	— ^c	LVSFAQQNMMSGQF	58–71	1585.8
	SASP-1	— ^c	PNQSGSNSSNQLLVPGAAQAIQMK ^b	2–26	2556.8
	SASP-2	— ^c	AQNSQNGNSSNQLLVPGAAQAIQMK ^b	2–27	2685.9
<i>B. halodurans</i>	SASP- γ	gi 15 613 502	TNAQEVK	14–20	789.9
	SASP- α/β	gi 15 613 701	LGSDDTTSR	32–39	836.9
	SASP- α/β	gi 15 613 701	LVQQSER	52–58	859.0
	SASP- γ	gi 15 613 502	QQQPQASK	6–13	914.0
	SASP- γ	gi 15 613 502	QQQAQQNR	57–64	1001.0
	SASP- γ	gi 15 613 502	QQNQQAQAEAK	47–55	1045.1
	SASP- α/β	gi 15 613 701	YEIAQEFVGR	22–31	1212.3
	SASP- α/β	gi 15 612 623	AIELAEQQLAER	46–57	1371.5
	SASP- α/β	gi 15 613 701	LLVPGVEQALNEMK	8–21	1541.8
	SASP- α	gi 25 299 033	LVAMAEQQLGGGFQK	53–67	1577.8
	SASP- α/β	gi 15 615 764	LVAMAEQQMGGFQQQ	54–68	1666.9
	SASP- α	gi 25 299 033	TEIAQEFVQLGADTTSR	23–40	1924.1
	SASP- α/β	gi 15 612 623	ELGFYDTVQEGWGGIR	19–35	1956.1
	SASP- α/β	gi 15 615 764	SNNNSQLVVPGVQQALDQMK	4–23	2171.4
	SASP- α	gi 25 299 033	ANNNSNQLLVPGVQQALDQMK ^b	2–22	2282.2
	SASP- γ	gi 15 613 502	QNQASEQNAGFATEFASSETNAQEVK	22–46	2700.8
	<i>B. megaterium</i>	SASP- γ	gi 134 245	NQNSGK	89–94
SASP- γ		gi 134 245	SQNAQASK	54–61	833.9
SASP- γ		gi 134 245	QQNAQASK	19–26	874.9
SASP- γ		gi 134 245	QQNAQAQAK	79–87	987.1
SASP- γ		gi 134 245	QQNAQAAANK	44–53	1044.1
SASP- γ		gi 134 245	TASGTSTQHVK	8–18	1117.2
SASP-A		gi 134 242	LVQMAEQQLGGK	51–62	1302.5
SASP-A		gi 134 242	LVAPGSAAAIDQMK	7–20	1372.6
SASP- γ		gi 134 245	NNFGTEFASSETSAQEVK	62–78	1887.9
SASP- γ		gi 134 245	NNFGTEFGSETNVQEVK	27–43	1901.0
SASP-A		gi 134 242	YEIASEFGVNLGPEATAR	21–38	1925.1
<i>B. mycoides</i>	SASP- γ	gi 29 378 307	SQSSSSNQ	85–92	824.8
	SASP- γ	gi 29 378 307	QNAQSEAK	39–46	875.9
	SASP- γ	gi 29 378 307	QNAQSAANK	76–84	932.0
	SASP- γ	gi 29 378 305	QNAQSAATQSQSSSSNE	77–93	1724.7
	SASP- γ	gi 29 378 307	AQASGAQSANASYGTEFATETDVQAVK	48–74	2702.3
	SASP- γ	gi 29 378 305	AQASAAQSANASYGTEFATETDVHAVK	49–75	2725.3
	SASP- γ	gi 29 378 297	AQTSGAQSANASYGTEFATETDVHAVK	49–75	2741.3
	SASP- γ	gi 29 378 307	ATSGASIQSTNASYGTEFATETNVQAVQK	10–38	2961.4
<i>B. stearothermophilus</i>	SASP-1	gi 80 225	PNQSGSNSSNQLLVPGAAQVIDQMK ^b	2–26	2584.9
<i>B. subtilis</i>	SASP- γ	gi 134 247	ANSNNFSK ^b	2–9	880.9
	SASP- α/β	gi 16 077 113	NGGWGEIR	28–35	889.0
	SASP- α/β	gi 16 077 113	GVMSEDFK	6–13	913.0
	SASP- α/β	gi 16 078 411	ANGSVGGEMTK	39–49	1051.2
	SASP- α/β	gi 16 077 113	DLGFYDTVK	19–27	1058.2
	SASP- α/β	gi 16 079 053	LAQQNMGGQFH	62–72	1231.4
	SASP- β	gi 16 078 040	LVSFAQQQMGGGR	54–65	1322.5
	SASP- α/β	gi 16 080 009	LVSFAQQNMGGGQF	56–69	1484.7
	SASP- α/β	gi 16 078 411	LVQQAQSQLNGTTK	51–64	1516.7
	SASP- α/β	gi 16 077 113	AIEIAEQQMAQNQNNR	46–61	1859.0
	SASP- α/β	gi 16 080 009	LEIASEFGVNLGADTTSR	26–43	1881.0

