RAPID DETERMINATION OF ENDOSPORE VIABILITY BY HYPERPSECTRAL REFLECTANCE FOLLOWING SURFACE DECONTAMINATION

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ABSTRACT

Bacterial spores, or endospores, such as those of *Bacillus anthracis*, are an asymmetrical threat. Decontamination following endospore release is costly and can be difficult and slow to confirm. Part of the constraint is because rapid and accurate means to differentiate live (viable) from dead (non-viable) bacterial endospores are lacking. Endospores have minimal metabolic activity and withstand many sterilization techniques, yet they can germinate and grow rapidly in favorable conditions, increasing the likelihood of disease. In scenarios such as post-infrastructure decontamination, a rapid and quantitative assessment of endospore viability is prerequisite to establishing health risks. Current and accepted methods for determining endospore viability require culturing, which takes several days. Scatter and depolarization signatures from polarized laser sources and micro- and nano-sensors show promise as a means of optically confirming endospore *presence*, but have not been shown to have the ability to distinguish viable from non-viable endospores. In the laboratory, we have exploited the oxidative alteration of the spore coat caused by decontamination solutions and used hyperspectral reflectance and appropriate post-processing to differentiate viable from non-viable endospores following endospore decontamination by oxidants.

1. INTRODUCTION

Currently there are no rapid and accurate means to differentiate live (viable) from dead (non-viable) bacterial endospores. We have exploited differences in hyperspectral reflectance signatures following oxidative decontamination to differentiate viable from non-viable endospores. The objective of this research was to find a rapid, non-destructive method to determine endospore viability. A "stand-off" approach does not require germination and growth and does not require the addition of dyes or stains.

The ability to identify viable endospores and track their die-off during decontamination of buildings and HVAC systems is important to the Army, DoD, and civilian infrastructure. Biological weapons of mass destruction (BWMD) are a high probability asymmetrical threat. Bacterial endospores, because of their ease of production coupled with genetic manipulations, are attractive as both crude and advanced vectors. In theory, a single cell can result in the onset of disease. As demonstrated by efforts to decontaminate buildings, rapid techniques to confirm endospore death do not exist. A means for rapidly determining endospore viability following decontamination would fill a serious and costly knowledge gap.

A bacterial endospore is a unique morphology that has evolved as a survival mechanism in certain bacteria such as *Bacillus anthracis* (Figure1). The intrinsic minimal metabolic activity of endospores makes determining their viability very difficult. Bacterial endospores have extreme tolerance to sterilization and exhibit minimal metabolic activity, yet they are capable of rapid growth in favorable conditions. Recent investigations (Hanna, 2004) have shown that *B. anthracis*, a known BWMD and bacterial endospore producer, cannot only survive, but can grow in non-mammalian systems such as soil. An optical confirmation of endospore non-viability under a range of conditions typical of buildings, tents, ventilation systems, and other surfaces would address the existing knowledge gap regarding endospore viability.

Current and accepted methods for determining endospore viability require growth on microbiological media, which takes days for verification due to the time requirement for growth to ensue. Micro- and nano-sensors show promise as a means of optically confirming endospore *presence*, but have not been able to distinguish between *live* and *dead* spores. Scatter and depolarization signatures from polarized laser sources can quantify aerosol particle size and shape and subsequently infer endospore presence but scattering, and in particular

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reflectance, have not been used to provide information on viability. Staining and other direct observation, while useful for determining viability of vegetative cells, do not differentiate viable from non-viable endospores (Figure 2). We have investigated endospore viability *in situ* by quantifying differences in composite hyperspectral signatures from both live and dead endospores and initiated a spectral library of those signatures.

