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ACTION SPECTRA IN ULTRAVIOLET WAVELENGTHS (150–250 nm) FOR INACTIVATION AND MUTAGENESIS OF *Bacillus subtilis* SPORES OBTAINED WITH SYNCHROTRON RADIATION

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Abstract—*Bacillus subtilis* spores were exposed *in vacuo* to monochromatic UV radiation from synchrotron radiation in the wavelength range of 150 nm to 250 nm. Survival and frequency of mutation to histidine-independent reversion were analysed for three types of spores differing in DNA-repair capabilities. UVR spores (wild-type DNA repair capability) exhibited nearly equal sensitivity to the lethal effects of far-UV (220 nm and 250 nm) and of vacuum-UV radiation (150 and 165 nm), but showed marked resistance to 190 nm radiation. UVS spores (excision-repair and spore-repair deficient) and UVP spores (a DNA polymerase I-defective derivative of UVS) exhibited similar action spectra; pronounced sensitivity at 250 and 220 nm, insensitivity at 190 nm and a gradual increase of the sensitivity as the wavelength decreased to 165 nm. In all strains, the action spectra for mutation induction paralleled those for the inactivation, indicating that vacuum-UV radiation induced lethal and mutagenic damages in the spore DNA. The insensitivity of the spores to wavelengths around 190 nm may be explicable by assuming that radiation is absorbed by materials surrounding the core in which DNA is situated.

INTRODUCTION

The effects of radiation upon bacterial spores have been investigated from several aspects. Extensive work with X-rays, γ-rays, and electrons have yielded parameters for the inactivation of *Bacillus megaterium* spores under various ambient conditions (Powers and Cross, 1970; Iwasaki *et al.*, 1974). The induction by X-rays of single-strand breaks in the DNA of *Bacillus subtilis* spores has been demonstrated (Tanooka and Terano, 1970), and also the involvement of DNA polymerase I in rejoining the breaks (Terano *et al.*, 1974). On the other hand, studies with 254 nm UV radiation revealed a unique pyrimidine photoproduct, identified as 5-thyminyl-5,6-dihydrothymine in spore DNA (Varghese, 1970) and genetically controlled processes of repair in *B. subtilis* (Munakata and Rupert, 1974). However, the effects of the vast wavelength region covering vacuum-UV radiation and low energy X-rays scarcely have been investigated due to the lack of appropriate radiation sources. The situation has been amended in recent years by technical developments aimed at using synchrotron radiation in many fields of physical and chemical research. Since 1976 we have been trying to apply this radiation source for radiobiological studies using several types of biological materials (Sasaki and Ito, 1979). We learned that bacterial spores offer several advantages over other organisms for such studies: (1) as dried samples on a membrane filter spores can be handled and transported with ease, (2) the spore can withstand an extreme vacuum (below 10^{-8} Torr) without any noticeable loss of viability, and (3) several repair-deficient mutant strains of *B. subtilis* are available for assays of survival and mutagenesis. The information obtained from them could be useful to deduce what types of molecular lesions are produced by different radiations. By constructing an irradiation chamber and a monochromator connected to a beam port of synchrotron radiation (Ito *et al.*, 1984), we obtained spectra for killing and mutagenic actions of UV radiation in the wavelength region of 150 to 250 nm. Although this is only the first step toward disclosing and defining the effects of monochromatic radiation in the vast range of vacuum-UV and low energy X-rays, nevertheless the results revealed unique structures in the action spectra and encourage future efforts.