Table 2. (Continued)

Species	SASP	Accession No.	Sequence	Span	[M + H] _{calc} ^a
<i>B. subtilis</i>	SASP- α/β	gi 16 078 411	LEVAQEFVNLGSDTVAR	21–38	1906.1
	SASP- α/β	gi 16 079 053	LEIASEFGVQLGAETTSR	29–46	1909.1
	SASP- β	gi 16 078 040	ANQNSSNDLLVPGAAQAIDQMK ^b	2–23	2286.5
	SASP- α/β	gi 16 080 009	ANNNSGNSNLLVPGAAQAIDQMK ^b	2–25	2442.7
	SASP- α/β	gi 16 079 053	SNNNDLLIPQAASAIEQMK	9–28	2172.4
	SASP- γ	gi 134 247	QNQQSAAGQQGQFGTEFASETNAQQVR	18–43	2783.9
	SASP- γ	gi 134 247	QNQQSAGQQGQFGTEFASETDAQQVR	45–70	2841.9
	SASP- γ	gi 13 676 636	QNQQSAAGQQGQFGTEFASETDAQQVR	18–44	2913.0
	SASP- γ	gi 13 676 636	QNQQSAAGQQGQFGTEFASETQVQVR	45–71	2941.0
<i>B. thuringiensis</i>			None based on available information.		
Kurstaki					

^a Average masses are calculated for peptide ions.^b Following post-translational removal of the N-terminal methionine.^c See Ref. 25.**Table 3.** *Cereus*-group tryptic SASP markers assigned from Table 1

Peptide Sequence	[M + H] _{calc} ^a	<i>B. anthracis</i> ^b	<i>B. cereus</i> ^b	<i>B. mycoides</i> ^b	<i>B. thuringiensis</i> ^b
NNSGSR	634.6	X	X	—	—
NEVLVR	729.8	X	X	—	—
QQGYNK	737.8	X	X	X	X
NELLVR	743.9	X	X	—	—
EGWGGIR	774.8	X	X	—	—
QNAQSAAK	817.9	X	X	X	—
GVMNQFK	911.1	X	X	—	—
QANAQSEAK	947.0	X	X	X	X
QSQSSSNQ	952.9	X	X	—	X
QSQSSSNE	953.9	—	—	X	X
SNGSVGGEVTK	1035.1	X	X	—	—
SNGSVGGEITK	1049.1	X	X	—	—
GAEQALDQMK	1091.2	X	X	—	—
LVALAQQLR	1140.4	X	X	—	—
ELGFYDVVQK	1198.4	X	X	—	—
LVAMAEQQLGGR	1273.5	X	X	—	—
AIEIAEQQLMK	1274.5	X	X	—	—
LAVPGAESALDQMK	1430.7	X	X	—	Y
LVAMAEQSLGGFHK	1488.7	X	X	—	Y
LVSLAEQQLGGFQK	1518.7	X	X	—	—
LVSLAEQQLGGYQK	1534.7	X	X	—	Y
LVAMAEQQLGGGYTR	1594.8	X	X	—	Y
NSNQLASHGAQAALDQMK	1885.1	X	X	—	—
YEIAQEFVSLGSNTASR	1930.1	X	X	—	—
YEIAQEFVQLGADATAR	1940.1	X	X	—	Y
YEIAQEFVQLGADSTAR	1956.1	X	X	—	Y
YEIAQEFVQLGADTTAR	1970.1	X	X	—	—
YEIAQEFVQLGADSTSR	1972.1	X	X	—	Y
ANQNSSNQLVVPGATAAIDQMK ^c	2258.5	X	X	—	Y
AQASGAQSANASYGTEFATETDVHAVK	2712.8	—	X	—	X
AQASGAQSANASYGTEFATETDVHVK	2728.8	—	X	—	X
ATSGASIQTNASYGTEFATETNVQAVK	2835.0	X	X	X	X
ATSGASIQTNASYGTEFSTETDVQAVK	2852.0	—	X	—	X
AQASGASIQTNASYGTEFATETDVHAVK	2942.1	X	—	—	X

