Anal Bioanal Chem (2015) 407:4757–4766
DOI 10.1007/s00216-015-8677-3

RESEARCH PAPER

Forensic differentiation of *Bacillus cereus* spores grown using different culture media using Raman spectroscopy

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Received: 17 March 2015 / Accepted: 2 April 2015 / Published online: 19 April 2015
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Abstract Some microorganisms have been shown to retain a chemical signature indicative of the medium used for culturing. However, the repeatability of medium-specific chemical signatures has not been demonstrated from samples of microorganisms produced in the same batch or in different batches by the same sporulation protocol. Here, the variation in Raman spectra of bacterial endospores repeatedly prepared by the same procedure is compared to the variation between Raman spectra of spores prepared using different media. *Bacillus cereus* T strain (*Bc*T) samples were correctly classified according to the medium used to induce sporulation for 100 % of spores grown in a controlled manner by the same scientist using Raman spectroscopy and multivariate data analysis. The proof-of-concept results from *Bc*T spores produced in 12 different sporulation media showed correct classification by medium for 98 % of samples (with 100 % classification accuracy for all but one sporulation medium in this data set). Spectral differences were discerned between spores that had been freshly prepared or freeze-dried and spores that had been frozen; however, the differences did not impact the classification of the sporulation medium. Latent variables reduced the classification accuracy of *Bc*T sporulated in G medium by different scientists using different media lots and stored for different periods of time and requires further study.

Keywords Bioanalytical methods · Bacterial spores · Chemometrics/statistics · IR spectroscopy/Raman spectroscopy

This article is dedicated to the memory of Diane Williams, who lost a valiant battle against ovarian cancer. Diane was a very effective mentor to many Visiting Scientist Fellows and always keen to share her expertise in chemometrics and spectroscopy and engage in both scientific and cultural discussions on numerous subjects. She will be missed.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-8677-3) contains supplementary material, which is available to authorized users.

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Introduction

The bioterrorism incidents of September 2001, when several letters containing endospores of *Bacillus anthracis* were mailed to US congressional offices and media outlets, have motivated the development of methods for the examination and identification of microbial evidence. Many recently developed techniques have focused on identifying phenotypic signatures that are unique to the method used to culture an organism. Differences between culture conditions could result in varying quantities of macromolecules (i.e., proteins, carbohydrates, and lipids) produced by the organism due to the presence or absence of particular nutrients and the amount and type of nutrients stored by the spore [1]. Identifying the sporulation medium or other details about the culture procedure may be of value to future investigations of bioterrorism incidents.

Discrimination of spores based on phenotypic chemical signatures grown in different culture conditions has been investigated using carbon and nitrogen stable isotope ratios [2–4], fatty acid methyl esters (FAMEs) [5], residual agar on spores [6], and heme for spores grown on blood-containing media [7]. Raman spectroscopy provides an attractive

combination of rapid analysis, minimal sample preparation, nondestructive analysis, and low detection limits. The analysis of small quantities of samples is possible when coupled with a Raman microscope, and the technology may be an additional tool for phenotypic characterization of bacterial spores. Changes in Raman spectra have been qualitatively observed from the same species grown in different media or culture conditions [8–10]. However, the variation in Raman spectra between spores arising from different sporulation media has not been compared to the variation in Raman spectra within a few batches of identically prepared spores with sufficient replication to determine if the medium leaves a reproducible chemical signature. Statistical significance is required if this technique is to be used by forensic scientists in a criminal investigation to identify the sporulation medium.

Demonstration of the variability of bacterial spore Raman spectra between different media formulations will also have implications for spectroscopic bacterial species identification [11–13]. Raman and Fourier transform infrared spectroscopy has been used in medical and food safety research to identify bacterial species by comparing the spectrum obtained from the microorganism to a reference library [14–16]. Changes in culturing conditions, such as growth media, time, and temperature, have been shown to affect the classification accuracy of *Bacillus* organisms [11]. Therefore, if culture conditions are not consistent between the microorganism studied and the reference library, the accuracy of identification of the bacterial species may decrease.

The purpose of this research was to compare the consistency in Raman spectra of *Bacillus cereus* T strain, a surrogate to *B. anthracis*, sporulated using the same medium (within-medium variance) with the changes in the bacteria sporulated using different media (between-media variance). Replicate batches of spores were prepared using selected culture conditions to assess the reproducibility of the Raman spectrum obtained from spores grown using the same procedure. A larger set of 12 *Bc*T samples sporulated using different media were analyzed to assess sporulation medium accuracy from a wider sample set. Although not included in this study, additional variables such as how the spores are preserved, the skill of the scientist preparing the spores, the use of different media lots, and spore storage time may impact the chemical state of the spore. Chemometric classification was used to elucidate the small differences between *Bc*T sporulated using different media formulations [17–20].

Materials and methods

Sporulation procedure

B. cereus T strain (*Bc*T) was chosen as a surrogate species to *B. anthracis* for safety reasons because it is genetically, biochemically, and structurally similar but not pathogenic [5]. Cultures of *Bc*T were maintained at 30 °C on trypticase soy agar (Becton Dickinson (BD), Franklin Lakes, NJ, USA). Prior to inducing sporulation, vegetative starter cultures were prepared by inoculating single colonies of *Bc*T into 125 mL of trypticase soy broth (BD) and incubating for 16 to 18 h at 30 °C and 300 rpm on an orbital shaker.

