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UV Resistance of *Bacillus anthracis* Spores Revisited: Validation of *Bacillus subtilis* Spores as UV Surrogates for Spores of *B. anthracis* Sterne

Wayne L. Nicholson* and Belinda Galeano

Department of Veterinary Science and Microbiology, University of Arizona, Tucson, Arizona 85721

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Recent bioterrorism concerns have prompted renewed efforts towards understanding the biology of bacterial spore resistance to radiation with a special emphasis on the spores of *Bacillus anthracis*. A review of the literature revealed that *B. anthracis* Sterne spores may be three to four times more resistant to 254-nm-wavelength UV than are spores of commonly used indicator strains of *Bacillus subtilis*. To test this notion, *B. anthracis* Sterne spores were purified and their UV inactivation kinetics were determined in parallel with those of the spores of two indicator strains of *B. subtilis*, strains WN624 and ATCC 6633. When prepared and assayed under identical conditions, the spores of all three strains exhibited essentially identical UV inactivation kinetics. The data indicate that standard UV treatments that are effective against *B. subtilis* spores are likely also sufficient to inactivate *B. anthracis* spores and that the spores of standard *B. subtilis* strains could reliably be used as a biodosimetry model for the UV inactivation of *B. anthracis* spores.

The October 2001 bioterrorist attack with *Bacillus anthracis* spores has sparked renewed interest in studying methods of bacterial spore inactivation and the mechanisms by which spores resist the lethal effects of various disinfection treatments. UV radiation at a 254-nm wavelength has been used as an efficient and cost-effective means of disinfecting surfaces (1, 4, 15, 16, 17), building air (3, 8, 13), and drinking water supplies (5). The most reliable method for testing the efficacy of UV disinfection equipment is biodosimetry, the use of a test organism to measure the biologically effective UV dose (2). Commonly used test organisms for UV biodosimetry studies are bacterial spores, usually spores of *Bacillus subtilis*, due to their high degree of UV resistance, reproducible inactivation response, and ease of use (reviewed in references 9 and 11). In particular, exhaustive testing of spores of the *B. subtilis* strain ATCC 6633 has resulted in this strain serving as the current European biodosimetry standard for 254-nm UV disinfection of drinking water (5).

Semilogarithmic plots of spore inactivation versus UV fluence (dose) produce a characteristic curve, consisting of a shoulder at low UV doses, followed by a curve reflecting exponential inactivation at higher UV doses (Fig. 1). Two parameters often used to describe spore resistance to UV are (i) the UV dose lethal for 90% of the population (LD_{90}) and (ii) the decimal reduction value (*D* value), defined as the UV dose which reduces spore viability by a factor of 10, measured from the exponential portion of the inactivation curve (6, 11). For example, from the published data of Hoyer (5) (Fig. 1), it can be calculated that spores of *B. subtilis* ATCC 6633 exhibit an LD_{90} and a *D* value of 260 and 120 J/m^2, respectively (Table 1).

In stark contrast to the extensively characterized UV inactivation response of *B. subtilis* spores, much less work has been performed to characterize the UV inactivation kinetics of *B. anthracis* spores. Perhaps the most complete work was a study which examined UV resistance of spores of the attenuated *B. anthracis* Sterne animal vaccine strain (7). From examination of the data presented in that report (7), we calculated that the *B. anthracis* Sterne spores exhibited LD_{90} and *D* values of 810 and 540 J/m^2, respectively (Table 1). Taken at face value, these data might be cause for concern, as they would suggest that *B. anthracis* Sterne spores are three to four times more resistant to 254-nm UV than are *B. subtilis* spores, and hence they may not be efficiently inactivated by standard UV disinfection treatments. However, the apparent UV inactivation response of a spore population can vary considerably, depending on the growth and sporulation environment and the methods used for spore purification, irradiation, and UV dosimetry (2, 5, 10). For this reason, the use of values from different studies to make comparisons of spore resistance to UV between different species is not valid. The most reliable method for testing intrinsic differences in spore resistance to UV between strains is to assay all strains in parallel, under identical conditions of growth, sporulation, spore purification, irradiation, dosimetry, and survival determination. In this article, we report that, when all such experimental variables were controlled, *B. anthracis* Sterne spores exhibited UV inactivation kinetics that were essentially indistinguishable from those of the spores of standard *B. subtilis* biodosimetry strains.