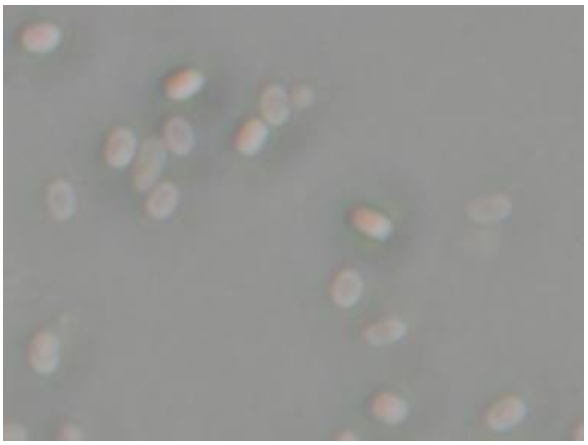

Fig. 1. Visible, light microscopy image of endospores.

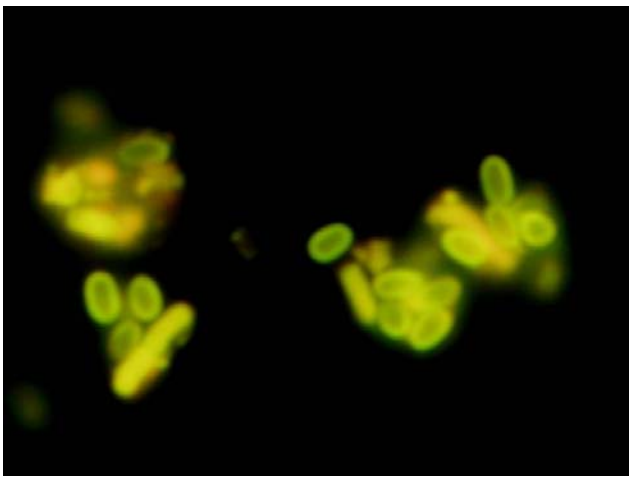

Fig. 2. *Bacillus globigii* viable and non-viable endospores mixed at a 50:50 ratio and stained with a "Live-Dead" stain developed for vegetative cells. Viability stains developed for vegetative cells do not differentiate viable from non-viable endospores.

We recognized that many sterilants, such as chlorine dioxide and hydrogen peroxide, alter the exosporium (Young and Setlow, 2003). We hypothesized that, because decontamination solutions use an oxidant to attack the outer coat of endospores, the reflectance signature of endospores killed by an oxidant would be measurably different from viable endospores, and that hyperspectral techniques would enable us to identify regions important in a composite signature. Figure 3 illustrates the concept (Reynolds et al., 2007). Further, we hypothesized that the reflectance differences might be subtle, and identifying key spectral regions would require multivariate and data reduction statistical techniques.

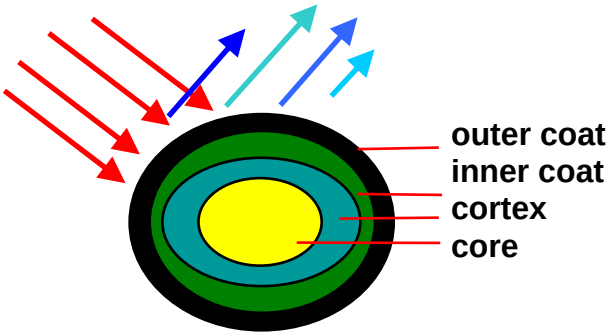

Fig. 3. Conceptual diagram of hyperspectral reflectance approach.

Advantages of using hyperspectral reflectance signatures to differentiate viability include 1.) speed, 2.) amenability to *in situ* techniques, and 3.) no requirement for stains or dyes. This more direct approach would reduce uncertainties associated with sampling, chain of custody, preparation, and growth analysis by removing the innate variability associated with time, temperature, and concentration that are inherent to staining and grow-out procedures.

2. EXPERIMENTAL

2.1 Experimental Design

We used three endospore-forming *Bacillus* species, *B. globigii*, *B. megaterium*, and *B. cereus* and three treatments. Treatments included a control, which were live (viable) endospores, and killed (non-viable) treatments using either hydrogen peroxide (H_2O_2) or chlorine dioxide ClO_2). Experimental units consisted of bacteria-treatment combinations. To provide sufficient precision for statistical analysis and to begin to develop a signature library, we used thirty replications for each experimental unit. We confirmed viability of the control endospores and sterilization efficacy using grow-out techniques.