The following sporulation media were used: casein acid digest broth (CAD) [5], brain heart infusion agar (BHI, BD), Columbia agar (CA, BD), Columbia agar with blood (CAB) [5], glucose broth (G) [21, 22], Lab Lemco agar (LL, Oxoid, Hampshire, UK), Leighton-Doi 1982 broth (LD82) [23], medium brain heart infusion G broth (MBG, G medium with 7 g BHI added), Mueller-Hinton agar (MHA, Oxoid) [24], N-Z Amine A broth (NZ) [25], Schaeffer's sporulation medium in both broth (SchBr), and agar (SchAg) formulations [26]. Media were autoclaved for 15 min at 121 °C and 1.5 kg cm^{-1} prior to addition of sporulation salts and starter culture. Sterilized MilliQ deionized water (18.2 MΩ cm resistivity) was used for sample preparations. A summary of the compositions of these sporulation media is given in Table 1.

Sporulation and spore harvesting were conducted, as described previously [5]. To remove residual media compounds adsorbed on the surface of the spores, the spore preparations were washed three times with separate 100 mL aliquots of cold water followed by an overnight wash at 4 °C on a rocking platform. Spore preparations were microscopically confirmed to contain ≥95 % phase bright spores with minimal residual cellular debris [5], washed with 50 mL of cold water, and diluted to approximately 30 mg/100 μL.

Unless noted, fresh spores were prepared by the same scientist and stored at 4 °C prior to sampling and Raman spectroscopy (fresh). To preserve spores for extended storage, fresh spores were wet-frozen or freeze-dried into a powder form. To wet-freeze, an aliquot of the spore suspension was pelleted, the supernatant was removed, and the spore pellet was frozen at −20 °C for storage. To freeze dry, the diluted spores were stored overnight in a −80 °C freezer, vacuum dried at −60 °C for 6 h using the Ultra-Dry Freeze-Dryer (Freezedry Specialties; Princeton, NJ, USA), and stored at room temperature. Prior to sampling for Raman spectroscopy, preserved spores were suspended in 100 μL of cold water with the vials on ice to prevent spore germination.

Preparation of spores for Raman spectroscopy

As discussed below, a closely packed monolayer of spores was determined to give optimal Raman signal-to-background ratios and between-spectrum measurement precision. Thus, spore samples for Raman microscopy were prepared by diluting the stock spore samples with cold water to 3–6 mg of dried spore weight/100 μL of deionized water for broth cultures and approximately 15 mg/100 μL for agar

Table 1 Composition of sporulation media (per liter of deionized water)

		Sporulation medium											
		CAD	BHI, BD	CA, BD	CAB	G	LL, Oxoid	LD82	MBG	MHA, Oxoid	NZ	SchBr	SchAg
Components (g/L)[a]	Acid hydrolysate of casein									17.5			
	Beef extract							6				3	3
	Beef infusion									300			
	Beef heart digest			3	3								
	Blood				50								
	BHI		6						7				
	Corn starch			1	1					1.5			
	Dextrose	0.75	3			1		0.9	1		7.5		
	Lab Lemco powder						3						
	Peptone			5	5		5	10				5	5
	Meat peptic digest		6										
	N-Z amine										5		
	Tryptone	8.5		10	10								
	Yeast	5		5	5	2			2		5		
	Agar		15	15	15		15						15

[a] Trace metal and sporulation salt components are listed in Electronic Supplementary Material Table S1

cultures with the dilutions determined empirically. Three 1-μL samples of each batch of spores were deposited onto a sterile, Raman grade CaF_2 slide (Crystran Ltd., Poole, UK) and allowed to air dry overnight. Variation in Raman spectra measured repeatedly from a quality control sample of MBG spores stored dry on a CaF_2 slide was on the order of the natural variation in Raman spectra between batches of freshly prepared MBG spores. Thus, the spores were considered to be unaffected due to dry storage at room temperature on a CaF_2 slide over the course of this study (~160 days).

Raman spectroscopy

An Almega Raman microscope (Thermo Scientific, Waltham, MA, USA) was used for data collection. A 50× objective delivered laser light (780 nm diode laser operated at ~15 mW with ~1.6-μm spot size) to the sample and collected the scattered light. The scattered light (collected from throughout the volume of the spores [10, 27, 28]) was passed through a 50-μm aperture into the spectrometer equipped with a 1200 grooves/mm grating and charge-coupled device detector. Spectra were recorded from 400 to 1700 cm^{-1} Raman shift with an estimated spectral resolution of 3.7–4.7 cm^{-1}. Peak positions were confirmed daily with a polystyrene standard [29].