The bacterial strains used in this study were *B. subtilis* strain WN624 (*amyE*::*spc*), an isogenic derivative of the standard laboratory strain 168; *B. subtilis* strain ATCC 6633, provided by David Battigelli and available from the American Type Culture Collection (ATCC; Manassas, Va.); and *B. anthracis* Sterne, available from the Colorado Serum Company (Denver, Colo.). All of the bacteria were sporulated by incubation in liquid Schaeffer's sporulation medium (SSM) (14) at 37°C for 48 h with vigorous aeration in a rotary shaker. The cultures

* Corresponding author. Mailing address: Department of Veterinary Science and Microbiology, Building 90, Room 102, University of Arizona, Tucson, AZ 85721. Phone: (520) 621-2157. Fax: (520) 621-6366. E-mail: WLN@u.arizona.edu.

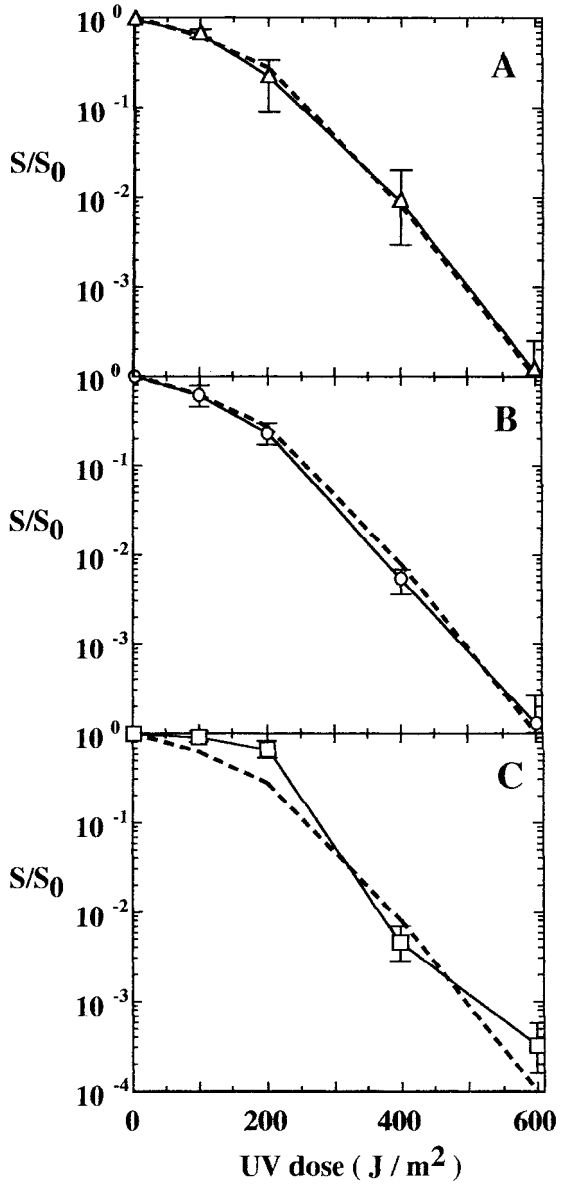

FIG. 1. UV inactivation curves of spores of *B. subtilis* ATCC 6633 (A) (triangles), *B. subtilis* WN624 (B) (circles), and *B. anthracis* Sterne (C) (squares). For comparison, the heavy dashed line in the background of each panel represents the UV inactivation curve of spores of *B. subtilis* ATCC 6633, as determined by Hoyer (5). The data points and error bars are the averages and standard deviations of results from four independent trials. S/S_0 represents the fraction calculated by dividing the viable spore titer at any given UV dose (S) by the spore titer obtained from the nonirradiated suspension (S_0).

were harvested by centrifugation (5,000 × *g*, 10 min, 25°C), and the spores were purified by using the lysozyme and buffer-washing method described by Nicholson and Setlow (12), heat shocked (80°C, 10 min), and stored in deionized water at 4°C. The viable titers of all spore preparations were determined by serial 10-fold dilution in phosphate-buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl, pH 7.4) and plating on SSM solidified with 1.7% agar.

