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Use of Fatty Acid Methyl Ester Profiles for Discrimination of *Bacillus cereus* T-Strain Spores Grown on Different Media[▽]

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The goal of this study was to determine if cellular fatty acid methyl ester (FAME) profiling could be used to distinguish among spore samples from a single species (*Bacillus cereus* T strain) that were prepared on 10 different medium formulations. To analyze profile differences and identify FAME biomarkers diagnostic for the chemical constituents in each sporulation medium, a variety of statistical techniques were used, including nonmetric multidimensional scaling (nMDS), analysis of similarities (ANOSIM), and discriminant function analysis (DFA). The results showed that one FAME biomarker, oleic acid (18:1 ω9c), was exclusively associated with spores grown on Columbia agar supplemented with sheep blood and was indicative of blood supplements that were present in the sporulation medium. For spores grown in other formulations, multivariate comparisons across several FAME biomarkers were required to discern profile differences. Clustering patterns in nMDS plots and *R* values from ANOSIM revealed that dissimilarities among FAME profiles were most pronounced when spores grown with disparate sources of complex additives or protein supplements were compared ($R > 0.8$), although other factors also contributed to FAME differences. DFA indicated that differentiation could be maximized with a targeted subset of FAME variables, and the relative contributions of branched FAME biomarkers to group dissimilarities changed when different media were compared. When taken together, these analyses indicate that *B. cereus* spore samples grown in different media can be resolved with FAME profiling and that this may be a useful technique for providing intelligence about the production methods of *Bacillus* organisms in a forensic investigation.

In September 2001, letters containing spores of *Bacillus anthracis*, the causative agent for anthrax, were mailed to television and print media outlets, as well as two U.S. congressional offices, in an act of bioterrorism. Genetic tests identified a single strain of *B. anthracis*, Ames, in all evidence samples. Since then, considerable effort has gone into developing techniques that can be used to analyze microbiological evidence recovered from a crime scene. Because the Ames strain used in the 2001 attacks was difficult to distinguish genetically from several commonly used Ames strains (20), many recently developed techniques have concentrated on nongenetic signatures associated with the cell that are unique to the methods that were used to culture an organism. Examples include assays that detect the presence of residual agar on spores (59), C/N isotope ranges for different medium components (22, 30, 31), and detection of heme in spores grown on blood-containing media (56). Phenotypic signatures such as these that indicate specific metabolic substrates, characteristic compounds, or defining features of an organism's production process could help in the attribution of a biocrime by providing leads or excluding suspects during an investigation (40, 59).

One phenotypic system that has not been fully tested in a forensic context is fatty acid methyl ester (FAME) analysis. FAMEs have long been recognized as useful biochemical markers for bacterial classification and characterization (38, 53, 54). The types and relative abundances of fatty acids produced within a cell are largely determined by an organism's genotype and can be used for identification of different species (50) and strains (34, 51) and for discriminating between free spores and vegetative cells (43, 47). Commercial systems that streamline fatty acid extraction and detection procedures (42) have facilitated the widespread use of fatty acid profiling to identify bacteria in clinical, agricultural, and biodefense settings (29, 53, 55).

Besides aiding in the identification of bacterial species, FAME profiling can potentially provide information on the methods used to grow microorganisms of forensic interest. Within the *Bacillus* group, the amino acid content or the type of complex additives used in the cultivation media can significantly affect the fatty acid composition of bacterial cultures. The relative proportions of branched fatty acids (iso-odd, iso-even, and anteiso), which are prevalent in *Bacillus* spp. (33, 36a), are heavily dependent on the ratio of amino acid precursors (leucine, valine, and isoleucine) and the corresponding α-keto acids present in the growth media (12, 27, 28, 32). Accordingly, the complex additives and protein sources that supply these amino acid precursors in growth media also affect the fatty acid compositions of *Bacillus* cultures. For example, it

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TABLE 1. Sporulation medium key

Sporulation medium	Abbreviation	Reference	Component(s)[a]
Chemically defined sporulation medium[b]	CDSM	16	Ammonium sulfate
G broth	G	6, 18	Yeast extract, ammonium sulfate
Modified G broth	MG	2	Yeast extract, ammonium sulfate (no supplemental sugar source)
Casein acid digest broth	CAD		Yeast extract, tryptone
Brain heart infusion agar	BHI		Meat peptic digest, brain heart solids, gelatin digest
Schaeffer's broth	Sch	34	Beef extract, meat peptone
Schaeffer's agar	SchAg	34	Beef extract, meat peptone
Lab Lemco agar	LL	9	Meat peptone, Lab Lemco powder
New sporulation medium agar	NSM	9	Yeast extract, tryptone, meat peptone, Lab Lemco powder
Columbia agar	CA		Yeast extract, beef heart digest, corn starch, tryptone, meat peptone
Columbia agar supplemented with sheep blood	CAB		Yeast extract, beef heart digest, corn starch, tryptone, meat peptone, defibrinated sheep blood

[a] Only complex carbon and nitrogen sources are listed for each medium. Trace metal and salt components are listed in Materials and Methods.
[b] Used in broth form.

has been reported that inclusion of components such as yeast extract, beef extract, or casein hydrolysate in growth medium formulations can change the relative ratios of iso and anteiso fatty acids in *Bacillus cereus* cultures (24, 25). Brain heart infusion (BHI) has been observed to have a similar effect on the relative proportions of branched fatty acids in *Bacillus caldolyticus* (52). Despite the clear relationship between the fatty acid compositions of vegetative cells and the formulations of growth media, no study has tested whether this could be exploited for investigative purposes by determining whether diagnostic FAME signatures for growth media exist within spores of a forensically relevant organism.

To test whether FAME signatures can be used to infer the compositional characteristics of the sporulation medium, we examined fatty acid profiles among *Bacillus cereus* T-strain (*Bc*T) spores grown on 10 different media, spanning nutrient formulations that varied primarily in the source of protein, either in the form of complex additives (yeast extract, beef extracts, brain-heart solids, etc.) or direct protein supplements (peptone, tryptone, or gelatin digest). Formulation pairs that differed in other medium attributes, such as the presence of supplemental sugars, the physical state (agar versus broth), or blood supplements, were included to compare the resulting FAME profile differences with the variation that derives from complex additive/protein components.

The effects that protein components in the sporulation medium have on FAME profiles were specifically targeted in the experimental design because of the direct biosynthetic relationship between amino acids and the three structure classes of branched fatty acids in *Bacillus* (24, 27). In addition, there are a limited number of common or commercially available complex additive and protein sources used for microbiological media. Identifying forensic signatures based on a reduced number of defining components rather than the myriad of possible medium formulations makes comprehensive surveys feasible, an added advantage for any potential forensic marker.