To enhance measurement precision and record a Raman spectrum representative of the entire dried spore sample, Raman spectra were recorded at eight locations on each sample. The eight spectra were collected at locations where, at 50× magnification, the spores appeared to be a single layer of tightly packed spores. This was to minimize variation between spectra due to incomplete filling of the laser spot with spores and due to a potentially unknown number of layers of spores arising from uncontrolled sample deposition. Additionally, spectra of more than one layer of spores (as estimated using reflected light microscopy) showed increased background signals. Because of this strict sampling requirement, mapping of the entire sample surface was not possible. Each of the eight Raman spectra was integrated for a total of 120 s (two 60 s spectra were recorded to allow the automatic removal of cosmic rays by the instrument software).

Spore photodamage due to laser exposure was assessed by collecting 20 spectra (each integrated for 60 s) sequentially at the same location on a sample of *Bc*T sporulated in MBG. The intensity of the four most prominent Raman peaks decreased <2 % over a 600-s laser exposure and <6 % over a 1200-s laser exposure. Thus, spore photodamage was not a significant concern even for integration times much longer than those used in these experiments.

Data analysis

Raw spectra were corrected by smoothing with a Savitzky-Golay filter (third-order polynomial with a seven-point smooth), baseline corrected using a linear correction, and transformed to z-scores (zero mean and unit standard deviation) to normalize all spectra to a common baseline and correct for different intensity scales/variances for spores grown in

different media (spores with strong fluorescence had much higher signal and correspondingly higher intensity variation than those with less of a fluorescence signal component). Each spectrum consists of 676 individual Raman scattered intensities from 400 to 1700 cm^{-1} Raman shift. Unless noted, the eight corrected spectra from each sample were averaged to increase measurement precision and to account for within-sample variation. Analysis of variance (ANOVA) and Tukey-Kramer honestly significant difference (HSD) tests were conducted in JMP Pro (SAS, Cary, NC, USA) using the average of the eight corrected spectra from each sample.

Statistical analysis was performed in The Unscrambler X (CAMO Software, Olso, Norway). To estimate the accuracy of sporulation medium classification using the information contained in the entire Raman spectrum, the number of variables were first reduced from 676 Raman scattered intensities to six weighted linear combinations (unless noted) of Raman scattered intensities (containing 99.7 % of the variation in the training set) by principal component analysis (PCA). Then, samples were classified according to the media used to sporulate the *Bc*T cells by linear discriminant analysis (LDA) [30–32]. Log posterior probabilities (similarity scores) were plotted with larger values indicating a higher probability of membership in the stated class (sporulation medium). Classification accuracy was determined from the percentage of samples for which the class with the largest log probability for each sample (predicted sporulation medium) was the correct sporulation medium for that sample.

For the data set containing samples of *Bc*T sporulated using each of the 12 different media, the eight corrected spectra from samples of the five media data set were averaged (for a total of nine samples from each of these sporulation media). The eight corrected spectra from each batch of the seven additional sporulation media were used without averaging (for a total of eight samples from each of these sporulation media). For complete classification of the sporulation media, two separate PCA-LDA models were constructed (tiered LDA [5, 33, 34]). *Bc*T samples classified with 100 % accuracy in the first PCA-LDA tier were not used to calculate the second PCA-LDA model.

Results and discussion

Proof-of-concept classification of *Bc*T sporulation medium using Raman spectroscopy

For successful discrimination of bacterial spores produced using different sporulation media, the variation in Raman spectra from spores produced in different sporulation media (between-media variation) must be large compared to the variation in Raman spectra from spores produced using the same sporulation medium (within-medium variation). To reduce the number of variables imparted by the sporulation process, spores were thoroughly washed prior to Raman analysis to remove residual culture medium components [35]. Both broth and agar-based sporulation media that cover a range of nutrient sources were selected to assess the relative magnitudes of the between-media and within-medium variations for a proof-of-concept study. The between-media variation was assessed by a comparison of five sporulation media (G, MBG, SchBr, SchAg, and CAB). Three sources of variation contribute to the overall within-medium variation: (1) within-sample variation (heterogeneity of the dried spore sample for Raman analysis, i.e., heterogeneity of each dot in Fig. 1), (2) within-batch variation (heterogeneity of a single batch of spores [36], i.e., each column of dots in Fig. 1 is three samples from the same batch), and (3) within-procedure variation (unavoidable changes between spores prepared separately by nominally identical and well-controlled procedures, i.e., each of the three rows of colored dots in Fig. 1 is an independently prepared batch of spores). All three sources of within-medium variation were addressed in these experiments: (1) unless noted, Raman spectra were collected and averaged from eight spatial locations within each dried spore sample (to minimize within-sample variation), (2) three samples of each batch were analyzed (to assess within-batch variation), and (3) each of the five sporulation media used to estimate between-media variation was prepared in triplicate (to assess within-procedure variation). This sampling scheme is diagrammed in Fig. 1. Figure 2 shows the average of all 72 spectra obtained for *Bc*T sporulated in five of the media formulations with the prominent peaks

