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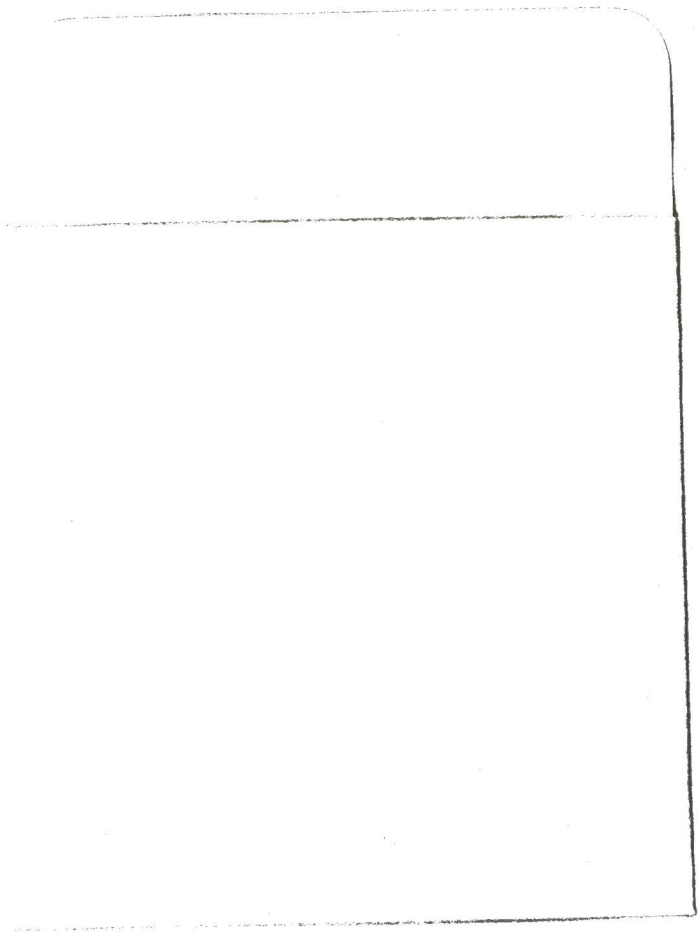