MATERIALS AND METHODS

Bacterial strains. Three strains of *Bacillus subtilis* were used: HA101 (*uvr*$^+$ *hisH101 metB101 leuA8*), TKJ6312 (*uvrA10 ssp-1 hisH101 metB101 leuA8*) and TKJ6321 (*uvrA10 ssp-1 polA151 hisH101 metB101*). The strain HA101 is wild-type with regard to DNA repair capability, and carries suppressible auxotrophic markers, *hisH101* and *metB101*, suitable for the assay of mutation induction (Okubo and Yanagida, 1968; Tanooka, 1977). The strains TKJ6312 and TKJ6321 are derivatives of HA101, and are doubly defective in excision repair and spore repair of spore photoproduct (Tanooka *et al.*, 1978). These defects in

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Abbreviations: UVR, UVS, and UVP stand for the strains, HA101, TKJ6312, and TKJ6321, respectively. c.f.u., colony-forming unit.

combination sensitize the spores more than twenty-fold to 254 nm UV radiation (Munakata, 1969). In addition, the strain TKJ6321 carries a mutation, *polA151*, that causes a defect in DNA polymerase I (Munakata and Rupert, 1975). We refer to the spores of HA101, TKJ6312, and TKJ6321 as UVR, UVS, and UVP spores respectively.

Sample for irradiation. Spores were produced and purified as described previously (Munakata and Rupert, 1974). The stock solution of the spores was kept as a suspension in water at 10^{10} c.f.u. per mℓ at 4°C. The suspension was diluted in water to 10^8 c.f.u. per mℓ before use, and the diluent was spotted onto membrane filter (Millipore, GSWP02500, pore size = 0.22 μm). To adjust the center of the filter, it was covered with a sheet of paper with a cross-shaped cut, 5 mm width. Filters were placed in a 12-place manifold apparatus (Millipore) under a weak vacuum, and a 10 μℓ suspension carrying 10^6 c.f.u. was spotted on the center of each filter. After drying in air the spores could be detected as a faintly refractile spread in a circle of about 3 mm-diameter. These filters were kept in a box, and used within one or two days. In preliminary experiments, we learned that more concentrated samples (over 5×10^6 c.f.u. per filter) produced a tailing in the survival curves after vacuum-UV irradiation, suggesting that there was shielding caused by overlapping spores.

Irradiation system. The instruments for the irradiation were installed at a beam port of an electron storage ring at Synchrotron Radiation Laboratory, Institute of Solid State Physics, University of Tokyo, Japan. The physical parameters of the ring and the irradiation system have been described in detail elsewhere (Ito *et al.*, 1983). In short, the beam line consisted of a premirror chamber with two pieces of Al-coated glass, a modified Wadsworth-type monochromator (Yanagihara *et al.*, 1979) with an Al+MgF_2 coated replica grating (Baush-Lomb, 35-52-47-410) and a sample chamber designed specifically for *in vacuo* irradiation. Two pieces of MgF_2 plate were used to separate the monochromator from the premirror chamber in order to protect the vacuum (below 2×10^{-9} Torr) in the ring, therefore the wavelength of the radiation entering into the monochromator was longer than 120 nm. The slit-width at the entrance of the sample chamber was 5 mm; at this slit-width, the bandwidth of wavelength at half maximum was 8.7 nm. Since the sample was spread vertically about 3 mm, the effective bandwidth of wavelength was estimated to be 5.2 nm. The photon intensity at the position of the sample was determined by measuring the fluorescence of sodium salicylate with a photomultiplier (R268, Hamamatsu Photonics Co.) (Ito *et al.*, 1983).

Irradiation procedure. Samples on filters were attached to a metal holder (the chamber has a hexagonal frame to insert six holders). Control samples could be placed in the chamber out of the lightpath. Repeated experiments showed that spores kept for several hours in the chamber evacuated below 10^{-5} Torr did not exhibit any subsequent loss in their ability to form colonies, nor any increase in mutation frequency. Sample positions were adjusted to the center of a rectangular light area (5 mm × 10 mm) so that the entire sample spot was exposed. The beam current during the exposure was monitored continuously. Exposure time was determined by considering the intensity, the beam current during the exposure, and the expected effectiveness of the radiation. Exposure time varied from 5 s to 1200 s according to the type of spores and the wavelength employed. The monochromator and the chamber were evacuated below 2×10^{-5} Torr before exposure started. The sample could be changed by turning a knob connected to the frame with sample holders: exposure was started or terminated manually using a shutter at the entrance of the chamber.