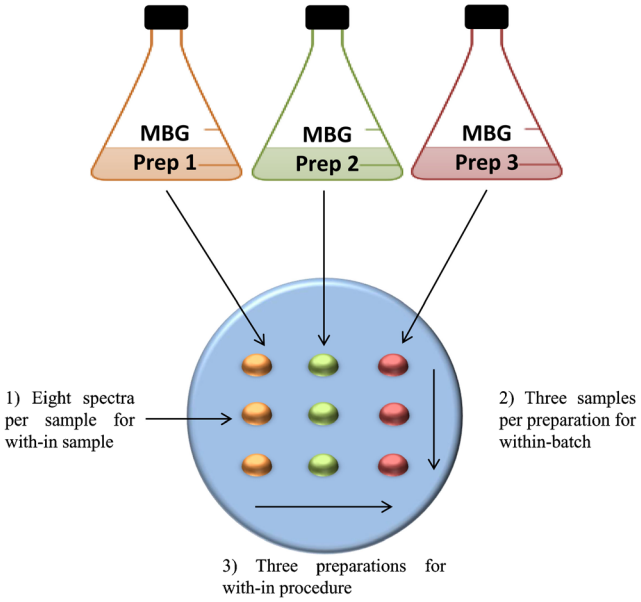

Fig. 1 Diagram of spore samples on a substrate illustrating samples and spectra used to assess the three levels of within-medium variation in Raman spectra of *Bc*T sporulated using a single medium (MBG in this example). This sampling scheme was used for SchAg, MBG, SchBr, G, and CAB with a separate substrate prepared for each sporulation media

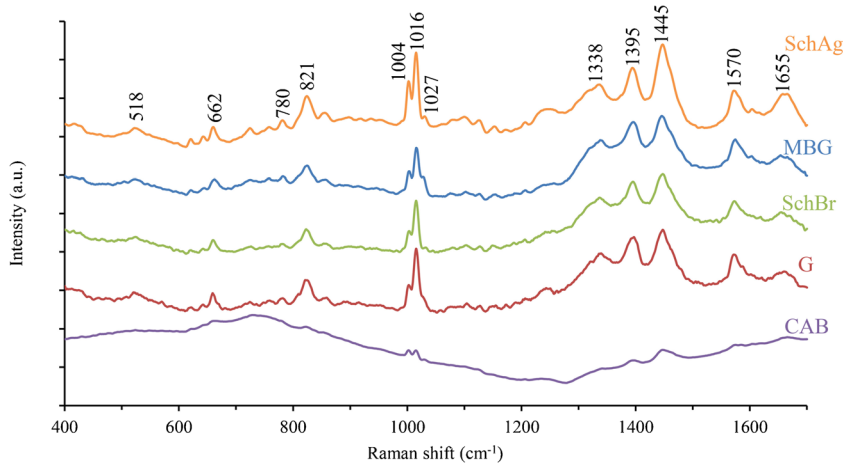

Fig. 2 Mean corrected Raman spectra for *Bc*T sporulated in five of the media studied with the most prominent peaks labeled. Peak assignments are from Table 2. Spectra are offset for visualization. Each spectrum is the average of 72 single spectra

tentatively assigned to vibrations/molecules (many from calcium dipicolinic acid (CaDPA)) in Table 2.

The peak assignments indicate the presence of a number of molecules expected a priori in a bacterial spore. CaDPA dominates the spectrum, as observed in the Raman spectra by previous experimenters [8, 37–40], due to high CaDPA concentrations (estimated to be 5–20 % of the dry spore weight [41]). Spores were analyzed after a lengthy incubation in sporulation media when levels of CaDPA are expected to be high [42].

The presence of amino acid amide I and III stretches from the backbone of proteins were observed [43], as well as peaks from side chains of specific amino acids such as tyrosine, phenylalanine, and a disulfide stretch, perhaps from cystine (cysteine dimer) [8, 13]. Protein is the major component of the spore coat, which serves as the outer barrier of the spore [44]. Peaks assigned to DNA or RNA were also observed: a symmetric stretch of PO_2^- likely from the phosphate backbone and stretches attributed to the nucleotides of adenine and uracil.

Table 2 Tentative assignments of prominent peaks present in the majority of Raman spectra from *Bc*T spores

Raman shift (cm^{-1})[a]	Assignment	Reference
518	S-S stretch	[8, 13]
662	CaDPA	[8, 38]
780	Uracil, citric acid	[8, 13, 40]
821	CaDPA, tyrosine	[8, 13, 40]
1004	Phenylalanine	[8, 13]
1016	CaDPA	[8, 38, 39]
1027	PO_2^- symmetric stretch	[8]
1338	Adenine, amide III	[39, 43]
1395	CaDPA, amide III	[8, 38, 39, 43]
1445	CaDPA	[8, 13, 38, 39]
1570	CaDPA, adenine	[8, 13, 38, 39]
1655	Amide I	[8, 13, 43]

[a] Peak positions are the average for spores across the five media studied

Upon close inspection, some visual differences between the spectra in Fig. 2 can be observed. *Bc*T sporulated in both agar-based media can be distinguished from broth samples based on the fluorescence background (CAB) or the presence of additional/more intense peaks in the 875–1130 cm^{-1} range (SchAg). These differences are reproducible for replications of the same sporulation procedure and significant due to the large number of spectra collected (and corresponding long effective integration times) for each average spectrum displayed in Fig. 2.