^a Average masses are calculated for peptide ions.^b X, Peptides determined from database searches in this study; Y, peptides observed in previous studies.^{15,16}^c Following post-translational removal of the N-terminal methionine.

Table 4. Database search results confirming identities of SASP peptides from MS/MS data

Sequence	[M + H] _{calc} ^a	Peptide type ^b	Species ^c	Score ^d
LVSFAQQQMGR	1322.5	S-s	Bs	38
LAVPGAESALDQMK	1430.7	CG-s	Ba, Bc, Bt	52
LVSFAQQNMGGGQF	1484.7	S-s	Bs	58
LVAMAEQSLGGFHK	1488.7	CG-s	Ba, Bc, Bt	71
SNNNSGSSNQLLVR ^e	1490.6	S-s	Ba	40
LVSLAEQQLGGFQK	1518.7	CG-s	Ba, Bc	74
LVSLAEQQLGGGVTR	1528.7	S-s	Ba	37
LLVPGAEQALDQFK	1529.8	S-s	Bc	36
LVSLAEQQLGGYQK	1534.7	CG-s	Ba, Bc, Bt	68
LEIASEFGVNLGADTTSR	1881.0	S-s	Bs	95
LEVAQEFVNLGSDTVAR	1906.1	S-s	Bs	24
YEIAQEFVQLGADATAR	1940.1	CG-s	Ba, Bc, Bt	88
YEIAQEFVQLGADSTAR	1956.1	CG-s	Ba, Bc, Bt	81
YEIAQEFVQLGADTSR	1972.1	CG-s	Ba, Bc, Bt	22
ANQNSSNQLVVPGATAIDQMK ^e	2258.5	CG-s	Ba, Bc, Bt	72
ANNNSGNSNLLVPGAAQIDQMK ^e	2442.7	S-s	Bs	28
QNQQSAAGQGFTEFASSETNAQQVR	2783.9	S-s	Bs	50
QNQQSAAGQGFTEFASSETDAQQVR	2913.0	S-s	Bs	90

^a Average masses are calculated for peptide ions.

^b S-s, species-specific peptides; CG-s, *cereus* group-specific peptides.

^c Ba, *B. anthracis*; Bc, *B. cereus*; Bs, *B. subtilis*; Bt, *B. thuringiensis*.

^d Scores obtained from MASCOT searches; search parameters detailed in Experimental section. Scores >35 indicate matches with >95% significance.

^e Following post-translational removal of the N-terminal methionine.

a peptide fragment resulting from trypsin autolysis. The use of immobilized trypsin for protein digestion is designed to minimize the occurrence of such peaks;¹⁶ nevertheless, this peak was occasionally observed.