To further frame our study in the context of forensic or investigative applications, we chose *B. cereus* as a target organism because of its genetic, structural, and biochemical similarities to *B. anthracis* (6, 19, 26). Also, since evidence from the 2001 anthrax mailings was predominantly composed of *Bacillus* spores (3), we used spore preparations of *B. cereus* for all FAME analyses.

Lastly, analysis of forensic signatures from fatty acid profiles is complicated by the large number of variables (typically >15) and complex interactions among different fatty acid structures during cellular biosynthesis (28). Therefore, FAME profiles were analyzed with orthogonal multivariate statistical techniques that first considered all variables simultaneously and analyzed the overall dissimilarities among spore FAME profiles and, second, maximized differentiation among groups using a subset of variables and extracted patterns in fatty acid differences that are diagnostic for specific medium formulations.

MATERIALS AND METHODS

Spore preparation. Cultures of *Bc*T were maintained at 30°C on tryptose beef extract agar (TBA) (10 g Bacto tryptose [Becton Dickinson DF0124-17], 5g NaCl, 3 g Bacto beef extract [Becton Dickinson DF0115-17], 16 g agar [Becton Dickinson DF0140-01]). Starter cultures were grown by inoculating single colonies of *Bc*T into 125 ml of tryptic soy broth (30 g Trypticase soy broth [Becton Dickinson 211771], 15 g agar [Becton Dickinson 211849]) and incubated for 16 to 18 h at 30°C and 300 rpm. The following sporulation media were used: a modified chemically defined sporulation medium (CDSM) identical to a previous formulation (17) but omitting 50 μg/ml isoleucine-tryptophan, G medium (7, 18), modified G medium (MG) (identical to G medium but excluding glucose [1]), CAD medium (8.5 g tryptone, 5.0 g yeast extract, 5.0 g dibasic potassium phosphate, 0.75 g dextrose, 0.025 g thiamine-HCl, 25 g $CaCl_2 \cdot 2H_2O$, 5 g $FeSO_4 \cdot 7H_2O$, 25 g $MgSO_4 \cdot 7H_2O$, 15 g $MnSO_4 \cdot H_2O$), brain heart infusion agar (BHI; Becton Dickinson 211059), Schaeffer's sporulation medium in both liquid and agar form (Sch and SchAg) (44), LL agar (23 g Lab Lemco agar [Fisher Scientific OXCM00115B]), NSM agar (10), Columbia agar (CA) (Becton Dickinson 279240), and Columbia agar with blood (CAB) (22 g Columbia agar base, 2.5% defibrinated sheep blood [Fisher Scientific 212390]). All weights are per liter of nanopure water (18 MΩ). A summary of the sporulation media used in this study is given in Table 1.

For spores grown in broth-based media, 20 ml of starter culture was inoculated into 250 ml of sporulation medium and incubated at 30°C and 300 rpm in an orbital shaker (VWR, PA). The cultures were monitored throughout the incubation period and harvested when the proportion of spores in the cell population reached ~90%. Incubation times varied between medium formulations but ranged between 24 and 48 h. For spores grown on solid media, a loopful of *Bc*T colonies from TBA plates was mixed in 10 ml of nanopure H_2O. From this solution, 100 μl was spread on 16 agar plates for each medium type and incubated for 72 to 120 h, depending upon the medium. The sporulation yield was monitored as before and harvested when the yield reached ~90%.

Spores grown in liquid media were harvested by centrifugation at 6,000 × *g* for 15 min. For spores grown on agar plates, colonies were scraped off with an

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TABLE 2. Fatty acid variables

Branched-odd[a]	Branched-even[a]	Anteiso	Normal
13:0 iso	14:0 iso	13:0 anteiso	14:0
15:0 iso	16:0 iso	15:0 anteiso	15:1 ω5c
17:0 iso		17:1 anteiso A	16:0
17:0 iso ω5c		17:0 anteiso	16:1 ω7c alcohol
17:1 iso ω10c			16:1 ω7c/16:1 ω6c
			18:1 ω9c

[a] "Odd" and "even" refer to the number of carbons in each fatty acid (from Kaneda [24]).

inoculating loop and placed in 50 ml of cold (4°C) H_2O. Once harvested, all spores were washed three times in 100 ml of sterile H_2O, including a final wash at 4°C on a rocking platform overnight. Following the overnight wash, spore preparations were usually free of any cellular debris or vegetative cells (>98% phase-bright spores). The spores were then washed two more times in 50 ml of H_2O and stored at −20°C until analysis was performed. Additional purification steps, including lysozyme treatment and density centrifugation, did not noticeably increase the spore yield. At least three replicate batches were grown for all media.

Gas chromatography and fatty acid profiling. Prior to chromatographic analysis, 6 to 10 mg of wet spore preparation was thawed and immediately distributed into separate 2-ml glass screw cap tubes (Fisher Scientific NC9174732). Samples were then vacuum dried at room temperature. The dried spore weight in each tube was typically 1 to 3 mg.

Fatty acid extraction and methyl ester generation were performed with the Instant FAME Method kit (part no. 7000 and 7020; MIDI, Inc., Newark, DE) according to the manufacturer's instructions. This protocol was chosen because it requires minimal starting material (~1 mg) and nominal processing time per sample (<15 min) and has been previously validated for other applications (49), all of which are advantageous features of a potential laboratory- or field-based forensic technique (37, 40). Briefly, the spore materials were first mixed with 250 μl of a KOH-MeOH solution. This step lysed the cells (data not shown) and replaced any polar head group on fatty acids with a methyl group, creating a FAME. The FAMEs were then partitioned into an organic phase with 200 μl of hexane. Approximately 150 μl of the hexane phase was transferred to a new screw cap vial and loaded onto an HP6890 gas chromatographic (GC) analyzer for FAME profiling. Two replicate GC samples from each spore preparation were processed and included in subsequent analyses to incorporate profile differences arising from variability between different fatty acid extractions and GC runs.

GC FAME profiling was performed with the MIDI Microbial Identification Sherlock software according to the manufacturer's instructions with one exception. In order to increase the sensitivity for the detection of fatty acids in low concentrations, the split ratio on the GC instrument was changed to 40:1 with a split flow of 50.1 ml/min. MIDI calibration standards were used (part no. 1300-AA) for identification and quantification of fatty acid peaks.