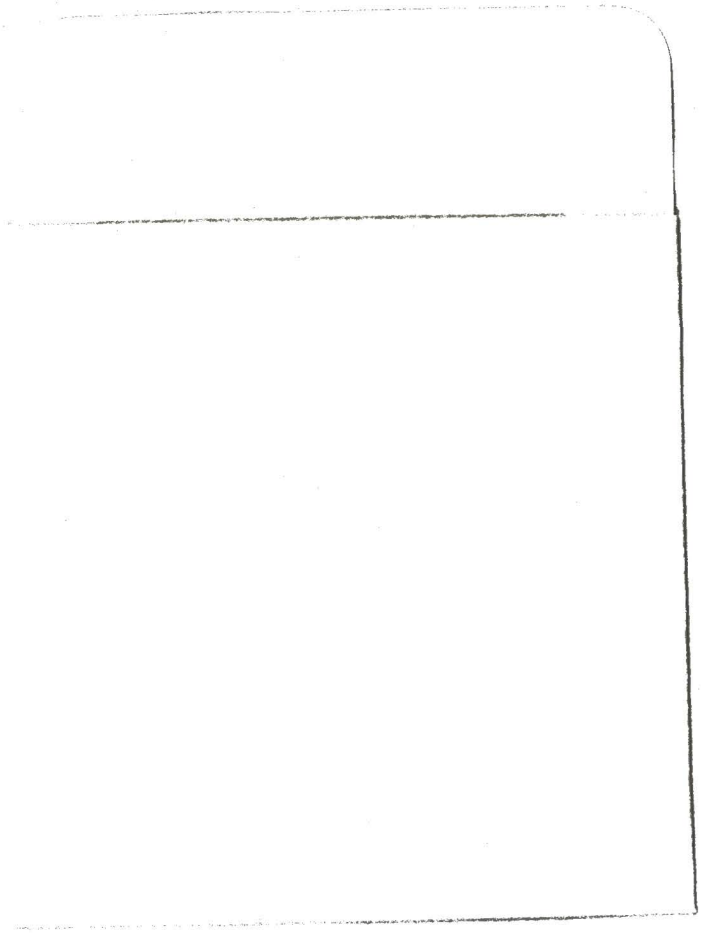

Xue, Yaming, Resistance of *Bacillus subtilis* spores lacking either nucleotide excision repair or spore photoproduct lyase to ultraviolet (UV) radiation from artificial or natural sources. Master of Science (Biomedical Sciences), June, 1996, pp., 4 tables, 12 illustrations, references, 38 titles.

Exposure of bacterial spores to UV radiation causes the accumulation of a unique pyrimidine dimer in the DNA, "spore photoproduct" (SP). In *Bacillus subtilis*, two distinct DNA repair pathways are used for removal of SP: general nucleotide excision repair (the *uvr* pathway), or the SP-specific enzyme SP lyase (the *spl* pathway). Spores of four strains of *Bacillus subtilis* differing in their repair capabilities were irradiated under either artificial or solar UV. To determine the biologically-relevant cumulative UV dose under each irradiation condition, a sporocidal dosimeter was constructed. The results showed: (i) Both *uvr* and *spl* pathways contributed to the survival of spores under all tested conditions. The *spl* pathway was more efficient than *uvr* pathway in repairing the DNA damage caused by UV-C and solar UV-A, but no significant difference was noted in repairing DNA damage caused by UV-B or full-spectrum solar UV. (ii) Exposure of spores to solar UV can cause cellular lethal damage which is reparable by neither repair pathway.

RESISTANCE OF *BACILLUS SUBTILIS* SPORES LACKING EITHER NUCLEOTIDE EXCISION REPAIR OR SPORE PHOTOPRODUCT LYASE TO ULTRAVIOLET (UV) RADIATION FROM ARTIFICIAL OR NATURAL SOURCES

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THESIS

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LIST OF ABBREVIATIONS

BGSC	*Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH
CFU	Colony-forming unit
Cm	Chloramphenicol
DSM	Difco sporulation medium
FULL SUN	Full-spectrum solar radiation
LD_{90}	The 90% lethal dose (The dose yielding 10% survival)
MLS	Erythromycin (1.0 μg/ ml) and Linconmycin (25 μg/ml)
NER	Nucleotide excision repair
NSD	No statistical difference
PVA	Polyvinyl alcohol
SASP	Small acid-soluble spore protein(s)
S.D.	Standard deviation
SDU	Sporocidal dosimeter unit
SP	Spore photoproduct (5-thyminyl-5,6-dihydrothymine)
spl	Gene encoding spore photoproduct lyase
tf	transformation
UV	Ultraviolet
UV-A	Ultraviolet A (wavelength > 320 nm)
UV-A SUN	UV-A portion of sunlight
UV-B	Ultraviolet B (wavelengths between 280-320 nm)
UV-C	Ultraviolet C (wavelength < 280 nm)
uvr	Genes encoding general nucleotide excision repair pathway

CHAPTER I

INTRODUCTION

The ultraviolet component of solar radiation is a major detrimental agent to living cells and tissues (Berger and Urbach, 1982). UV light acts on the primary structure of DNA and produces DNA photoproducts, which can block DNA replication, induce mutations and ultimately result in cell death (Tyrell, 1978; Yasbin et al., 1990). Dormant bacterial endospores are cellular structures which are particularly resistant to the effects of UV, being one to two orders of magnitude more resistant to the killing effects of UV radiation than the corresponding vegetative cells of the same strain (Stuy, 1956; Donnellan and Setlow, 1965; 1992). Two interrelated factors account for elevated spore UV resistance:

(i) There is a difference in spore DNA's UV photochemistry *in vivo*. Decreased water content in the spore core together with the binding of spore DNA by unique α/β-type small, acid-soluble spore proteins (SASP) contribute to the spore's unique DNA photochemistry (Nicholson et al., 1990; 1991; Nicholson and Setlow, 1990b; Setlow, 1988a). Upon UV irradiation, the major DNA photoproduct within the spore is not cyclobutane pyrimidine dimers (a four-member ring structure resulting from saturation of the 5,6 double bonds of each pyrimidine) which is the predominant photoproduct in UV-irradiated DNA of vegetative cells. Instead the predominant photoproduct in spore DNA is the specific "spore photoproduct" (SP), 5-thyminyl-5,6-dihydrothymine (Varghese, 1970).

(ii) *Bacillus subtilis* spores have two major DNA repair systems capable of correcting SP (Munakata, 1969). One is the general nucleotide excision repair (NER or

uvr) pathway, which closely resembles the analogous system that has been well-characterized in *Escherichia coli* (for review, see Friedberg et al., 1995). Another is the SP-specific DNA repair enzyme called SP lyase, encoded by the *spl* gene (Munakata, 1969; Munakata and Rupert, 1972; Fajardo-Cavazos et al., 1993).

Much research has been done regarding these two pathways on bacterial spores during the past few decades. *Bacillus subtilis* strains carrying mutations of genes (such as *uvr*A) in the *uvr* pathway produce UV-sensitive vegetative cells, but their spores are only slightly more UV-sensitive than wild-type spores (Munakata and Ikeda, 1968; Munakata, 1977). UV-irradiated spores of mutants lacking SP lyase activity, in contrast, exhibit a lethal dose for 90% of the population (LD_{90}) of about 1/2 to 1/3 of that observed in wild-type spores (Munakata and Rupert, 1975; Fajardo-Cavazos et al, 1993), indicating that SP lyase makes a larger contribution to spore UV resistance than does NER (*uvr*). Mutant strains lacking either SP lyase (*spl*) or NER (*uvr*) still produce spores which are more UV resistant than vegetative cells of the same strain (Munakata and Rupert, 1975). NER is a general repair pathway present in spores as well as in vegetative cells, and it can repair the DNA damages that generate bulky base aducts, which can cause significant distortion of the DNA helix (such as SP, cyclobutane dimers and 6-4 photoproducts). However SP lyase is a purely sporulation-associated enzyme (Pedraza-Reyes et al., 1994), which constitutes an SP-specific error-free repair pathway (Setlow, 1992). When UV-irradiated spores germinate, SP lyase directly reverses SP *in situ* back to two thymines (Munakata and Rupert, 1972; 1974; Wang and Rupert, 1977).

Much of the research into spore DNA photochemistry and spore UV resistance has been conducted in laboratories using monochromatic 254 nm (UV-C) radiation. Sunlight, however, reaching the earth's surface is a mixture of visible, UV, and infrared radiation, the UV portion of which spans approximately 290 nm to 400 nm (UV-B and UV-A); UV-C is almost completely excluded by the stratospheric ozone layer (Urbach and Gange,

1986). This polychromatic solar spectrum, possibly complicated by other environmental conditions (such as heating), may result in a biological response to solar UV which differs considerably from the response to monochromatic UV-C used in the laboratory. Considerable effort has therefore been directed towards investigation of spore UV resistance and DNA repair under a variety of environmental conditions. A solar UV biological dosimetric system was developed, and was used to monitor solar UV changes at various global sites including: Rio de Janeiro, Brazil (Tyrell, 1978); Tokyo, Japan (Munakata, 1981); Dallas, Texas, USA (Wang, 1991); and Antarctica (Quintern et al; 1994). In all cases, the dosimeter consisted of mutant *Bacillus subtilis* spores which lack both NER (*uvr*) and SP lyase (*spl*), and survival of the spores to solar exposure over time was used to calculate the relative dose of radiation received. Using a series of UV cutoff filters, it has been determined that the most effective portion of the solar spectrum for dosimeter inactivation consists of UV-B radiation in the 305-325 nm range (Munakata, 1989; Wang, 1991).