Recovery of the spore and the assay of survival and mutation. The irradiated samples were brought back to the laboratory, the center part of the filter was cut out and placed in a test tube containing 1 mℓ of water. The next day extraction of the spores was performed using a microultrasonic cell disrupter (Kontes) with a 10 cm-probe. Sonication for 5 s was sufficient to elute the spores completely from the filter. The suspension was heated at 75°C for 15 min. Then it was diluted appropriately and colony formation was determined in duplicate on a medium consisting of Spizizen salt solution (Spizizen, 1958), 0.5% glucose, 50 mg/ℓ casein hydrolysate enzymatic (ICN Pharmaceutical), 50 mg/ℓ L-alanine, 50 mg/ℓ L-leucine, 50 mg/ℓ L-methionine, and 1.5% Bactoagar (Difco). After incubation at 37°C overnight, the number of colonies was counted. To estimate His^+ reversion frequency, a precalibrated amount (10^5–10^6 c.f.u.) of the surviving spores was poured on to the same medium. Since this medium contained a limited amount of histidine, revertant colonies overgrew on a lawn of the cells after 2-day incubation at 37°C. The number of spontaneous His^+ colonies was determined as a function of the number of spores plated with the spore stocks and this value was subtracted from the number of His^+ colonies produced in the irradiated sample. The value was divided by the number of the viable spores plated in order to obtain the induced mutation frequency.

RESULTS

Action spectra for survival

Colony-forming abilities of three types of spores, UVR, UVS, and UVP after the exposure to 150, 165, 175, 190, 220, and 250 nm radiation are plotted *vs* fluence (J/m^2) in Fig. 1. In all cases, the inactivation appears approximately exponential, with a small shoulder. A suitable procedure to obtain the inactivation constant (k_1) from the survival (N/N_0) and the fluence (F) would be to calculate the k_1 and y-axis intercept (n) in a relationship, $N/N_0 = n \exp(-k_1F)$ (Powers and Cross, 1970). However, the scatter and the scarcity of data points in some sets do not allow us to estimate these two parameters independently in each case. Therefore, as a compromise, first we drew eye-fitted curves for each set of survival data, and found that most of the curves could be extrapolated to y-axis at between 1.0 and 1.5. Thus, we have chosen arbitrarily 1.2 for n. Then, in the relationship, $N/N_0 = 1.2 \exp(-k_1F)$, inactivation constants (k_1) were calculated for each datum point. From this, the average value of the inactivation constant (k_1) with standard deviation was calculated for a set of data points for one of the three types of the spores exposed to a particular wavelength. Resultant inactivation constants were used to draw the straight lines in the figure. The inactivation constants, as expressed by (photon/m^2)$^{-1}$, were taken as measures of the sensitivity of the spore to the killing action of UV radiation; these values were used to draw the action spectra in Fig. 2.

Action spectra for mutation induction

The induced mutation frequency of His^+ reversion was calculated by subtracting the spontaneous frequency, as described in Materials and Methods. Calculated values of the frequency are plotted *vs* fluence (J/m^2) for UVR, UVS, and UVP spores in Fig. 3. Although the numbers of data points are