Statistical analysis. All fatty acid structures recognized by the MIDI Sherlock software that were common to every profile within a particular medium type were defined as the fatty acid variables in statistical analyses and are listed in Table 2. Values for all variables represent the relative percent contribution of individual fatty acids to the GC profile and are shown in Table 3. Mean and standard deviation values were calculated from the six replicate profiles generated for each spore group (Table 3). For certain comparisons among spore samples, fatty acid variables were grouped into four categories (columns in Table 2 labeled "branched-odd," "branched-even," "anteiso," and "normal") that correspond to the biochemical structure classes and associated biosynthetic pathways in *Bacillus* spp. previously described (24). These categorical variables were used because the relative abundance of each of these structure classes is directly affected by the concentrations of amino acid precursors (27, 28) that are likely to be specific for each growth medium.

nMDS and ANOSIM. Multivariate differences in FAME profiles for all spore samples were analyzed by nonmetric multidimensional scaling (nMDS) and analysis of similarities (ANOSIM). Both techniques were performed in Primer v6.0 (Primer-E Ltd., Plymouth, United Kingdom [2004]). The Bray-Curtis similarity measure was used to generate the distance matrix for nMDS. Kruskal fit scheme—option 1, along with 50 restarts, was used for the two-dimensional (2D) plot. Spore media groups (defined in Table 1) spanning both agar and broth forms were compared in the nMDS and ANOSIM analyses. Spores grown on the

TABLE 3. Relative abundances of fatty acid markers

Medium	Relative abundance[a]																																															
	13:0 iso		13:0 ante		14:0 iso		14:0		15:0 iso		15:0 ante		15:1 ω5c		16:1 ω7c OH		16:0 iso		SumFeat2[b]		16:0		17:1 iso ω10c		17:1 iso ω5c		17:1 ante A		17:0 iso		17:0 ante		18:1 ω9c	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CDSM	1.9	0.60	1.09	0.41	13.24	1.62	6.94	2.16	11.47	2.87	6.13	2.16	0.71	0.21	2.04	0.42	16.96	2.96	18.89	3.57	7.31	1.16	1.18	0.25	2.31	0.57	1.03	0.42	3.31	0.60	2.20	0.87	0.45	0.50
G	6.89	0.53	1.50	0.27	6.62	0.17	4.31	0.34	30.68	1.44	6.77	0.66	0.89	0.13	1.01	0.10	6.80	0.16	13.76	0.92	1.97	0.32	3.03	0.29	5.60	0.36	1.10	0.13	6.21	0.52	1.57	0.34	0.02	0.07
MG	6.15	0.52	1.27	0.13	6.09	0.37	3.86	0.20	32.24	0.43	6.22	0.65	0.58	0.25	0.76	0.08	7.27	0.50	12.22	0.79	2.46	0.51	2.64	0.15	6.19	0.37	1.08	0.09	8.26	0.90	1.69	0.23	0.00	0.00
CAD	8.56	0.26	0.88	0.05	4.81	0.16	3.46	0.37	34.13	2.09	3.92	0.31	0.84	0.03	0.41	0.08	5.28	0.54	11.54	0.96	2.07	0.32	1.54	0.20	7.67	1.17	1.14	0.11	11.27	0.52	0.83	0.07	0.04	0.08
BHI	5.82	0.30	0.54	0.06	4.40	0.38	4.46	0.17	34.85	0.88	3.36	0.34	0.04	0.10	0.46	0.04	6.67	0.45	11.76	0.67	5.78	0.87	1.78	0.33	4.81	0.68	0.64	0.20	11.04	0.49	1.68	0.29	0.59	0.32
Sch	5.65	0.42	1.01	0.05	7.56	0.71	5.59	0.44	25.03	1.75	5.63	1.18	0.50	0.33	0.95	0.13	7.16	0.61	18.85	2.33	2.96	0.17	2.42	0.21	7.16	0.35	1.83	0.87	5.42	0.48	1.54	0.28	0.00	0.00
SchAg	6.10	1.23	0.98	0.19	5.53	0.37	5.02	1.16	32.40	2.70	5.71	0.60	0.80	0.21	0.52	0.23	4.82	0.45	16.85	3.32	3.17	1.05	1.50	0.22	5.74	0.62	0.99	0.12	6.83	2.77	1.44	0.45	0.23	0.21
LL	4.45	0.53	1.05	0.18	7.73	0.36	3.72	0.35	23.68	1.65	5.54	0.23	0.23	0.27	0.20	0.22	11.29	0.83	12.15	1.04	6.11	1.08	1.11	0.07	5.74	0.24	0.91	0.11	10.62	1.32	2.90	0.37	0.07	0.16
NSM	5.69	0.88	0.81	0.06	5.35	0.89	4.76	0.30	27.84	1.80	4.29	0.69	0.64	0.07	0.19	0.21	6.74	0.55	12.74	0.36	8.17	2.09	1.15	0.08	5.30	0.39	0.92	0.06	10.57	0.55	1.89	0.22	0.05	0.13
CA	5.54	0.63	0.71	0.16	5.46	0.94	3.97	0.41	35.52	3.23	4.25	0.85	0.32	0.30	0.44	0.16	7.25	0.63	8.52	2.64	5.53	0.70	1.61	0.60	5.07	0.84	1.09	0.07	10.59	2.39	1.78	0.41	0.82	0.46
CAB	5.97	0.76	0.23	0.28	4.31	0.75	4.56	0.43	33.34	3.69	3.07	1.75	0.13	0.22	0.34	0.21	6.02	0.44	7.43	2.04	5.97	0.83	1.46	0.17	3.37	0.88	0.61	0.19	9.48	0.33	1.72	0.96	7.90	5.09

[a] Mean values are calculated from the relative abundance in each profile determined from GC.
[b] SumFeat2 represents fatty acids 16:1ω7c/16:1ω7c.

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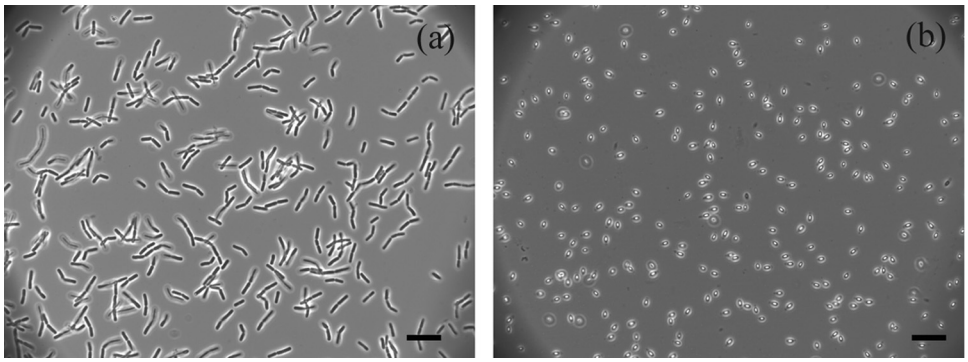

FIG. 1. Vegetative and spore forms of *Bacillus cereus* T strain. (a) Vegetative starter cultures were used to inoculate 12 different sporulation media. (b) After 24 to 48 h of incubation in sporulation media, the cell cultures were >90% phase-bright spores. Scale bar = 5 μm.