UV irradiation was performed by using a commercial low-pressure mercury vapor lamp (model UVGL-25; UV Products, Upland, Calif.) with the filter removed, producing predominantly 254-nm-wavelength UV radiation. Throughout the experiments, the lamp was placed at a constant height of 42 cm above the target. The UV output was measured with a UVX radiometer fitted with a UVX-25 filter (UV Products), which was recently calibrated and traceable to the National Institute of Standards and Technology standard. Before the experiments were performed, UV fluence rates were measured at 0.5-cm intervals from the center of the target area along both the *x* and *y* axes, the values obtained were entered into UVCalc (a Microsoft Excel program developed by James Bolton and posted on the website of the International UV Association [http://www.iuva.org]), and the relative UV fluence rate pattern throughout the target area was computed. Once the relative UV intensity pattern was obtained, the UV fluence rate at the center of the target was measured before each experiment and entered into UVCalc to obtain the average UV fluence rate over the surface of the target for each experiment. Purified spores were diluted to a final concentration of 10^6/ml in 10 ml of PBS. The absorbance at 254 nm (A_{254}) of the resulting spore suspension was determined in a Beckman DU-6 UV/visible spectrophotometer, and the results were entered into UVCalc. Factors accounting for reflectance and depth of the spore suspension were also entered into UVCalc. The resulting exposure times needed for the desired UV doses were computed. The spore suspension was pipetted into the bottom of an uncovered 6-cm-diameter petri dish, a sterile stir bar was added, the dish was placed on a magnetic stir plate, and the samples were removed from the suspension at UV doses of 0, 100, 200, 400, and 600 J/m^2. The samples were serially diluted 10-fold in PBS, plated in duplicate on solid SSM, and incubated at 37°C for 48 h, and the resulting colonies were counted. The surviving fraction of spores was calculated by dividing the viable spore titer at any given UV dose (S) by the spore titer obtained from the nonirradiated suspension (S_0). Each irradiation experiment was repeated four times, and the averages and standard deviations of each set of data points were calculated by using Minitab version 10.5 software.

TABLE 1. UV (254 nm) resistance parameters of *B. subtilis* and *B. anthracis* spores

Strain	LD_{90} (J/m^2)	*D* value (J/m^2)	Reference or source
B. subtilis ATCC 6633	260	120	5
	245	130	This study
B. subtilis WN624	245	120	This study
B. anthracis Sterne	810	540	7
	275	140	This study

The UV inactivation curves obtained from the spores of *B. subtilis* ATCC 6633, *B. subtilis* WN624, and *B. anthracis* Sterne are presented in Fig. 1. For purposes of visual comparison of results among strains, we superimposed our inactivation curves onto the UV inactivation curve of the European standard biodosimetry strain ATCC 6633 that was reported by Hoyer (5) (Fig. 1). In our hands, the UV inactivation curves of the *B. subtilis* ATCC 6633 spores were very similar among the four replicate experiments and very closely matched the published data (5) (Fig. 1A), indicating that our spore purification