The analysis of *Bacillus cereus* str. T (Fig. 1(b)) shows that the species-unique peptides observed were those with m/z 1156.1 and 1529.8, corresponding to a major SASP- α/β protein (Tables 1 and 2). In addition to these peaks, several *cereus* group-specific peaks were observed, with m/z 1139.5, 1430.4, 1487.9, 1534.4, 1594.9, 1884.9, 1939.3, 1956.1, 1971.6, 2258.4, 2712.1 and 2851.6. Most of these *cereus* group-specific peaks were also observed in the spectrum of *Bacillus anthracis* str. S-Sterne. One exception is the SASP peptide peak at m/z 2712.1; this peak is predicted to be present in the spectra of *Bacillus thuringiensis*, but not *Bacillus anthracis* (Table 3).

With *Bacillus globigii* (Fig. 1(c)), all three of the expected species-specific peptides were observed, at m/z 1584.9, 2556.9, and 2684.9 (corresponding to the proteins SASP-1 and SASP-2), whereas with *Bacillus subtilis* str. 168 (Fig. 1(d)), six specific peptides were observed, at m/z 1322.4, 1483.8, 1880.6, 2442.5, 2783.9 and 2840.9 (corresponding to one SASP- β protein, two SASP- α/β proteins and one SASP- γ protein (Tables 1 and 2)).

Examination of the spectrum obtained for *Bacillus thuringiensis* str. Kurstaki (Fig. 1(e)) revealed that several *cereus* group-specific SASP peptides were observed (m/z 1272.8, 1429.6, 1488.8, 1533.5, 1593.8, 1885.2, 1940.2, 1955.9, 1971.6, 2257.7, 2729.2 and 2851.8). None of these peaks were unique to *Bacillus thuringiensis*.

Analysis of *Bacillus* mixtures

Once it had been established that species-specific or *cereus* group-specific peptides could be identified and observed in the spectra of spore extracts, *Bacillus* mixtures were prepared in 1:1 and 1:10 ratios. Specifically, 1:1 mixtures of *Bacillus anthracis* str. S-Sterne and either *Bacillus cereus* str. T, *Bacillus globigii*, *Bacillus subtilis* str. 168 or *Bacillus thuringiensis* str. Kurstaki were analyzed using the same method as above.

Figure 2(a) shows that *Bacillus anthracis* str. S-Sterne could readily be distinguished from *Bacillus subtilis* str. 168 in a 1:1 mixture by the appearance of their species-specific peaks (for *Bacillus anthracis*, the peaks at m/z 1490.9 and 1528.2; for *Bacillus subtilis*, the peaks at m/z 1321.9, 1881.4, 2442.1, 2783.9, and 2841.3). In addition, several *cereus* group-specific peaks were observed in the spectrum (m/z 1518.7, 1595.9, 1939.9, 1955.8, 1971.6, 2258.3). The identities of these peaks were confirmed by MS/MS analysis; Figure 2(b) shows the tandem mass spectrum and interpretation for the *Bacillus anthracis* peak with m/z 1528.1, and Fig. 2(c) shows the tandem mass spectrum and interpretation for the *cereus* group-specific peak with m/z 1940.6. MS/MS data for the *Bacillus subtilis*-specific peak with m/z 1881.3 are shown in Fig. 2(d). Using this strategy, species could be identified in all other 1:1 mixtures that were tested (data not shown). Where possible, the tandem mass spectra were evaluated using MASCOT database searching. The results of these searches are shown in Table 4.

Next, it was of interest to evaluate whether the present strategy could be applied to mixtures with more extreme component ratios. Mixtures with 1:1 and 1:10 ratios of