CDSM formulation were excluded, since this modified recipe is not commonly used for growing/sporulating *Bacillus* organisms.

Discriminant function analysis. Discriminant function analysis (DFA) was used to assess the relative contributions of individual fatty acid biomarkers to the multivariate patterns observed among medium groups and to isolate a subset of variables that would maximize sample group discrimination. DFA was performed in SPSS v.16.0 (SPSS Inc., Chicago, IL [2008]). In contrast to nonparametric techniques, such as nMDS or ANOSIM, the effectiveness of DFA can be influenced by the characteristics of the input variables. Ideally, variables in DFA are normally distributed, have homogeneous variances, and are uncorrelated with each other (21). Therefore, a subset of FAME markers that minimized these data structure effects was chosen. Variables were first excluded based on the magnitude of correlation with every other variable. Correlations were determined with Pearson's correlation coefficient (r), which was calculated for all FAME variable pairs. Those variables with high correlations ($|r| > \sim 0.650$) were grouped into categories. Only one FAME variable from each category was included in subsequent analyses so that all remaining variables had approximately equivalent standard deviations. Lastly, uncorrelated variables were subjected to a preliminary round of DFA to identify the FAME biomarkers with the greatest discriminatory power for medium formulations with disparate protein sources. This was judged by calculating an "F-to-remove" statistic for each FAME variable (21, 48). The eight FAME variables with the largest F-to-remove values and therefore the highest contributions to group separations were included in the final analysis. (See Fig. 4 for the variable subset used for DFA.)

RESULTS

Sporulation yields in different medium formulations. All *Bc*T cultures showed sporulation yields greater than 90%. Cultures grown in broth generally showed higher spore yields before water washing/purification (>95%) than cultures grown on agar plates (generally between 90 and 95%). Despite this discrepancy, the washing and purification steps increased the proportion of phase-bright spores in all preparations to above 95% (Fig. 1). Spores grown on Columbia agar and Columbia agar supplemented with blood showed the highest proportion (5 to 10%) of vegetative cells prior to and immediately following the first three water washes. The proportion of vegetative cells was minimized after subsequent water washes (<3%).

Effect of the sporulation medium on the fatty acid composition of *Bc*T spores. To investigate the effects of amino acids in the sporulation media on the fatty acid composition of spores, *Bc*T cultures were grown in a modified CDSM broth that lacked an exogenous amino acid source compared to other sporulation media that contained either defined or complex protein sources (Table 1). The relative proportions of the four fatty acid structure classes in spores grown in each medium are shown in Fig. 2. While variation existed across each medium preparation for every fatty acid, the largest differences were observed with spores grown in CDSM. The relative proportions of branched-odd fatty acids for spores grown in this medium were generally half of the percentages observed in other sporulation media (Fig. 2a). Conversely, the overall proportion of branched-even fatty acids typically was doubled in CDSM samples compared to spores grown in other media (Fig. 2b). CDSM spore profiles also showed a slight increase in the ratio of normal fatty acids (Fig. 2d). Although the relative percentages of anteiso fatty acids varied considerably between different batches of CDSM spores (between 7 and 16%), the range showed significant overlap with the proportions obtained for other media, including G, LL, and Sch (Fig. 2c).

Multivariate and individual biomarkers in *Bc*T FAME profiles. Comparison among cultures grown in media with different types and combinations of complex additives showed more subtle variations in the relative proportions of fatty acids. For example, medium groups containing higher concentrations of tryptone or gelatin digests, such as BHI, CAD, and CA, generally had elevated levels of branched-odd fatty acids (~58 to 63%) compared to certain medium formulations with meat peptone as the sole nitrogen source (i.e., Sch and LL, with ~44% branched-odd) (Fig. 2a and Table 3). In addition, spores grown in G, MG, LL, and Sch media had slightly elevated proportions of anteiso fatty acids, ranging between 10 and 11% compared to other medium groups (BHI, CAD, and CAB), with anteiso fatty acids ranging between ~5 and 7% (Fig. 2c and Table 3). However, the trends in anteiso FAMEs did not directly correlate with the presence of any particular constituents in the sporulation medium.

In addition to differences in the relative proportions of FAME markers, one FAME marker was associated uniquely with one medium. Oleic acid (18:1 ω9c) was present in appreciable quantities (>1%) only when spores were grown on Columbia agar supplemented with sheep blood (CAB). The concentration varied between 5 and 20% of the total fatty acid composition. Oleic acid was not evident in spores grown on Columbia agar (Table 3). The results from GC-mass spectrometry analysis were consistent with the presence of oleic acid in CAB spore extracts (data not shown).

Multivariate differentiation of *Bc*T spore FAME profiles. FAME profiles are complex due to the high number of variables and the fact that the total dissimilarity among profiles may arise from unequal contributions from all the variables.

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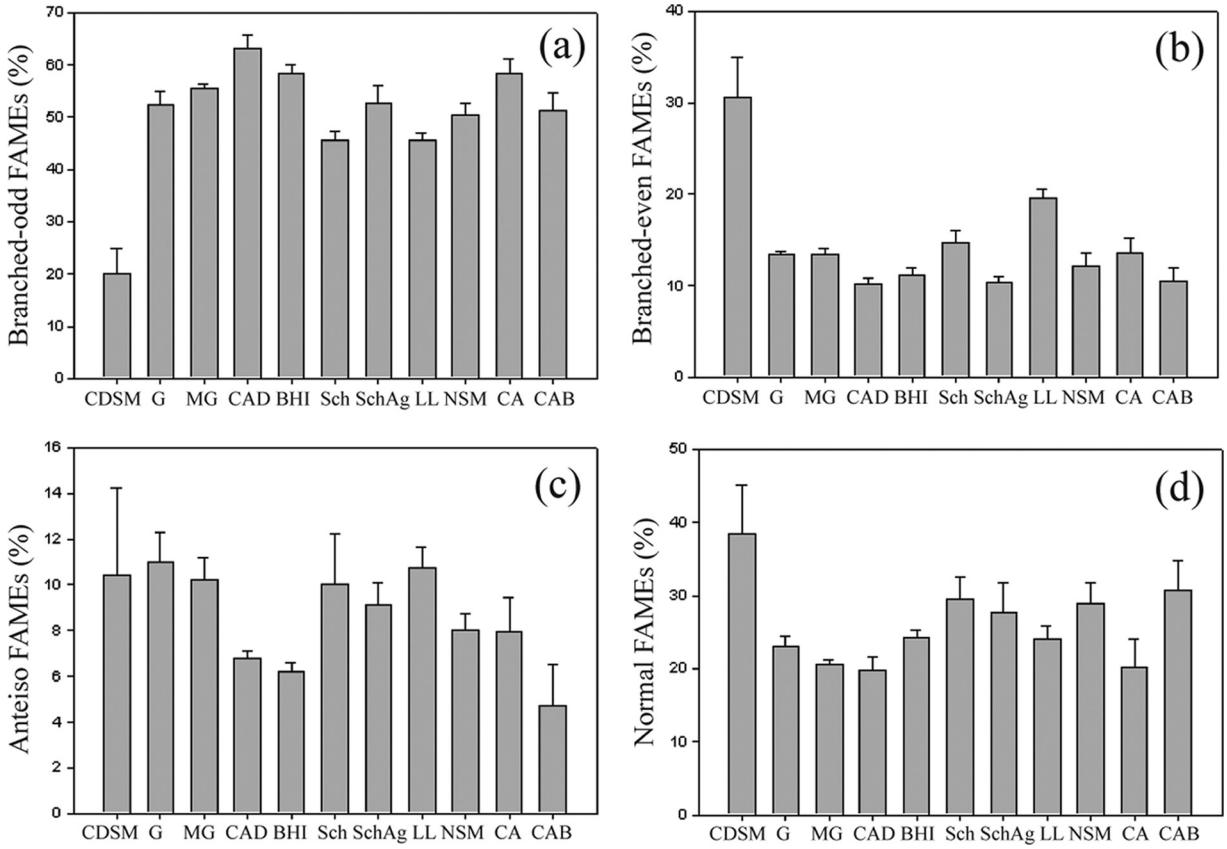