2.2 Bacterial Growth, Sporulation, and Endospore Kill

In brief, Bacillus cells were streaked onto either modified Schaeffer's sporulation agar containing glucose or Nutrient agar and incubated at 35ºC for 2 days to induce sporulation. Cells from each plate were harvested and transferred to individual 50-ml centrifuge tubes containing sterile water. Harvested endospores were washed multiple times using sterile distilled water,

overnight shaking, and centrifugation. Following washing, we used direct microscopy to ensure that washing removed vegetative cells and cell debris. To ensure statistical homogeneity, each sample then was split into three equal parts for treatment.

Endospores were killed by adding H_2O_2 or ClO_2 to glass tubes containing washed endospores to final concentrations of 10% and 250 ppm of H_2O_2 and ClO_2, respectively.

2.3 Endospore "Target" Preparation

To obtain targets for hyperspectral reflectance, we coated filters with a layer of treated endospores. We used black membrane filters having a 0.2-µm pore size. Endospores were transferred to the filters using a Buchner funnel and mild suction. Funnels were bleached and rinsed between transfers to avoid cross contamination. Following endospore transfer, filters were placed in labeled Petri dishes and stored in a desiccator prior to obtaining reflectance signatures.

2.4 Viability Confirmation

To ensure that spores were killed by the two decontamination treatments and remained viable in the control treatment, aliquots of endospores from each treatment were spread plated onto tryptic soy agar. Following 48 h incubation, no growth occurred on the plates of the killed spores. Growth did occur on the plates of viable spores. Additionally, following filtration and imaging, several of the filters were plated by direct contact onto agar to transfer the endospores to the agar surface. Again no growth was observed for the killed spores, while growth occurred for the filters of viable spores.

2.5 Hyperspectral Signatures

An Analytical Spectral Device (ASD) visible/near-infrared (VNIR) spectrometer was used to acquire reflectance data in the spectral range of 350 nm to 2500 nm with 1 nm resolution, from the surface of each endospore coated filter. In general, the ASD was programmed to average 100 measurements, each having a 1s integration time for each spectrum acquired.

To obtain spectra, endospore-coated filters were dried for a minimum of 24 h in a desiccator and the spectra were obtained with the ASD using an 8˚ field of view lens and a consistent light source of 3200K. With the lens positioned at approximately 7 cm from the read surface the diameter of the read area was approximately 1 cm. Filters were randomized prior to reading and each filter was read three times with the filter moved slightly prior to reading so that a different area on the filter was measured. Blank filters were read in the same manner. Three such spectra and accompanying errors were acquired for each endospore treatment, averaged, and placed into a spectral library. Appropriate controls for filters and sterile media were included.

2.5 Data Analyses

Spectral data were normalized to a spectralon standard and expressed as percentage reflectance for each wavelength. To reduce dimensionality and identify patterns suggesting important wavelengths for composite signatures, we used principal components analysis (PCA). Spectral resolution was reduced from 1 to 10 nm, prior to statistical analysis. The data set we developed had 30 replications, three species (*B. globigii*, *B. megaterium*, and *B. cereus*), three treatments (live, killed using peroxide (H_2O_2) or chlorine dioxide (ClO_2)) Reduced dimensionality and resulting composite signatures also suggested key wavelength bands for later multispectral (rather than hyperspectral) applications.

3. RESULTS

3.1 Hyperspectral Reflectance Signatures

Figure 4 shows a typical signature for an individual experimental unit representing an endospore species/treatment combination. Although regions of stronger and weaker absorbance can be seen, separating endospore viability was more challenging and, as foreseen, identifying differences among treatments and hence viable and non-viable endospores by direct comparison of these spectra was difficult. Using direct comparison of the data, differences were suggested, but not statistically defensible.