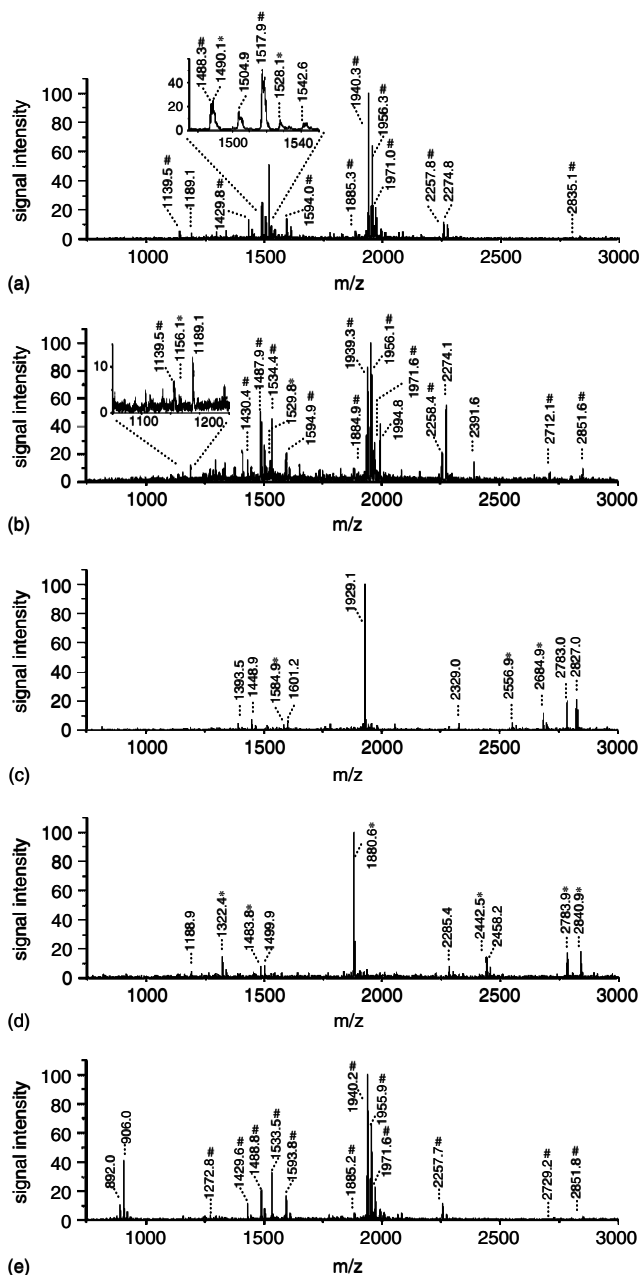


Figure 1. Partial mass spectra of individual *Bacillus* spore samples treated with acid and digested with immobilized trypsin *in situ*. (a) *Bacillus anthracis* str. S-Sterne; (b) *Bacillus cereus* str. T; (c) *Bacillus globigii*; (d) *Bacillus subtilis* str. 168; (e) *Bacillus thuringiensis* str. Kurstaki. Asterisks indicate species-specific SASP peptides and *ceres* group-specific peptides are indicated by the symbol #.

Bacillus anthracis str. S-Sterne and the closely related *Bacillus cereus* str. T were prepared and analyzed as above. The survey scans for the 1:1 and 1:10 mixtures are shown in Figs. 3(a) and (b), respectively. Whereas species-specific peaks from both *Bacillus anthracis* and *ceres* are observed in the 1:1 mixture, in the 1:10 mixture the only species-specific peak observed is that from *Bacillus cereus* with m/z 1529.6. The tandem mass spectrum and interpretation for this peak are shown in Fig. 3(c). Several *ceres* group-specific peptides are also evident in the spectrum in Fig. 3(a), with