Additional discrimination is possible by comparing peak intensity ratios. For example, the ratios of the 1004, 1016, and 1027 cm^{-1} peak heights appear different in Fig. 2. The uncorrected average peak height from each spore sample was background subtracted (linear correction based on background intensities at 985 and 1041 cm^{-1}), and all six ratio permutations were calculated for each spore sample. Statistically significant differences in peak height ratios ($p<0.0001$) were detected using ANOVA tests between *Bc*T sporulated in one or more media using four of the six ratios. As shown in Fig. 3, discrimination of *Bc*T sporulated in each medium was possible using two Tukey's HSD tests on 1004/1016 and 1027/1004 (or 1027/1016 which also provided equivalent discrimination). Arbitrary letters were assigned based on the results of the two separate Tukey-Kramer HSD tests to show the classification of all of the *Bc*T sporulation media in this data set (Table 3).

As illustrated in Fig. 3, similarities exist between the 1004/1016 ratios (dependent on the tyrosine and CaDPA concentrations in the spores) of MBG and SchAg (unable to be statistically distinguished via a Tukey-Kramer HSD test and thus grouped by the letter A in Fig. 3) as well as between SchBr and G (connected by letter C). Both the A and C groups were statistically distinguished from each other and from CAB (group B). The 1027/1004 ratios, indicative of the ratio of

Table 3 *p* values obtained from Tukey-Kramer HSD tests of 1004/1016 and 1027/1004 intensity ratios

1004/1016	MBG	G	CAB	SchBr	1027/1004	MBG	G	CAB	SchBr
MBG					MBG				
G	<0.001				G	0.082			
CAB	<0.001	<0.001			CAB	<0.001	<0.001		
SchBr	0.003	0.899	<0.001		SchBr	<0.001	<0.001	0.998	
SchAg	0.242	<0.001	<0.001	<0.001	SchAg	<0.001	<0.001	0.653	0.466

nucleic acid to protein contents, are similar for CAB, SchBr, and SchAg (connected by letter Z in Fig. 3) as well as MBG and G (connected by letter Y). The unique combination of letters for each sample in Fig. 3 shows that the medium used to sporulate *Bc*T can be classified for these samples using the two peak intensity ratios selected. Thus, Raman peak intensity ratios may provide clues about the type of sporulation medium used to prepare spores recovered as evidence. Peak intensities and intensity ratios may also provide a biomarker of broth- or agar-based media, but further study is required prior to use as a phenotypic metric.

Multivariate data analysis was also conducted to discriminate *Bc*T sporulation media. In the first analysis, a limited five medium data set was examined (G, MBG, CAB, SchBr, and SchAg). Although these five formulations can be discriminated using peak intensity ratios, this method ignores a vast majority of the spectrum and the contained information. After correction of raw spectra as described above, PCA was performed to reduce the number of variables (Raman shifts) describing the data. Then, LDA was used to select variables that would minimize the spread within each medium formulation and maximize the separation between different media formulations. The grouping and discrimination using PCA-LDA is shown in Fig. 4.

Note that the apparent pattern in this figure is not a trend [20]. The variables plotted are the relative similarity of each sample to two of the five media (MBG on the *x*-axis and SchBr on the *y*-axis). For example, samples that have similar values to the MBG samples from LDA are closest to zero on the *x*-axis and samples that are increasingly different have increasingly negative values on the *x*-axis. The apparent "horseshow" pattern is a coincidence due to the dissimilarity of the SchAg samples to both MBG and SchBr and the similarity of the G samples to both MBG and SchBr samples. Note that classification accuracy is based not only on the two variables plotted but also on the other three dimensions (similarity to G, SchAg, and CAB, respectively).

The within-procedure variation is larger than the within-batch variation (compare the overall spread for each medium to the spread within each of the batches [different symbols] for each medium). This may be important when the spores of interest are a minor component in a mixture, such as when the spores of one species are a minor contaminant in spores of another species (after immunomagnetic isolation [45, 46]) or in a different matrix such as milk, food, sugar, etc. [12, 47, 48].

The medium used for *Bc*T sporulation was correctly classified for 100 % of the known spore samples. This indicates that the within-medium variation is sufficiently small compared to the between-media variation for effective discrimination of *Bc*T spores in the five media studied using Raman spectroscopy. This PCA-LDA model could be used as a training set to identify the media used for spores collected as evidence. Based on this training set, the unknown sporulation medium of *Bc*T spores recovered from a crime scene could