FIG. 2. Comparison of spore cultures grown on different medium formulations. (a and b) The spores grown on CDSM showed the most significant differences in the relative proportions of branched-odd and branched-even fatty acids. (a to d) Variation in each of the four fatty acid structure classes was also observed among all the other medium formulations. The error bars indicate 1 standard deviation.

Typically, data from every detected FAME marker are used to generate multivariate distance matrices (47) or functions representing linear combinations of the original variables (16, 57), which are used to analyze dissimilarities among all profiles.

For this study, FAME data were first analyzed in a similar manner by incorporating the relative proportion of every FAME marker identified by GC into the multivariate distances that were calculated for spore profiles. The distances were then analyzed with nMDS and ANOSIM to examine the relationships among spores grown on different medium types and to test for significance between spore-medium groups. These two nonparametric techniques made no assumptions about data structure or sample groupings prior to analysis. In this way, they provided an unbiased snapshot of the total dissimilarity in the whole FAME composition of each spore sample.

More specifically, nMDS offers a mathematically robust yet conceptually simple method for visualizing multivariate relationships between individual samples and among groups of samples (36). In a two-dimensional nMDS plot, distances between points reflect the relative rank order dissimilarities among all samples. The spatial arrangement of sample points and the resulting distances among sample groups, therefore, can be interpreted as a measure of the relative dissimilarity in FAME profiles among spores grown in different media. In addition, the goodness of fit between the underlying distance matrix and the observed spatial arrangement of sample points in each nMDS plot is measured with a "stress" value (8).

Figure 3 shows the nMDS plot for spores grown on 10 sporulation media (Table 1, excluding CDSM). Spores grown on media with disparate chemical compositions exhibited well-defined clusters with high intergroup distances with respect to each other, suggesting significant multivariate dissimilarity. These media included LL, G, Sch, CAD, NSM, and BHI. Compositionally similar media, such as G and MG (Table 1), showed closely associated (but separated) clusters with each other. Noncompositional features also appeared to affect the FAME profiles of *Bc*T spores. For example, SchAg samples (agar based) did not show a close association with Sch samples (broth based), exhibited high intragroup variation, and overlapped with other medium groups in the center of the nMDS plot (Fig. 3). Similarly, spores grown on CA and CAB showed higher intragroup variation and, in the case of CA, completely overlapped with BHI samples. The stress value reported in Fig. 3 (0.11) indicates that the nMDS plot is an accurate representation of intersample relationships (8).

To complement the descriptive assessments of multivariate dissimilarities provided by nMDS, ANOSIM was used to test for the statistical significance of grouping spore samples according to the medium formulation. The ANOSIM test statistic, R, is a measure of the magnitude of dissimilarity within and

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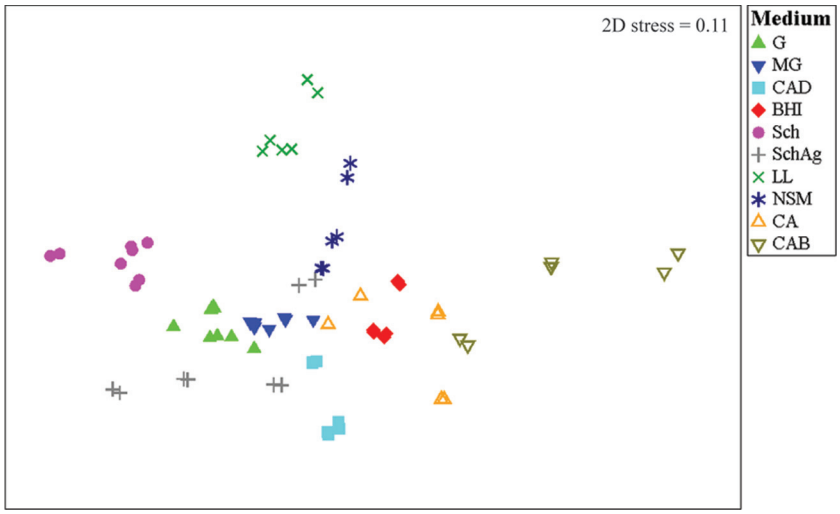

FIG. 3. Two-dimensional nMDS plot of spore FAME profiles grouped by growth medium. Multivariate dissimilarities were generated using every fatty acid variable in the FAME profile. Many of the medium groups showed distinct clusters that were clearly differentiated from other groups (G, MG, Sch, and LL). Some spore groups showed overlapping or poorly defined clusters (BHI, CA, SchAg, and CAB).

between sample groups and ranges between 0 and 1 (8, 9). *R* values near 0 indicate that dissimilarities between sample points within one group are equivalent to the dissimilarities found between different groups. *R* values closer to 1 indicate strong differences between two groups relative to intragroup variation among FAME profiles. Statistical significances in the form of *P* values were calculated for each pairwise *R* value.