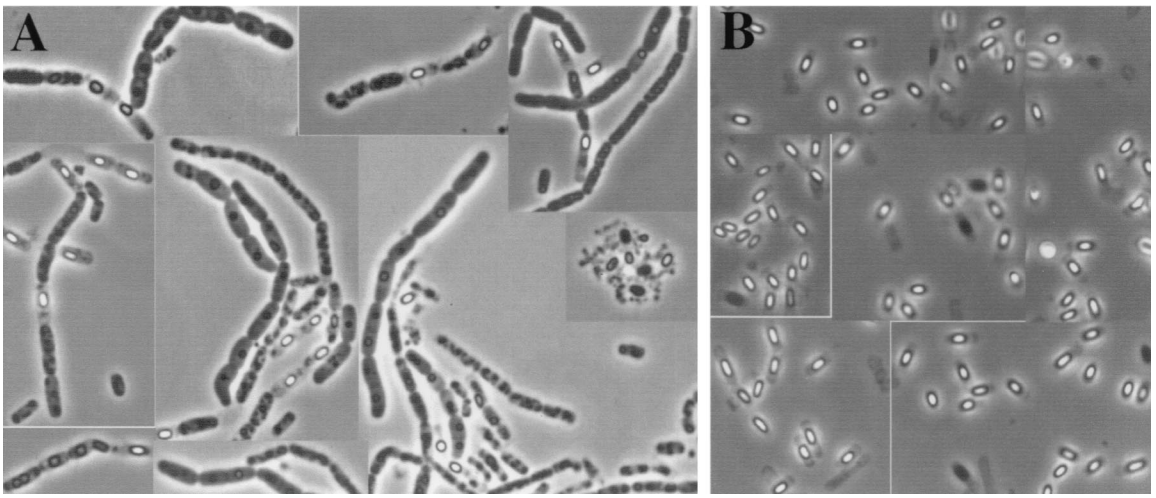

FIG. 2. Microscopic appearance of *B. anthracis* Sterne spores prepared by the method described in references 7 (A) and 12 (used in this study) (B). The images are of wet mounts prepared in water and photographed with phase-contrast optics. Original magnification, ×1,000. Several representative fields are presented in each panel.

and UV dosimetry protocols are highly reproducible, both in repeated trials in our laboratory and between our laboratory and that of Hoyer (5). The UV inactivation curve of our wild-type UV biodosimetry strain, *B. subtilis* WN624, also closely matched that of *B. subtilis* ATCC 6633 and exhibited high reproducibility among the four replicated experiments (Fig. 1B). The UV inactivation curve of the *B. anthracis* Sterne spores (Fig. 1C) differed slightly from that of the *B. subtilis* standard in exhibiting a slightly more pronounced shoulder at UV doses of 0 to 200 J/m^2 and a slight tail at UV doses between 400 and 600 J/m^2. These differences were minor, and overall the *B. anthracis* inactivation curve closely matched those obtained with the *B. subtilis* ATCC 6633 and WN624 spores. From the UV inactivation curves for all three spore types, we calculated the LD_{90} and *D* values for comparison with the literature values (Table 1). Under our experimental conditions, the *B. anthracis* Sterne spores exhibited LD_{90} and *D* values of 275 and 140 J/m^2, respectively, which are very close to the values computed for *B. subtilis* spores under the same controlled conditions (Table 1) and which are certainly not three to four times greater as previously reported (7).

As discussed above, some of the experimental factors which can lead to considerable variation in the apparent resistance of bacterial spores to UV are (i) the method of spore preparation, (ii) the irradiation conditions, and (iii) the dosimetry method used. We examined these factors in order to better understand how they influence the apparent UV resistance of a spore population.

Spore preparation. In an earlier benchmark study (7), spores were prepared by growing *B. anthracis* Sterne for 3 to 4 days on a blood agar plate, washing the harvested cells twice in sterile distilled water, and treating them with heat (60°C, 30 min). When we prepared *B. anthracis* Sterne spores by this method, we noted that sporulation did not occur at a high frequency, the majority of the cells present in the final suspension had not sporulated, and the few spores formed were often not completely liberated from the characteristic chains in which they had developed (Fig. 2). This situation could lead to an overestimate of *B. anthracis* spore resistance to UV for two reasons. First, 1 CFU can consist of multiple spores, all of which must be UV inactivated in order to result in the destruction of that particular CFU, thus leading to an artificial inflation of the apparent UV resistance. We also noted that spores were not completely liberated from spore chains when we allowed *B. anthracis* Sterne to sporulate in liquid SSM (data not shown). To correct for this, we subjected our *B. anthracis* Sterne spore preparations to further purification by lysozyme treatment and a series of buffer and detergent washes (12). Microscopic examination of the final spore suspension after further purification revealed that the spores had been liberated from chains and that the amount of nonsporulated cells and other debris had been reduced substantially (Fig. 2B).

Irradiation conditions. In the earlier benchmark study, *B. anthracis* Sterne spores were resuspended in phenolized gel-phosphate buffer (28 mM sodium phosphate, dibasic; 0.2% gelatin; 1% phenol [pH 7.2] [7a]) and UV irradiated at a relatively high density of spores (2×10^7 CFU/ml) (7). These conditions could lead to an artificial overestimate of the spore resistance to UV due to absorption of UV by the gelatin and phenol in the buffer and shielding by the additional turbidity provided by nonsporulated cells as discussed above (Fig. 2A). In the present work, spores were irradiated at a 20-fold-lower density (10^6/ml), at which no visible turbidity was evident and which resulted in low A_{254} values of the spore suspensions (0.022 ± 0.0093). Further, to take even these low A_{254} values into account, a correction factor for A_{254} of the suspension was programmed into UVCalc. This is an important consideration, as discussed below.