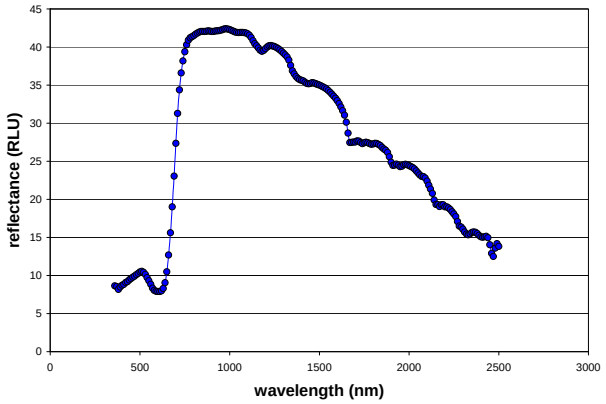

Fig. 4. A typical reflectance signature from an endospore coated filter.

3.2 Principal Component Analysis – Treatments within Species

Differences in reflectance patterns can be seen better when spectra from all replications of all treatments for a single *Bacillus* species are plotted as a response surface (Figure 5). In Figure 5, the x-axis represents 90 experimental units, or 30 replications of each of three treatments (viable, H_2O_2 killed, and ClO_2 killed), the y-axis represents the reflectance wavelength, and the z-axis represents the response. From this visual analysis, patterns become more apparent and differences among treatments are suggested, but clearly the strength of the similarities or contour pattern differences among treatments is partially dependent on the interpolation scheme and granularity used to develop the contours. Reflectance results for each treatment applied to each *Bacillus* species, or for like treatments applied to different species, allow creation of analogous data sets for other comparisons.

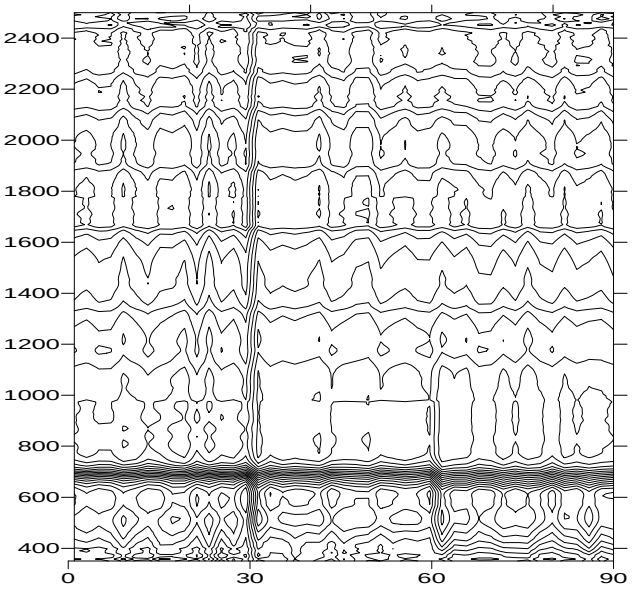

Fig. 5. Reflectance spectra from all replications of all treatments for a single *Bacillus* species expressed as a response surface and plotted as a contour plot. Left to right on x-axis, data are 30 replications of H_2O_2-killed, viable, and ClO_2-killed.

To address these vagaries, we used principal component analysis. Figure 6 shows PCA results from the three treatments applied to *B. cereus* endospores. Treatment differences were adequately defined with the first two principal components. Live or viable endospores differentiated readily from killed, non-viable endospores. We observed similar treatment results for *B. megaterium* and *B. globigii* (data not shown).

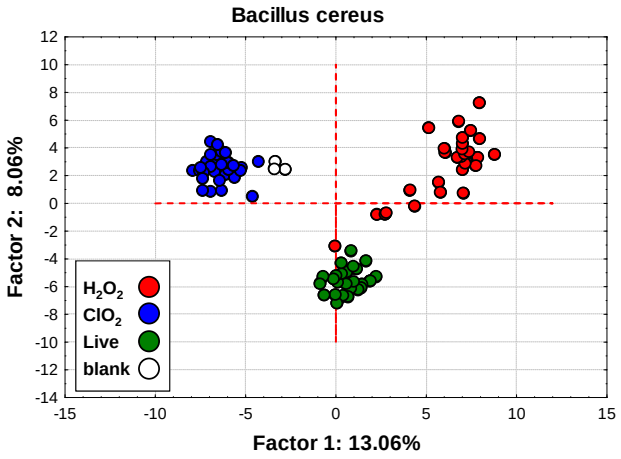

Fig. 6. Principal component analysis of reflectance spectra from all replications of all treatments for *B. cereus*. An analogous approach yielded similar results for *B. megaterium* and *B. globigii* (data not shown).