The results from the ANOSIM analysis for individual FAME markers were consistent with the spatial trends observed in the nMDS plot (Table 4 versus Figure 3). The *R* values for most pairwise medium group comparisons showed *P* values of less than 0.01, indicating that all medium groups had statistically significant dissimilarities in their composite FAME profiles (BHI-CA was the one exception). However, the magnitudes of the *R* values did show variation across the medium groups. SchAg samples exhibited the lowest *R* values (<0.8) with all other medium groups, excluding SchAg-LL and SchAg-CAB ($R \geq 0.8$ for both). The *R* values for G-MG and BHI-CA were also relatively low: 0.39 and 0.30, respectively.

TABLE 4. ANOSIM[a] on spore-medium group comparisons

Medium	Individual fatty acid variable (global $R = 0.84$)[b]									
	G	BHI	CA	CAD	NSM	Sch	SchAg	MG	LL	CAB
G		1	0.94	1	1	0.83	0.61	0.39	1	1
BHI			0.3*	1	0.97	1	0.77	0.99	1	0.75
CA				0.83	0.61	0.96	0.48	0.75	0.93	0.87
CAD					0.98	1	0.54	1	1	0.96
NSM						1	0.57	1	0.90	0.91
Sch							0.62	0.75	1	1
SchAg								0.48	0.80	0.96
MG									1	1
LL										1

[a] The matrix lists the *R* value for each pairwise medium group comparison. The asterisks indicate that the *R* value was not significant at the $P = 0.01$ level.
[b] Global *R* was calculated from simultaneous comparisons of the rank dissimilarities for all samples and groups.

Differentiation of FAME profiles using DFA. While nMDS and ANOSIM are useful for detecting differences among spore profiles, these techniques do not provide criteria for discriminating between different spore samples. DFA can complement these analyses by identifying individual FAME biomarkers that differentiate spores grown in different medium formulations. In DFA, linear functions (i.e., "discriminant functions," or "canonical variate [CV] functions") that maximize the variation among user-defined sample groups and minimize the variation within each sample group are derived from the original variables. The relative contribution of each variable to the observed group differentiation is proportional to the absolute values of the discriminant function coefficients (21).

The main advantage of DFA for forensic studies is that it incorporates prior data structure into the analysis and allows the user to define sample groupings so that different levels of variation in the data set can be examined. In this way, differences among FAME profiles that may be due to experimental error among replicates or small variations in medium chemistry can be minimized and changes in FAME variables that are driven by distinguishing characteristics of the sporulation medium (e.g., complex additives, supplemental protein, or unusual compounds) can be identified. For example, Sch and SchAg have identical chemical compositions but differ in the physical state (broth and agar, respectively), which results in non-compositionally based variation in FAME profiles (Fig. 3 and Table 4). If all samples grown in either Sch or SchAg are defined in one group, then DFA will generate linear functions that minimize variation between the Sch and SchAg samples and maximize variation between these and other sporulation medium groups. The variables with the largest coefficients in these functions will then represent FAME biomarkers reflecting compositional differences, rather than the total dissimilarity between Sch/SchAg and other media.

To identify FAME variables that are diagnostic for differences in protein content among sporulation media, dis-

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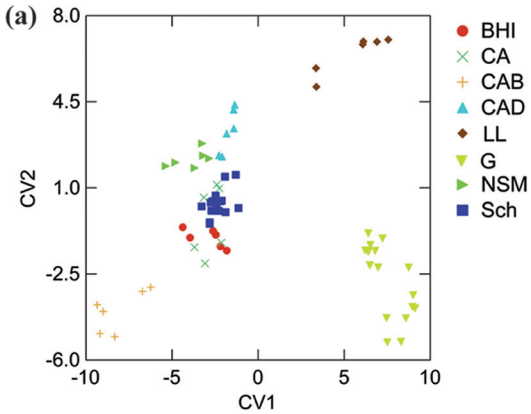
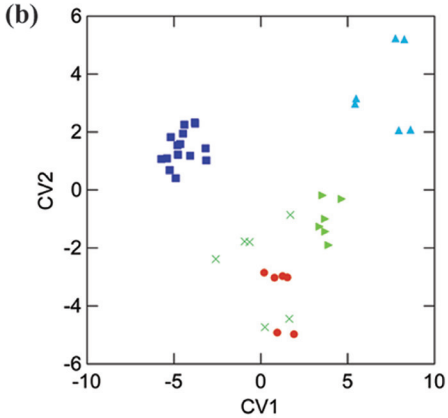
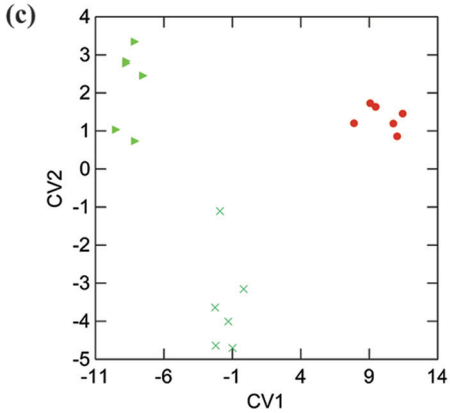


	CV1	CV2
13:0 iso	1.70	0.475
14:0 iso	-1.29	-0.694
14:0	-0.247	0.907
15:0 anteiso	3.21	0.402
16:0 iso	2.25	1.28
17:1 iso w10c	2.33	-3.79
17:1 iso w5c	0.309	1.82
17:1 anteisoA	-4.39	-0.590
Proportion of dispersion (%)	57	76



	CV1	CV2
13:0 iso	0.646	0.850
14:0 iso	-2.73	0.026
14:0	0.867	0.271
15:0 anteiso	-1.11	1.16
16:0 iso	2.41	0.235
17:1 iso w10c	-3.12	-0.549
17:1 iso w5c	2.45	1.34
17:1 anteisoA	0.821	-0.733
Proportion of dispersion (%)	68	89



	CV1	CV2
13:0 iso	5.82	1.42
14:0 iso	-3.22	-3.58
14:0	-2.37	1.20
15:0 anteiso	3.91	-0.333
16:0 iso	-0.279	1.13
17:1 iso w10c	4.00	-2.43
17:1 iso w5c	-5.86	0.672
17:1 anteisoA	-2.61	0.143
Proportion of dispersion (%)	90	100

FIG. 4. Discriminant function analysis of spore FAME profiles. In each panel, spore samples are plotted against two CV functions. The equation for each CV function is shown on the right of the plot. The cumulative proportion of the total dispersion among groups is included for each CV. (a) Distinct clusters were observed for the G, LL, and CAB groups across the first two CV functions. (b and c) Successive CV functions separated the remaining medium groups.

criminant functions were built to analyze eight composite medium groups reflecting different complex protein sources. Six of these groups were identical to the sample groups used for nMDS. However, spore samples for MG were grouped with G samples, since both represent media with yeast as the sole protein source (Table 1). Similarly, Sch and SchAg samples were combined into one group. The equations for the first two discriminant functions (or CVs), along with a 2D plot of the distribution of each sample in CV space, are shown in Fig. 4a.

