Absorption Edge Imaging of Sporocide-treated and Non-treated Bacterial Spores

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When deprived of nutrients, spore forming bacilli produce endospores which are remarkably resistant to chemical sterilization.^{1, 2} Little is known about the morphology and response of these spores following exposure to sporocidal agents. Light microscopy does not provide sufficient resolution for studying the rupture of the spore coat and fate of intracellular material. Transmission and scanning electron microscopy offer superior resolution but require specimen preparation methods that induce physiologic as well as morphologic changes in the spores, thereby making accurate interpretation of micrographs difficult. To eliminate the possible artifacts induced by chemical fixation, dehydration, embedment, staining and sectioning, treated and non-sporocide-treated endospores of B. thuringiensis and B. subtilis were imaged by x-ray contact microscopy using monochromatic x-rays.

Soft x-rays (1-10 nm wavelength) offer the biologist distinct advantages for imaging samples that are particularly sensitive to electron or proton beams, or to conventional specimen preparation procedures.³ These x-rays produce less specimen damage than electrons and protons, and they penetrate the biological specimen more easily than electrons without necessitation of a vacuum environment.⁴ By using monochromatic x-rays for exposure, it is possible to augment selective absorptivity and produce x-ray contact replicas that reveal the location of specific elements within the tissue.⁵ With the advances in synchrotron technology, it is now possible to obtain monochromatic x-rays with sufficient coherence, brightness and dose for the brief exposure times required with biological hydrated samples. Although quantitative x-ray microanalysis of frozen biological tissue can also identify specific elements within the sample, soft x-ray absorption edge microscopy offers a less damaging method that requires little specimen preparation or skill; has an inherent contrast mechanism (selective x-ray absorptivity) which eliminates the need for staining or chemically fixing a specimen;³ and the ability to image elements (such as oxygen, nitrogen, carbon, etc.) that cannot readily be viewed or identified by conventional x-ray microanalysis methods. This paper represents some of our first attempts at absorption edge imaging and in no way presents this methodology as a quantitative trace analysis technique.

1. METHODS

1.1 Endospore Preparation

Bacillus subtilis var niger ATCC 7932 was obtained from American Type Culture collection and Bacillus thuringiensis was obtained from Dipel, Abbot Laboratories. Spores were grown on slants of AK Sporulating Agar No. 2 (BBL, Cockeysville, MD) in 16 oz prescription bottles at 35°C aerobically. After maximal sporulation, the spores were harvested in deionized water and heated

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to 80°C for 20 min to kill any remaining vegetative cells. The spores were washed three times with sterile deionized water and stock suspensions were stored at 2-8°C until needed. For comparison, spore samples were either vacuum dried onto aluminum scanning mounts or fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, followed by acetone dehydration and critical point drying in liquid CO₂. These samples were rotary coated with 7-10 nm of gold-palladium in an Edwards vacuum evaporator prior to examination in an AMRAY 1000A SEM at 20 or 30 keV accelerating voltage. Spores treated with a vanadium-containing sporocide for 30 sec, 10 min and 50 min were either vacuum dried or prepared in the aforementioned way and viewed by scanning electron microscopy.

1.2 X-ray Detector and Exposures

Polymethyl methacrylate (PMMA) dissolved in chlorobenzene was spun at 1 µm thickness onto silicon wafers and baked for 1 hr at 156°C. B. thuringiensis and B. subtilis spores were smeared as a monolayer onto the photoresist and excess fluid wicked-off with filter paper. These spores were exposed to monochromatic x-rays near the nitrogen absorption edge (30.99 nmλ) and above and below the V L_{III} absorption edge. B. thuringiensis and B. subtilis spore preparations treated with sporocide for 30 sec, 10 min and 50-80 min were exposed to monochromatic x-rays near the nitrogen edge and above and below the V L_{III} edge. For this study the exposures were made at a dedicated soft x-ray beamline (U15) at the high brightness synchrotron radiation facility at Brookhaven National Laboratory, National Synchrotron Light Source. A toroidal grating monochromator provided a monochromatic beam at the 750 MeV VUV storage ring. All of the x-ray exposures ranged from 20.7 to 28.3 µA minutes.

Following x-ray exposure, the spores were washed off the photoresist and the photoresist developed in 1:1 isopropanol/methyl-isobutyl ketone (MIBK). The x-ray contact replicas were rotary coated with 5-8 nm gold-palladium in an Edwards vacuum evaporator and viewed at 60-75° tilt, 20 or 30 keV accelerating voltage.