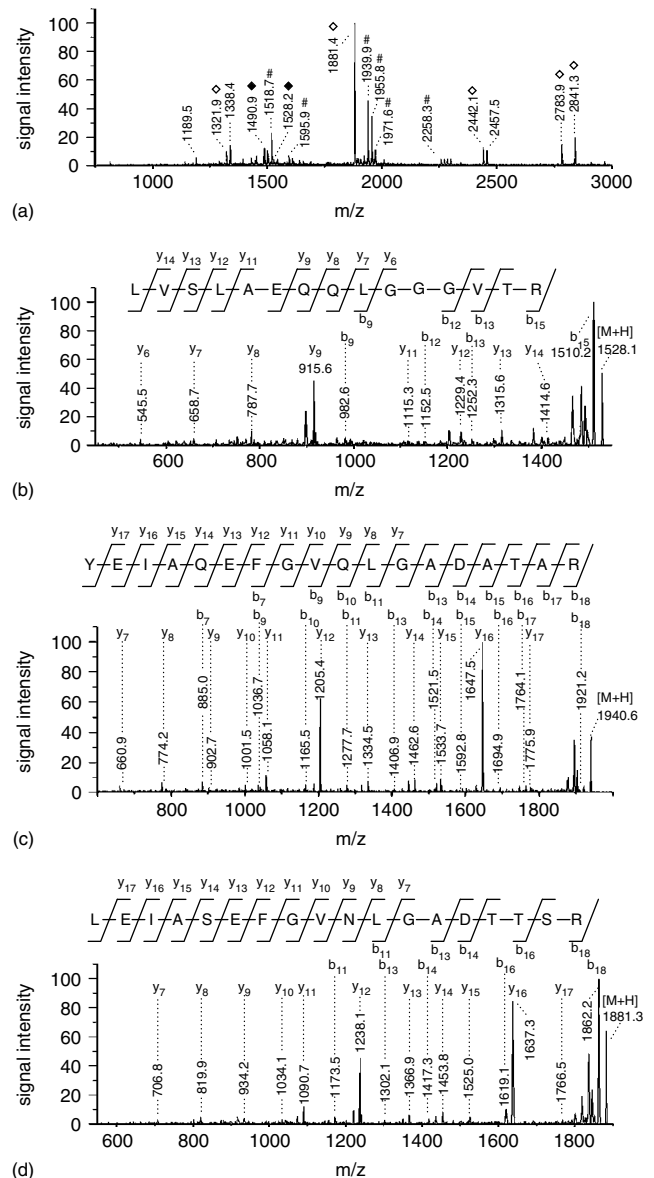


Figure 2. Partial mass spectra from a 1:1 mixture of *Bacillus anthracis* str. S-Sterne and *Bacillus subtilis* str. 168 treated with acid and digested with immobilized trypsin *in situ*. (a) Survey scan of the mixture. SASP peptides specific for *Bacillus anthracis* and *Bacillus subtilis* are indicated by closed and open diamonds, respectively. Peptides specific to the *ceres* group are indicated by the symbol #. (b) Tandem mass spectrum and interpretation of the *Bacillus anthracis*-specific peptide with m/z 1528.1 (c) Tandem mass spectrum and interpretation of the *ceres* group-specific peptide with m/z 1940.6. (d) Tandem mass spectrum and interpretation of the *Bacillus subtilis*-specific peptide with m/z 1881.3.

m/z 1429.9, 1488.8, 1518.5, 1534.9, 1594.9, 1939.6, 1955.9, 1972.3, 2257.9, 2712.2 and 2851.6. Although most of these could have originated from either species, the peaks with m/z 2712.2 and 2851.6 were not predicted to be present in *Bacillus anthracis* (see Table 3) and, therefore, must have originated from *Bacillus cereus*.

To verify the presence of *Bacillus anthracis* in the 1:10 mixture, MS/MS analysis was attempted directly on one