Samples belonging to the LL, G, and CAB groups are clearly differentiated from each other and from the remaining groups in the center of the plot (CA, CAD, NSM, BHI, and Sch). Separation between CAB and G occurs primarily along CV1, whereas the distances between the G and LL groups are found along CV2. The CAB and LL groups are separated along both CV1 and CV2. The coefficients for each CV function (on right of the plots) indicate that different sets of variables drive separation along each axis. CV1 is dominated by the relative proportions of 15:0 anteiso, 17:1 iso ω10c, and 17:1 anteisoA,

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and CV2 is dominated by the relative proportions of 17:1 iso ω10c and 17:1 iso ω5c.

Because little differentiation was observed among BHI, CA, CAD, NSM, and Sch samples (Fig. 4a), new discriminant functions were constructed using only these groups (Fig. 4b). Similar to the previous plot, two of the sample groups (CAD and Sch) can be easily distinguished from each other along CV1. However, the variables with the largest contribution to the differentiation among these groups are 14:0 iso, 16:0 iso, 17:1 iso ω10c, and 17:1 iso ω5c. The remaining groups (CA, BHI, and NSM) cannot be clearly discriminated from each other along CV1 or CV2 but are separated from CAD and Sch by both CV functions.

When discriminant functions are built for the last three groups (BHI, CA, and NSM) (Fig. 4c), CV1 accounts for nearly all the variation among groups (~90%) and clear separation in the CV plot is observed for every group. Unlike the previous functions, only branched-odd variables, 13:0 iso, 17:1 iso ω10c, and 17:1 iso ω5c, account for the variation along CV1.

DISCUSSION

The influence of exogenous protein sources in the sporulation medium on FAME profiles. The relationship between the ratio of amino acid precursors in the culture medium and the proportion of each fatty acid structure in vegetative *Bacillus* cells is well established (24, 27, 28), The fact that spores grown in CDSM showed obvious changes in the proportions of branched-odd, branched-even, and, to a lesser extent, normal fatty acids compared to spores grown in other media surveyed in this study indicates that amino acid precursors (specifically, leucine and valine) are utilized directly from the sporulation medium. Consequently, the absence of a direct protein source or any complex additive in the formulation can have a significant effect on the types and abundances of branched FAME markers associated with *Bacillus* spores.

Less drastic variation in branched fatty acid composition was also evident among non-CDSM groups, indicating that FAME profiles are also affected by different types of protein sources and complex additives used in the sporulation medium. However, with the exception of spores grown on meat peptone-based formulations showing decreased branched-odd proportions, FAME abundances did not show clearly defined ranges that corresponded to specific chemical constituents of any growth medium (Table 1). Also, transitions in the relative abundances of FAME biomarkers were small and incremental among the different medium preparations (Fig. 2 and Table 3). Such patterns have been observed previously with bacteria grown on different media (16), suggesting that forensic signatures based on FAME profiles depend on more complex multivariate relationships between variables and samples of interest.

Oleic acid as a biomarker for media with blood supplements. The only fatty acid biomarker exclusive to any of the surveyed media was oleic acid (18:1 ω9c) from spores grown on Columbia agar supplemented with sheep blood. Oleic acid is predominantly associated with eukaryotic organisms (13) but can be introduced exogenously through blood supplements (45) or surfactants, such as Tween 80, during the preparation process (15). Also, oleic acid is a common feature in commercial FAME libraries that contain organisms grown on blood-supplemented substrates (G. Jackoway, MIDI Inc., personal communication). This, combined with the observation that oleic acid did not appear in significant quantities in *Bc*T spores grown on the Columbia agar base (CA) (Table 3) but was present when spores were grown on other media containing blood products (tryptic soy agar with blood) (data not shown) suggests that this fatty acid is likely derived from the eukaryotic supplements in the CAB medium. Regardless of the origin, our results indicate that oleic acid may be a promising biomarker for *Bacillus* cultures grown on media that are supplemented with sheep blood products.

Differentiation among medium groups using whole FAME profiles. Fatty acid analyses based on all FAME biomarkers showed that many of the 10 medium cultures in this study could be differentiated by their FAME profiles on the 2D nMDS plot (Fig. 3) and with pairwise *R* values (Table 4). The largest dissimilarities in FAME profiles were found among spores grown on media with distinctly disparate protein and nitrogen sources (yeast, meat peptone, yeast/casein peptone, brain heart infusion/gelatin digest, and beef extract/meat peptone for G, LL, CAD, BHI, and Sch, respectively). The dissimilarities in FAME profiles likely reflect the distinct differences in fatty acid precursors (amino acids and α-keto acids) inherent in each of the above-mentioned medium formulations.

Other media (BHI, CA, CAB, and SchAg) exhibited less distinct differences, as evidenced by cluster overlap (BHI-CA) or large intersample distances (CA, CAB, and SchAg). Overlapping sample groups could, in part, be indicative of the protein/amino acid composition of the sporulation medium. Both BHI and CA contain a variety of meat digest and beef infusion components (Table 1). Since different beef-derived supplements can have comparable ratios of leucine and isoleucine (35), the similarity in BHI and CA samples may reflect overlapping concentrations of these fatty acid precursors in each medium.

Variation in FAME profiles was also observed between spores grown on media with identical substrate compositions but different physical states (Sch and SchAg). FAME differences between *Bacillus* cultures grown in agar and broth-based media with identical compositions have been observed previously (26, 41) and could be related to microenvironments that are created during sporulation on agar media. In *Bacillus* organisms, the synthesis of unsaturated fatty acids is mediated by desaturase enzymes that are oxygen dependent (14, 26). The relative proportions of unsaturated fatty acid markers, such as 16:1ω7c, which is abundant in *Bc*T FAME profiles (Table 3), is primarily affected by the concentration of the saturated fatty acid precursor (16:0) and oxygen availability (26). During growth on agar plates, the oxygen concentration may show spatial heterogeneities within *Bc*T colonies that can affect the proportions of saturated and unsaturated fatty acid spore profiles compared to organisms grown in liquid media (26, 58). Similarly, metabolic substrates can show heterogeneities within agar colonies (39). This hypothesis would be consistent with shifts in the observed proportions of 16:1 ω7c (SumFeat2), 16:0 iso, and 15:0 iso for Sch and SchAg samples (Table 3). Consequently, statistical analyses (nMDS) based on all individual fatty acids would be prone to these variations, since each

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marker contributes independently to the calculated dissimilarity among samples. The observed discrepancies in FAME profiles between Sch and SchAg samples indicate that forensic discrimination of spores should incorporate different physical states of the medium.