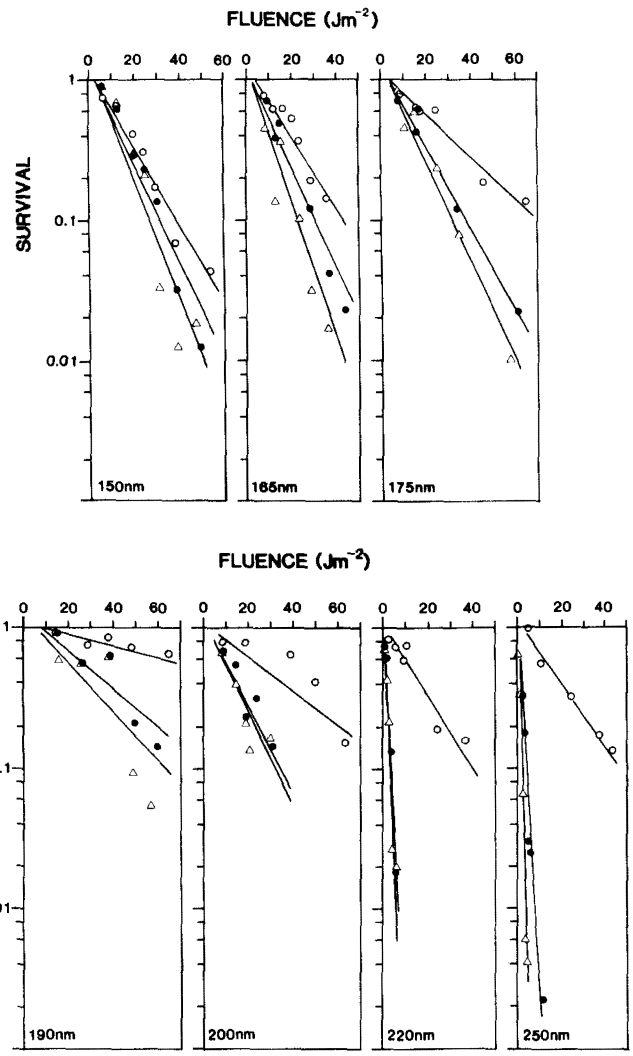

Figure 1. Survival of the spores of UVR (○), UVS (●), and UVP (△) after exposure to monochromatic radiation of 150, 165, 175, 190, 200, 220, and 250 nm. Lines were drawn from the average values of k_1 in the relationship, $N/N_0 = 1.2 \exp(-k_1 F)$ as described in the text.

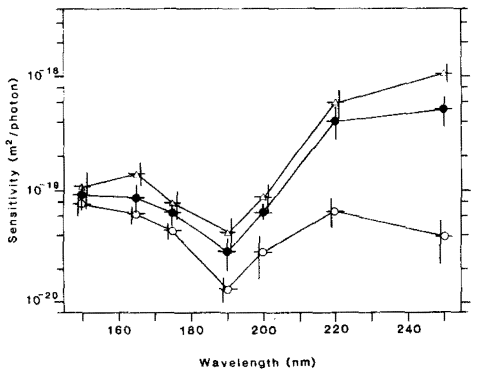

Figure 2. Action spectra for killing of UVR (○), UVS (●), and UVP (△) spores (see text for details). Horizontal bars represent the half-maximum width of the wavelength upon the sample spot. Vertical bars represent the standard deviations for each set of the determinations, and the bars for UVR and UVP spores are displaced to the left and right of the center wavelength, respectively, to avoid the overlap.

limited, most could be fitted relatively well to linear lines at least in the initial portion of the curves. Eye-fitted straight lines were drawn for each set of data points and the fluence yielding 100 induced mutants per 10^6 c.f.u. was read from the figure. The reciprocal of the fluence was termed the mutagenicity constant. In the case of UVR spores exposed to 190 nm or 200 nm radiation, the lines are extrapolated to 10^{-4} to obtain the mutagenicity constant. The mutagenicity constants as expressed by (photon/m^2)$^{-1}$, were taken as measures of the sensitivity of the spore to UV radiation, and were used to draw the action spectra in Fig. 4.