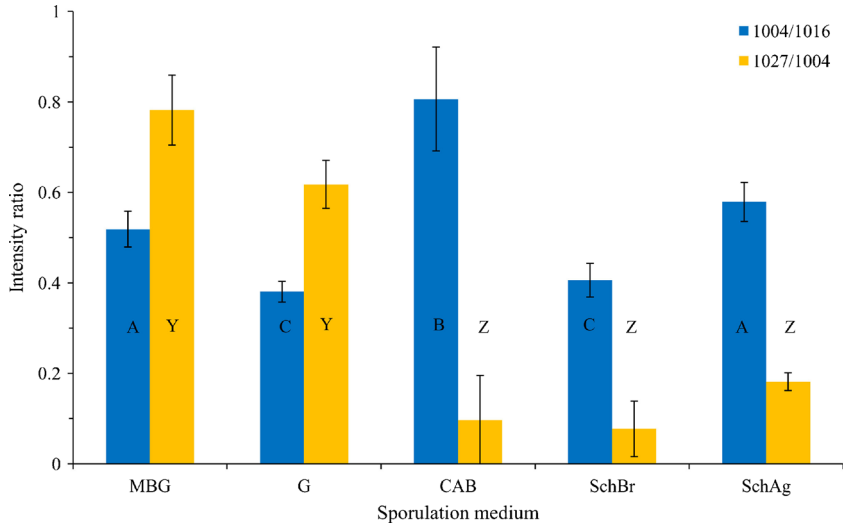

Fig. 3 Intensity ratios with *letters A*, *B*, *C*, *Y*, and *Z* to indicate spores grouped/discriminated with separate Tukey-Kramer HSD tests for each intensity ratio. Intensity ratios from *Bc*T sporulated in each medium with different letters (i.e., A and Y) are significantly different at $p<0.01$. The *error bars* indicate one standard deviation

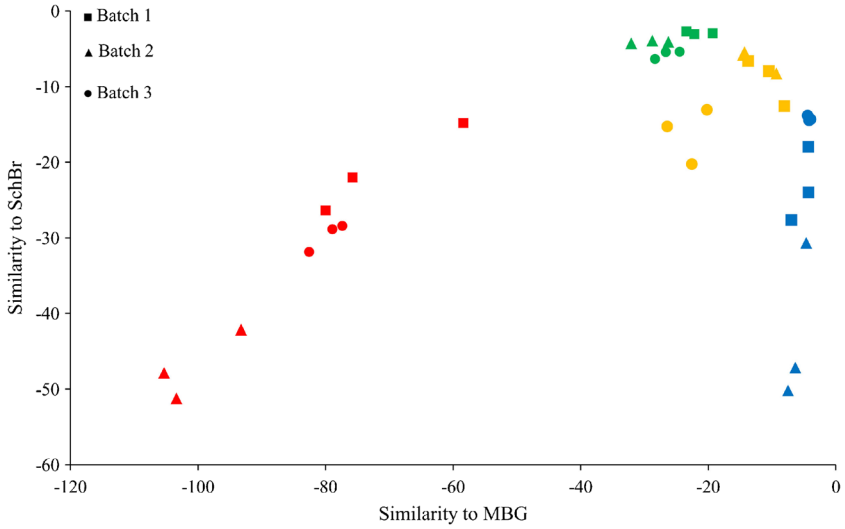

Fig. 4 PCA-LDA of Raman spectra of *Bc*T sporulated in different media formulations (SchAg [*red*], SchBr [*green*], MBG [*blue*], and G [*orange*]) with three replicate batches (denoted by *different symbols* for different batches) prepared for each medium and three samples analyzed per batch. The relative similarity of each sample to MBG and SchBr, respectively, is plotted with a value of 0 indicating a high degree of similarity to *Bc*T sporulated in the stated medium. The *Bc*T sporulated in CAB are off-scale and centered at coordinates of (x=−875, y=−1000)

be identified as one of the five media formulations in Fig. 4 using Raman spectroscopy.

To potentially increase the number of sporulation media that can be identified, Raman spectra from *Bc*T sporulated using an additional seven media formulations (for a total of 12 media formulations) were also measured. These were single batches prepared by different scientists at different times which may add additional, uncontrolled variation in the Raman spectra; however, the classification of additional samples by sporulation media was investigated to assess the value of this method at this proof-of-concept stage. The cross-validation classification accuracy for the tiered PCA-LDA is shown in Table 4.

Table 4 Accuracy of classification of *Bc*T sporulation medium using a two-tiered PCA-LDA approach

	Tier 1 (%)	Tier 2[a] (%)
MBG	100	–
G	67	100
CAB	100	–
SchBr	89	100
SchAg	100	–
BHI	88	100
CA	100	–
CAD	100	–
LD82	88	100
LL	100	–
MHA	88	75
NZ	100	–

[a] Raman spectra from *Bc*T sporulated using the media that were identified with 100 % accuracy were held out from the second PCA-LDA tier

In this data set, all 12 samples except MHA can be classified by sporulation medium with 100 % accuracy using the tiered PCA-LDA approach. The first tier allows the classification of MBG, CAB, SchAg, CA, CAD, LL, and NZ samples. The second tier classifies G, SchBr, BHI, and LD82 with only MHA not fully separated. The overall classification accuracy was 98 % (100 % other than MHA). These encouraging results indicate a significant chemical signature that persists even after thorough spore washing. After analysis of replicate batches of the additional seven media types, a training set could be generated and used for identification of the medium used for *Bc*T sporulation. A more comprehensive database of bacterial Raman spectra should be developed to further test and potentially widen the applicability of this approach to other bacterial species (e.g., *B. anthracis*) and additional sporulation media of interest in forensic science, medical diagnosis, and food safety.