3.3 Principal Component Analysis – Separating Species

Using the same PCA approach applied to data across species but within treatments, we separated all three species, *B. cereus*, *B. megaterium*, and *B. globigii*. Figure 7 illustrates results for the three live *Bacillus* spp. An analogous PCA approach applied across species for the two killed treatments also showed species separation (data not shown).

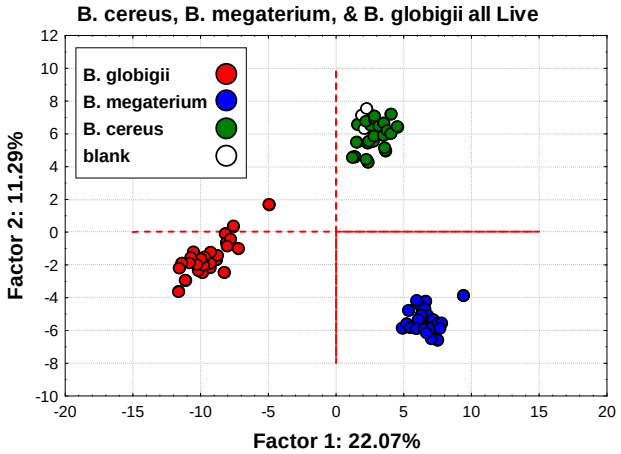

Fig. 7. Principal component analysis of reflectance spectra from all replications of all species for live (viable) endospores. An analogous approach yielded similar results for H_2O_2 killed and ClO_2 killed treatments (data not shown).

4. DISCUSSION

Grow-out tests confirmed our hyperspectral results. In subsequent experiments using endospore washing, lysozyme treatment, and gel electrophoresis, we have shown protein coat differences among the viable and killed endospores (data not shown). These data further suggest that changes in the protein coat are associated with reflectance differences and support our hypothesis, that the oxidative decontamination alters the exterior of endospores, which in turn alters the reflectance signature in measurable ways. To date, we have not characterized the robustness of hyperspectral reflectance with respect to growth media or conditions, species, and other decontamination techniques. Our data suggest reflectance signature changes due to altered endospore coat is a useful phenomenon on which to develop a real-time sentinel to monitor decontamination efficacy, and more broadly may form the basis for the development of a rapid and portable capability for answering the fundamental question "is a bacterial endospore alive or dead". In subsequent analysis, we also have used hyperspectral imaging so that the full range of data present in a single field of view was exploited on a pixel-by-pixel basis (Anderson et al., 2008). Viable and non-viable endospores reflected characteristic composites of wavelengths similar to those obtained using the hyperspectral radiometer, demonstrating the feasibility of a multispectral image-based technique for the *in situ* identification of individual viable and non-viable endospores.

CONCLUSIONS

To the best of our knowledge, hyperspectral reflectance signatures coupled with PCA as an indicator of endospore viability have not been considered previously. Importantly, grow-out tests confirmed our findings. These results provide a strategy for a phenomena-based signature library that would provide a distribution or error term associated with the signature for both live and dead endospore populations, a prerequisite for use in both research and real-world applications.

These data show that hyperspectral reflectance has utility for *rapidly* determining endospore viability, that oxidative decontaminant solutions alter the endospore hyperspectral signatures differently, and that the *species* also had different hyperspectral signatures. Advantages of using hyperspectral reflectance signatures to differentiate viability include 1.) speed, 2.) amenability to *in situ* techniques, and 3.) no requirement for stains or dyes. This more direct approach would reduce uncertainties associated with sampling, chain of custody, preparation and growth, and analysis by removing the innate variability associated with time, temperature, and concentration that are inherent to staining and grow-out procedures.

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