Similarities and differences in medium composition or physical state would not explain why the SchAg, CA, and CAB groups had larger dissimilarities among replicate samples. This FAME profile heterogeneity displayed within CA and CAB could reflect slight variation in the proportion of vegetative cells in these spore preparations (see Results). Alternatively, the period of incubation prior to spore harvesting varied more for CA, CAB, and SchAg than for broth-based media, which would affect the number of metabolic conversions and potentially the relative proportions of FAMEs. However, this idea does not hold for all agar groups, since BHI and LL did not show comparable levels of intragroup variation.

Other medium pairs, mainly G-MG, showed distinct but closely related clusters on the nMDS plot and smaller *R* values for ANOSIM. Some dissimilarity between these two groups was expected, since the presence of different sugars in growth media has been found to affect the fatty acid profiles of *Bacillus* organisms (4, 11, 24, 52). Less dissimilarity between G and MG spore profiles suggests that small variations in supplemental sugar concentrations in the sporulation medium do not affect FAME profiles as significantly as other changes in the growth medium formulation.

Ultimately, these results suggest that using raw calculations of dissimilarity generated from every variable constituting a FAME profile is insufficient to differentiate all spore groups. While the analysis does indicate that FAME differences are most pronounced when spores are prepared on media with different types of complex additives and nonoverlapping protein sources, it also shows that total profile dissimilarities can be affected by variations in other, nondefining attributes of the medium or by the intrinsic variability of certain spore-medium groups. To compensate for these effects, profiles need to be analyzed with a statistical technique that can minimize variation that is nonspecific to the protein/amino acid content of each medium and can extract signatures that are unique to its defining compositional characteristics.

Differentiation of FAME profiles using DFA. The FAME biomarkers that responded most significantly to variation in the protein source were isolated by combining all spore samples that were grown in media with similar protein components and analyzing the resulting groups with DFA. A comparison of the clustering patterns in Fig. 3 and 4 indicates that non-compositionally based variation is minimized with DFA. For example, all Sch samples (composites of Sch and SchAg) showed a well-defined cluster with low intersample variation (Fig. 4a and b). Other groups exhibiting heterogeneities among replicates in nMDS (CA and CAB) displayed smaller intragroup variation in CV plots that were comparable to that of other spore groups (Fig. 4a and c). With non-compositionally based variation reduced, canonical variate functions and the corresponding high-magnitude FAME variables can be used to identify promising biomarkers for spore discrimination.

Figure 4a shows that CV1, which was heavily influenced by the proportions of 15:0 anteiso and 17:1 anteisoA, clearly separated G and CAB samples from other media. However, CV1, and therefore these two FAME biomarkers, could not clearly distinguish G samples from LL, or any samples grown in BHI, NSM, Sch, CAD, or CA from each other. Different FAME biomarkers were responsible for differentiating each of these groups. Branched-even FAMEs 14:0 iso and 16:0 iso and branched-odd FAMEs 17:1 ω5c and 17:1 ω10c drove the separation for CAD and Sch groups (Fig. 4b). However, only branched-odd FAMEs were significant contributors to the CV functions differentiating NSM, BHI, and CA groups (Fig. 4c).

These results suggest that FAME variation among all spore groups cannot be captured with a single set of equations but requires multiple, successive CV functions to discriminate among all spore groups. Also, the varying contributions of individual FAMEs to CV-based differentiation indicate that the same set of FAME variables cannot differentiate all spore groups simultaneously. For example, 15:0 anteiso and 17:1 anteisoA distinguished G-medium samples from other groups but poorly discriminated Sch and CAD from other samples. Conceptually, this is reasonable, since medium formulations may show variation across different sets of amino acid precursors, and spores grown on these media should vary across different sets of FAME variables.

Due to these observations, DFA-based discrimination of spores grown on different media may necessitate a hierarchically structured analysis similar to that portrayed in Fig. 4. In such a system, FAME profiles would be subjected to a cascading system of discriminant functions that model variation across separate subsets of reference groups (23). Taking Fig. 4 as an example, the FAME profile of an unknown microbial sample would first be run through an initial set of discriminant functions (Fig. 4a) and classified as belonging to either G, CAB, LL, or the unresolved group composed of BHI, CA, Sch, NSM, and CAD. If the sample was most closely related to the unresolved group, the FAME profile would be analyzed with the second set of discriminant functions (Fig. 4b), classifying it as either Sch, BHI, or the unresolved group composed of BHI, CA, and NSM. If the sample again was most closely related to the unresolved group, subsequent discriminant functions (Fig. 4c) would be used until a singular identification was acquired. Tiered classification systems with DFA have been used successfully in other forensic systems (23, 46) and could be applied to microbial samples provided that the discriminant functions are built from a comprehensive reference database (in this case, a library of FAME profiles from spores grown in various media).

Conclusions and future work. Overall, the results indicate that variations in *Bc*T FAME profiles can be used to discriminate among spores grown on different media. While individual biomarkers, such as oleic acid, may be diagnostic for certain components of the growth medium, the majority of variation across FAME profiles is driven by differences in the protein or amino acid sources in each sporulation medium. Other characteristics of the medium, such as the physical state or the concentration of glucose, do affect FAME profiles to an extent. Nevertheless, these profile variations can be reduced with DFA so that the FAME signatures that are specific for formulations with unique combinations of complex additives or supplemental protein sources can be detected.

Although this is an important first step for determining whether FAME profiling can be a forensic tool, much work

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remains before the observed differences among cultures grown on different media can be translated into forensically relevant biosignatures. First, the hypothesis that FAME variation is diagnostic for the combination of complex additives and protein sources that are present in each medium formulation should be explicitly tested by expanding a FAME data set to include formulations that vary in the concentrations of identical protein sources. Second, the effects that environmental factors, such as growth temperature, pH, or dissolved oxygen, have on FAME profiles should be compared to the compositionally based variation reported here. Third, different subsets of FAME variables should be identified with alternative statistical techniques, such as stepwise DFA (48) or Bayesian variable selection (2), and compared to the discriminatory power of the FAME subset used in this study.

Most significantly, multivariate strategies that allow sample profiles to be matched statistically to FAME databases are needed. The discriminant function analyses shown here are descriptive in nature (21) and are not intended to be robust classification schemes. A separate statistical procedure termed "predictive DFA" can be used for this purpose and is a promising strategy for classification of unknown samples (48). However, this technique still needs to be tested on a microbial data set. Another possibility is "Bayesian network analysis" (23), which would allow FAME databases to be combined with other orthogonal data sets (isotope, SIMS, etc.) to characterize a microbial sample of unknown origin. Future strategies may need to incorporate a combination of the above-mentioned approaches in order to successfully reduce the intrinsic complexity of FAME profiles and to help this technique become a viable tool in forensic microbiology.

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