Effect of spore storage procedure on Raman spectra

Variables other than the sporulation medium formulation may also impact the spore Raman spectrum. For example, freezing has been demonstrated to have no direct impact on the viability of *Bacillus* spores but does have an impact on their resistance to inactivation by chlorine exposure (which removes proteins from the spore coat) [49, 50], perhaps by weakening the protein-based spore coat. The results shown in Fig. 4 were obtained using spores that had been stored at 4 °C, spotted/dried overnight on a CaF_2 slide, and analyzed shortly after sporulation (i.e., freshly prepared). For longer term storage, spores may be wet-frozen or freeze-dried as described in the "Materials and methods." To assess the impact of these

storage procedures, wet-frozen and freeze-dried samples of *Bc*T sporulated in MBG were analyzed and the sporulation medium was determined using the training set in Fig. 4 (which contains nine freshly prepared MBG samples).

All wet-frozen and freeze-dried test samples were correctly classified as having been sporulated in MBG. Based on Fig. 4, freeze-drying had little impact on the chemical makeup of *Bc*T sporulated using MBG, as evidenced by their proximity to freshly sporulated *Bc*T. Although no visual microscopic changes such as cell lysing were detected after wet-freezing (in agreement with previous results [49, 50]), small differences in Raman spectra were observed between fresh or freeze-dried and wet-frozen spores (as also demonstrated in Fig. 5). When a separate PCA-LDA analysis was conducted with only the fresh, wet-frozen, and freeze-dried *Bc*T cells sporulated in MBG, these changes allowed wet-frozen spores to be discerned from fresh and freeze-dried spores (results not shown). Even though the differences in Raman spectra did not cause any misclassification in Fig. 5, there are small differences in Raman spectra between fresh or freeze-dried and wet-frozen samples which may be an issue when comparing spores preserved by different methods. This data indicates freeze-drying results in spores that appear to be chemically similar to fresh ones and thus should be preferred by researchers for long-term spore storage over suspension in water and freezing.

Potential for comparison with archived spore samples

*Bc*T sporulated using the same procedure, but by different scientists with different media lots and/or different storage times, adds a number of latent variables to the spore preparation process that may impact the Raman spectra. Thus, *Bc*T cells sporulated in G and freeze-dried for storage by three different scientists at different times were examined: scientist I in Oct. 2007, scientist II in May 2008, and scientist III in Sept. 2013. The potential impact of these latent variables on the discrimination of different media types was assessed by using the PCA-LDA model from Fig. 4 as a training set to classify the nine additional *Bc*T samples sporulated in G by scientists I, II, and III. The classification is shown in Fig. 6.

Only two of the nine additional samples of *Bc*T sporulated in G and freeze-dried were correctly classified. Three samples were classified as originating from SchBr and four samples from MBG. This may be a result of the close grouping of SchBr, G, and MBG with G at the center. The changes in Raman spectra between latent variables are impacting the Raman spectra in these spore preparations and require further study for identification of the cause. Some of the potential latent variables in the spore preparation process which may explain these results include small variations from the nominal sporulation procedure between scientists, differences in chemical composition between-media lots, and changes in the spore makeup over time. However, analyzing a training set which includes replicate preparations covering these potential latent variables may result in increased classification accuracy. For example, when a new PCA-LDA analysis was conducted with the additional nine samples of G sporulated *Bc*T included in the original training set shown in Fig. 4, only the three scientist III samples are misclassified as SchBr (data not shown).

Conclusions

Raman spectroscopy is a fast, nondestructive method for analyzing bacterial spores to discern physiochemical properties related to growth and sporulation. In this proof-of-concept study, *Bc*T samples were correctly classified according to the medium used to induce sporulation for 100 % of spores grown in a controlled manner by the same scientist using the initial

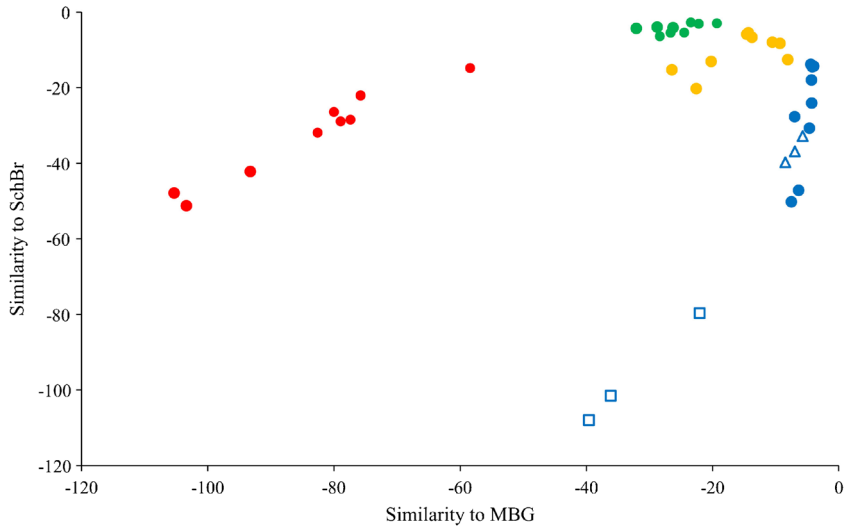

Fig. 5 Effect of storage procedure on PCA-LDA classification. Three wet-frozen (*squares*) and three freeze-dried (*triangles*) samples of *Bc*T sporulated in MBG were classified using a training set (*circles*) consisting of formulations (CAB (off-scale), SchAg [*red*], SchBr [*green*], MBG [blue] (nine fresh samples), and G [*orange*])