DISCUSSION

We have presented the action spectra for cellular inactivation and mutation in the wavelength region (150 to 250 nm) covering longer vacuum-UV and

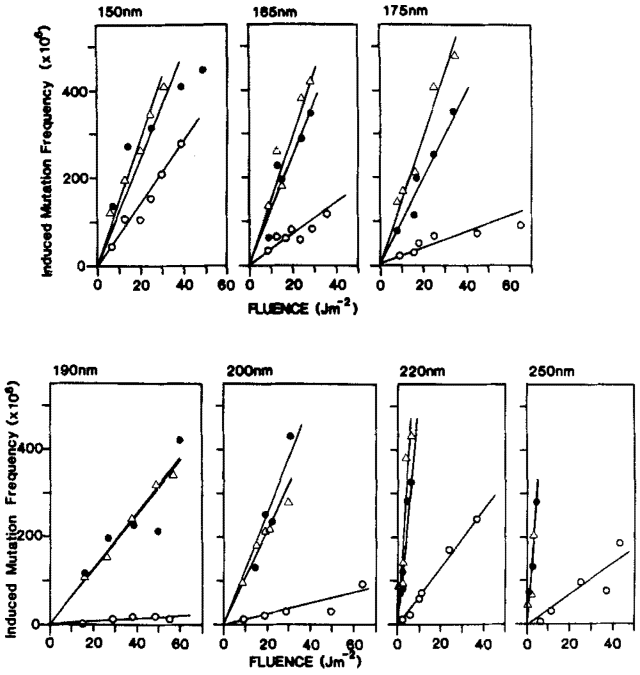

Figure 3. Induced mutation frequency as determined by His^+ reversion of the spores of UVR (○), UVS (●), and UVP (△) after exposure to monochromatic radiation of 150, 165, 175, 190, 200, 220, and 250 nm. Lines were drawn by eye.

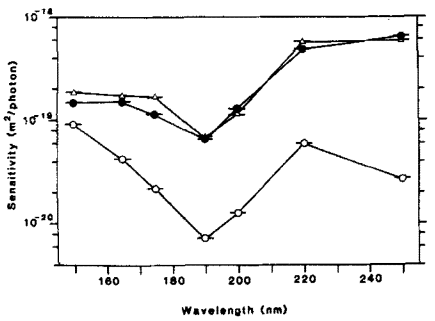

Figure 4. Action spectra for mutation induction of UVR (○), UVS (●), and UVP (△) spores (see text for details).

far-UV radiation. Some features of the action spectra are prominent and unmistakable, though reservations due to technical limitations should be kept in mind. One conspicuous feature is a deep depression of the action spectrum around 190 nm; in all spectra, 190 nm radiation is the least effective. This "valley" in the spectra is almost symmetrical toward the shorter and the longer wavelengths. It seems reasonable to discuss the action of the radiation in this region in two parts, corresponding to far-UV (longer than 190 nm) and vacuum-UV (shorter than 190 nm) region.

In far-UV region at 250 nm, there is a big difference in sensitivity between UVS (and UVP) and UVR spores. In our irradiation system, the spore photoproducts of 5-thyminyl-5,6-dihydrothymine type are likely to be the major DNA damages at this wavelength. We can make dosimetric comparisons with the inactivation constants of these spores. One-hit lethal fluence (k_1^{-1}) for UVS and UVR spores corresponds to 1.4 J/m^2 and 15 J/m^2, respectively. The 37% survival fluence of dried spores on membrane filters were reported to be 2.0 J/m^2 and 45 J/m^2 for UVS and UVR spores, respectively, when irradiation was performed in air with a germicidal lamp (Munakata, 1981). Thus, monochromatic synchrotron radiation at 250 nm is 1.4 and 3.0 times more effective than germicidal UV radiation with UVS and UVR spores, respectively. Horneck *et al.* (1984) reported that the ratios of the fluences of 254 nm radiation in atmosphere and in vacuum causing 10% survival of UVS and UVR spores were 1.2 and 4.1, respectively. These values agree well, and therefore it is likely that a major factor causing these differences is the ambient condition of the exposure.

The decrease in the action spectra for UVS and UVP spores at 220 nm, indicates a decrease in the formation of the spore photoproduct at this wavelength. For UVR spores, however, the sensitivity is somewhat higher at 220 nm than 250 nm. This suggests that other types of DNA damages, lethal and mutagenic, that can not be repaired in the UVR spores are formed at this wavelength. It would be interesting to identify and determine the action spectrum for the formation of the spore photoproducts at these wavelengths.