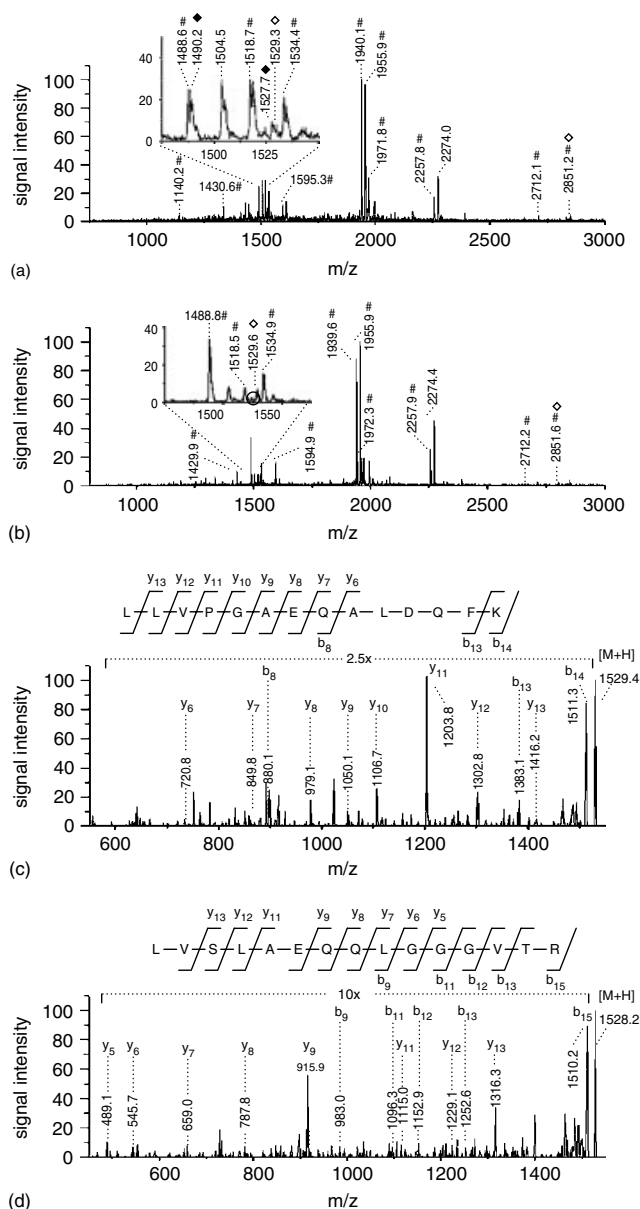


Figure 3. MS analysis of 1 : 1 and 1 : 10 mixtures of *Bacillus anthracis* str. S-Sterne and *Bacillus cereus* str. T treated with acid and digested with immobilized trypsin *in situ*. (a) Survey scan of the 1 : 1 mixture. (b) Survey scan of the 1 : 10 mixture. SASP peptides specific for *Bacillus anthracis* and *Bacillus cereus* are indicated by closed and open diamonds, respectively. Peptides specific to the *cereus* group are indicated by the symbol #. (c) Tandem mass spectrum and interpretation of the *Bacillus cereus*-specific peptide with *m/z* 1529.4. (d) Tandem mass spectrum and interpretation of the *Bacillus anthracis*-specific peptide with *m/z* 1528.2. The circle in the inset in (b) indicates the position where the *Bacillus anthracis* peptide with *m/z* 1528.2 would be present in the survey scan.

of its unique peptides. Despite the fact that the precursor ion at *m/z* 1528.2 is not observed in the survey scan as plotted (Fig. 3(b)), the region highlighted by the circle in the inset, targeted precursor selection for MS/MS analysis and subsequent interpretation of the spectrum revealed its presence (Fig. 3(d)). This result, in addition to demonstrating

the successful identification of *Bacilli* in this type of mixture, also demonstrates the advantage of unit mass resolution for precursor ion selection (i.e. *m/z* 1528.2 in Fig. 3(b) and *m/z* 1529.4 in Fig. 3(c)).

CONCLUSIONS

In this work we have developed and evaluated a strategy for the rapid identification of *Bacillus* spores, individually and in mixtures, by targeting SASP peptides that are specific to each organism. A list of species-specific peptides was constructed. In the case of members of the *cereus* group of bacteria, group-specific peptides were also identified that could augment the analysis, particularly for *Bacillus thuringiensis* and *Bacillus mycoides*, whose genomes (and SASPs) have not been fully characterized. The results were obtained with an ion trap mass spectrometer, which allows for facile unit mass selection of precursor ions for MS/MS analysis and atmospheric advantages. The strategy was shown to be successful in the identification of *Bacillus* spores in various mixtures. This approach can potentially be applied to other microorganisms in mixtures and to contaminated samples.

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