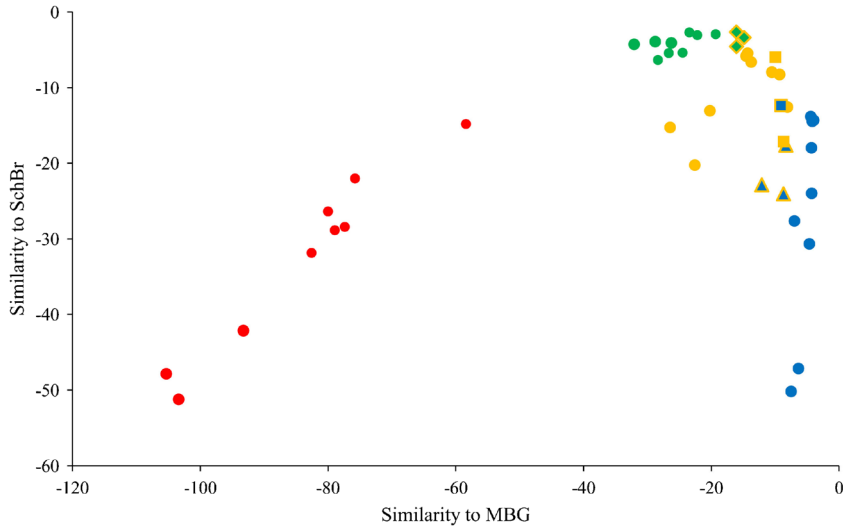

Fig. 6 PCA-LDA classification of *Bc*T sporulated in G by scientists I (*triangles*), II (*squares*), and III (*diamonds*) using different media lots and stored for different periods of time using the training set (*circles*) based on CAB (off-scale) SchAg [*red*], SchBr [*green*], MBG [*blue*] and G [*orange*]) in Fig. 4. The actual sporulation medium is denoted by the *outline color* and the sporulation medium predicted by PCA-LDA is denoted by the *filled color*

five media. To our knowledge, the identification of forensic signatures present in Raman spectra due specifically to the sporulation medium formulation has not been previously demonstrated with sufficient replication to make statistical inferences. *Bc*T spores produced using a set of 12 media formulations were also analyzed and resulted in 98 % classification accuracy (with all but one medium correctly classified with 100 % accuracy). Since the sporulation medium has a reproducible impact on the Raman spectrum of a *Bc*T spore, this technique may be able to provide leads in an investigation of a criminal release of microorganisms.

Other variables such as the spore storage procedure, the scientist preparing the spores, the media lot, and the spore storage time did have an impact on the spore Raman spectrum. Differences were discerned between spores that had been freshly prepared or freeze-dried and spores that had been wet-frozen, although the differences did not impact the PCA-LDA classification of the sporulation medium. Latent variables did impact the classification of *Bc*T sporulated in G by different scientists using different media lots and stored for different periods of time.

In addition to the forensic utility, these results with *Bacillus* spores support the concerns of other experimenters [10, 35, 51] using Raman spectroscopy for species identification in the clinical and food science fields. For example, when clinical samples are examined, the clinical specimen from a human body fluid is cultured on a growth medium, the spectra are collected, and the data is compared to reference samples of a known bacterial species. According to the results presented here, small changes in the Raman spectrum and an increased error rate of species misidentification may result if the medium used to culture the reference and patient samples are not the same.

Because variables such as growth time and temperature have been shown to influence bacterial spore Raman spectra [10, 11, 51], further studies still need to be performed. Additional variables that warrant investigation include different brands and lots of media components, long-term spore storage under different environmental conditions, more media formulations (including variation of the vegetative starter culture medium), purification processes, and spore inactivation procedures. The within-medium variation resulting from spore preparations by different scientists also deserves further experimentation. Before implementation of this method for attribution, this proof-of-concept work must be followed up with collection of data obtained on the actual target organism of interest, such as *B. anthracis*. Based on the results of this study, Raman spectroscopy may be able to provide an orthogonal means for identification of the medium used to sporulate *Bc*T, which may be transferable to *B. anthracis*.

Acknowledgments We are very grateful to Jack Hietpas for independent statistical analysis and confirmation of the results presented here. We would also like to thank Jason Brewer (FBI Laboratory, Chemistry Unit) for providing assistance with the Raman instrument and Lindsay Lundberg for spore preparations. This research was supported in part by an appointment to the Visiting Scientist Program at the FBI Laboratory Division, administered by the Oak Ridge Institute of Science and Education, through an interagency agreement between the US Department of Energy and the FBI.

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