2. RESULTS AND DISCUSSION

X-ray contact replicas of B. subtilis and B. thuringiensis endospores made following exposure to x-rays near the nitrogen edge revealed details of the spore coat and exosporium. Nitrogen absorption maps of the spores were used to monitor spore morphology and general protein distribution on the silicon wafer. At 30 sec after sporocide treatment, nitrogen x-ray maps of B. thuringiensis revealed alterations in the spore coat and at 5 min post-treatment complete disruption of the spore coat. B. subtilis treated with sporocide for 1-5 min produced nitrogen x-ray maps with wrinkling and blebs on the spore coat but complete disruption of the spore coat was not found even after 80 min of sporocide treatment.

X-ray exposures of sporocide-treated B. subtilis spores made below the absorption edge of vanadium (V) produced x-ray contact replicas with no clearly intact spores. The outline of the disrupted spore coat could be seen surrounded by a puddle of material with numerous globular inclusions (Fig. 1). Areas of the photoresist lacking the hazy images of the spores also lacked the puddles of globular material, suggesting that the sporocide treated B. subtilis spores may exude or lose their intracellular contents without the total destruction of the spore coat. Above the absorption edge of V the same sporocide-treated B. subtilis spores revealed the hazy image of the spore coat and

a surrounding puddle of material, however, in this case the formerly globular inclusions in the puddle now appear dendritic with six or more arms radiating from a central core (Fig. 2). These rosette-like arrays surrounding the spore coats were only seen in exposures made just above the V L_{III} absorption edge. The nitrogen x-ray replicas and replicas made below the V L_{III} edge using the same specimen showed no rosettes but rather indistinct globular material in the puddles surrounding the spore coats. One would expect areas of the specimen originally containing V to be strongly imaged when exposed to monochromatic x-rays just above the absorption edge of the V L_{III} line and these same structures to be poorly imaged at x-ray wavelengths below the absorption edge of the element in question.⁶

Fig. 1. Sporocide treated B. subtilis x-ray contact replica taken below the absorption edge of vanadium shows hazy image of spore coat (s) surrounded by a region of material with elliptical inclusions.

Fig. 2. Same sample as figure 1 when imaged with x-rays above the absorption edge of vanadium shows same puddle surrounding the spore coat, but the inclusions appear crystalline or as rosettes (arrows).

X-ray contact replicas of sporocide treated B. thuringiensis spores revealed such extensive fragmentation of the spore coat and cellular debris at 30 sec or more post-sporocide treatment that it was not possible to identify individual structures in the micrographs. This was also the case with the B. thuringiensis glutaraldehyde fixed and vacuum dried control preparations.

The capability to reveal compositional information from the same specimen by imaging it with different monochromatic x-rays poses many exciting possibilities. The fact that the rosette-like images seen above the V absorption edge may represent the V in the sporocide (bound to endogenous dipicolinic acid) suggests that it may be possible to learn how these sporocides may attack and kill the more resistant bacterial spores. Although it might be considered that this type of analysis could be done by electron or x-ray induced x-ray microanalysis, it is not possible to produce images of, or analyze these puddles around the disrupted spores following most specimen preparation procedures. By x-ray fluorescence spectrometry it would be impossible to obtain this spatial resolution, and by electron microprobe analysis the problems of mass loss, and orientation of an unstained sample, would make this type of analysis extremely difficult. The ability to image partially hydrated, unstained unfixed biological material and gain immediate information about the possible localization of a specific element without the beam damage and specimen trauma experienced with other analytical techniques, is an exciting step forward in the realm of biological analysis.

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4. REFERENCES

1. P. Fitz-James and E. Young: In The Bacterial Spore, ed. by G. Gould and A. Hurst, (Academic Press, NY, 1969) p. 39.
2. C. R. Phillips: Bact. Rev. 16, 135 (1952).
3. B. J. Panessa-Warren and J. B. Warren: Ann. NY Acad. Sci. 342, 350 (1980).
4. J. Kirz and D. Sayre: In Synchrotron Radiation Research, ed. by S. Doniach and H. Winick, (Plenum Press, NY, 1980) p. 277.
5. J. Kirz: Ann. NY Acad. Sci. 342, 273 (1980).
6. L. Beese et al., Biophys. J. 49, 259 (1986).

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