UV dosimetry. In the benchmark study (7), UV fluence at the surface of the suspension was correctly measured with a commercial UV meter but no correction was made for the shielding by nonsporulated cells, absorption of UV by the resuspension medium itself, or the depth of the suspension. These general considerations have recently been addressed by James Bolton, who developed UVCalc, a Microsoft Excel spreadsheet designed to take these factors into account in

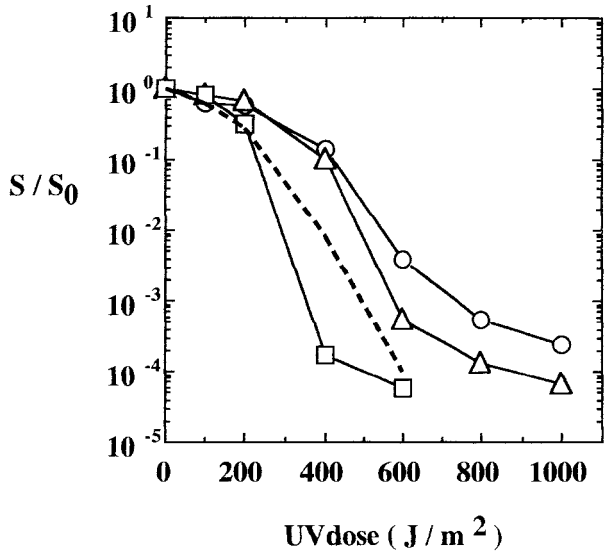

FIG. 3. Effect of turbidity of, and absorbance of UV by, buffer on the apparent UV resistance of *B. anthracis* Sterne spores. Spores were prepared and irradiated as described previously (7) and in the text. Survival curves were plotted based on dosimetry calculated by using UVCalc with no correction (circles) or with correction for the A_{254} of the buffer used (triangles) or for the A_{254} values of both the buffer and the cell suspension (squares). For comparison, a heavy dashed line represents the UV inactivation curve of spores of *B. subtilis* ATCC 6633, as determined by Hoyer (5).

calculations of the biologically effective UV fluence in aqueous suspensions. In order to determine the extent to which the turbidity of, and absorbance of UV by, the phenolized gel-phosphate buffer affects the apparent UV resistance of *B. anthracis* Sterne spores, we undertook the following experiment. *B. anthracis* Sterne spores were prepared as described elsewhere (7) by growth on blood agar plates and resuspension in phenolized gel-phosphate buffer, diluted to 3×10^5 CFU/ml in PBS, and UV irradiated. The UV fluences used were calculated by using UVCalc with (i) no correction factor, (ii) correction for the A_{254} value of the buffer, and (iii) correction for the A_{254} values of both the buffer and the spore suspension. The results of this experiment (Fig. 3) strongly support the notion that UV absorption by the buffer and by the turbid suspension leads to an overestimate of the apparent spore resistance to UV. Indeed, when these factors are taken into account, it appears that *B. anthracis* Sterne spores prepared from blood agar plates (Fig. 3) may be slightly less UV resistant than comparable spores prepared by growth in SSM (Fig. 1C).

In conclusion, to establish unambiguously the level of UV resistance of *B. anthracis* Sterne spores, we prepared, purified, and assayed the resistance of the spores in parallel with spores of standard *B. subtilis* biodosimetry strains whose UV-inactivation characteristics are well established. When all of the variables associated with spore production, purification, irradiation, and UV dosimetry were accounted for, we found that *B. anthracis* Sterne spores exhibited essentially the same resistance to 254-nm-wavelength UV as do spores of standard, currently available UV biodosimetry strains of *B. subtilis*. Therefore, in the absence of evidence that spores of virulent *B. anthracis* strains are more UV resistant than are *B. anthracis* Sterne, we conclude that *B. subtilis* spores can serve as safe and accurate substitutes for *B. anthracis* spores in validating the efficacy of 254-nm-wavelength UV-disinfection devices.

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