Below 190 nm, the sensitivity increases as the wavelength decreased to 165 nm. The sensitivities of the three types of the spores are closer, although

small differences persist throughout; UVP is the most sensitive, followed by UVS and UVR spores. The difference between UVP and UVS spores indicates the involvement of DNA polymerase I. However, it is not possible to specify a particular wavelength region where there is differential inactivation, indicating that the damage due exclusively to the action of DNA polymerase I is not induced in the wavelength region we surveyed. From 165 nm to 150 nm, sensitivity does not change much, and, for UVR spores, it now matches the level of 220 nm radiation.

The mutagenic action spectra almost parallel the inactivation spectra, suggesting that the relative effectiveness for inducing mutation and lethality does not exhibit significant variations in this wavelength range, and that DNA damages are a major cause for these actions upon the spores. The UVS (and UVP) spores are more easily mutagenized than the UVR spores at any wavelength due to the presence of *uvrA10* mutation.

A *B. subtilis* spore is elliptical, with a length of about 1.3 μm and the width of about 0.7 μm; the DNA is situated in the core, the periphery of which is 0.1–0.3 μm from the outer surface (Korch and Doi, 1971). Thus, vacuum UV radiation should be attenuated after being absorbed by several layers of coat, cortex, germ cell wall, and plasma membrane before reaching to DNA. By assuming that DNA is surrounded by a layer (0.1 or 0.25 μm) of protein molecules, we can construct hypothetical DNA absorption spectra from the published values of absorption coefficients for DNA film (Inagaki *et al.*, 1974) and albumin film (Inagaki *et al.*, 1975). The resultant DNA absorption spectra simulates the prominent depression observed in the action spectra, since peptide bonds strongly absorb 190 nm radiation (Fig. 5). Thus, it is plausible that the ineffectiveness of radiation around 190 nm is due to its absorption by proteinaceous materials. More generalized discussions of the absorption of vacuum-UV radiation by biological materials have been presented (Ito *et al.*, 1983).

Several other organisms have been studied for *in vacuo* inactivation in the same irradiation system, and the action spectra obtained for bacteriophage T1 and *Saccharomyces cerevisiae* cells. For phage T1, the sensitivity is relatively flat between 250 nm to 190 nm and increases sharply to 150 nm (Maezawa *et al.*, 1984). For wild-type yeast cells 150 nm is the most effective wavelength followed by 170 nm, 250 nm, and 220 nm (Hieda *et al.*, 1984). Gene conversion in yeast cells was not induced by 170 nm or 150 nm radiation, indicating that the target molecules for the inactivation are not DNA. This is probably due to the intense absorption of vacuum-UV radiation by cellular constituents before reaching chromatin. Thus, each organism is quite unique in the response to vacuum-UV radiation, and bacterial spores offer the opportunities to study genotoxic effects of vacuum-UV radiation with lethal and mutagenic consequences.

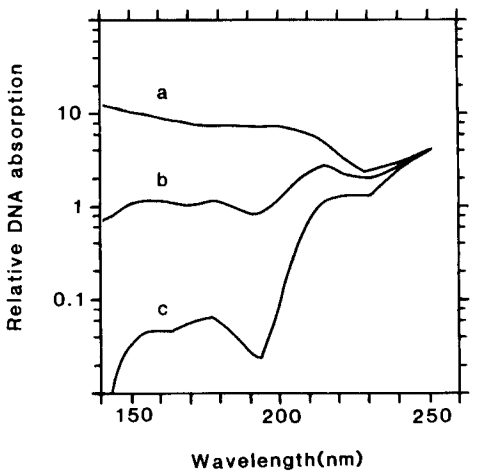

Figure 5. Hypothetical absorption spectra of DNA covered by a layer of albumin with thickness of (a) 0 μm, (b) 0.1 μm or (c) 0.25 μm. In the latter two cases, the absorption coefficient of DNA was multiplied by the transmittance of the albumin layer, as described in the text.

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