To date, however, the relative contribution of the NER (*uvr*) or SP lyase (*spl*) pathway to solar UV resistance has not been determined. In the experiments described below, the main emphasis was placed on determining the relative contributions of each of the two major DNA repair pathways, NER (*uvr*) and SP lyase (*spl*), to the survival of *Bacillus subtilis* spores exposed to either artificially-produced UV-C or UV-B radiation, and to either full-spectrum of sunlight or the UV-A portion of sunlight. Measuring the relative contribution made by NER (*uvr*) or by SP lyase (*spl*) to UV resistance of spores under a variety of exposure conditions may provide clues as to the nature of the photodamage which spores experience under natural environmental conditions.

CHAPTER II

MATERIALS AND METHODS

Strains, growth conditions and spore preparation

All *Bacillus subtilis* strains used in this study originated from strain 168 (*trpC2*) and are listed in Table 1. Four kinds of strains which differed in their DNA repair capacities were used: WN170 represented the wild-type strain (containing both *uvr* and *spl* repair systems), WN171 represented the *uvr* mutant strain (with only *spl* repair), WN174 represented the *spl* mutant strain (with only *uvr* repair), WN252 represented the double mutant strain (with neither *uvr* nor *spl* repair). The growth and sporulation medium used throughout was Difco Sporulation Medium (DSM; Schaeffer et al., 1965). When appropriate, antibiotics were added to DSM at the following final concentrations: chloramphenicol (Cm), 3.0 μg/ml; or a combination of erythromycin (1.0 μg/ml) and lincomycin (25.0 μg/ml), hereafter referred to as MLS. Liquid cultures were incubated with vigorous aeration, and all cultivations were at 37°C unless otherwise indicated.

After two days of incubation either on solid or in liquid DSM, spores were purified by washing with buffer and heat shocked at 80°C for 10 min as previously described (Nicholson and Setlow, 1990a). Spores were kept as suspensions of 10^9-10^{10} colony forming units (CFU)/ml in water at 4°C until use.

UV transmission spectra

Various materials were used in this study either as UV cutoff filters or as UV-transparent coverings. They include: Saran Wrap (Dow Brands L.P., Indianapolis, IN); polystyrene sheets constructed from Petri dish lids (Fisher Scientific, Cat. No. 8-757-12);

Table 1. ***Bacillus subtilis*** **strains used in this study**

Strain [orig. code]	Genotype or phenotype	Source (reference)
168	*rpC2*	Laboratory stock
WN170	Trp^+, Cm^R transformant of 168	pWN162-->168 (tf)[a]
WN171 [1A345]	*metC14, sul, trpC2, thyA1, thyB1, uvrA42*	BGSC
WN173 [1A488]	*metC14, spl-1, sul, trpC2, thyA1, hyB1*	BGSC
WN174	as WN173, but Trp^+, Cm^R	pWN162-->WN173(tf)
WN252	*metC14, sul, Δ(ptsI-spl)::ermC1, trpC2, thyA1, thyB1, uvrA42,* MLS^R	Laboratory stock

[a]Abbreviations used: BGSC, *Bacillus* Genetic Stock Center; tf, transformation

and 1/2 inch thick plate glass obtained from a local glass supplier (The Edge of Art Glass, Ft. Worth, TX). Each of the above materials were placed into the beam of a visible-UV spectrophotometer (Ultrospec III, LKB), and their UV transmittance spectra in the 200-400 nm range obtained. The spectra are reported as the percent transmittance of the material at a given wavelength, normalized to its transmittance at 400 nm (Fig. 1).

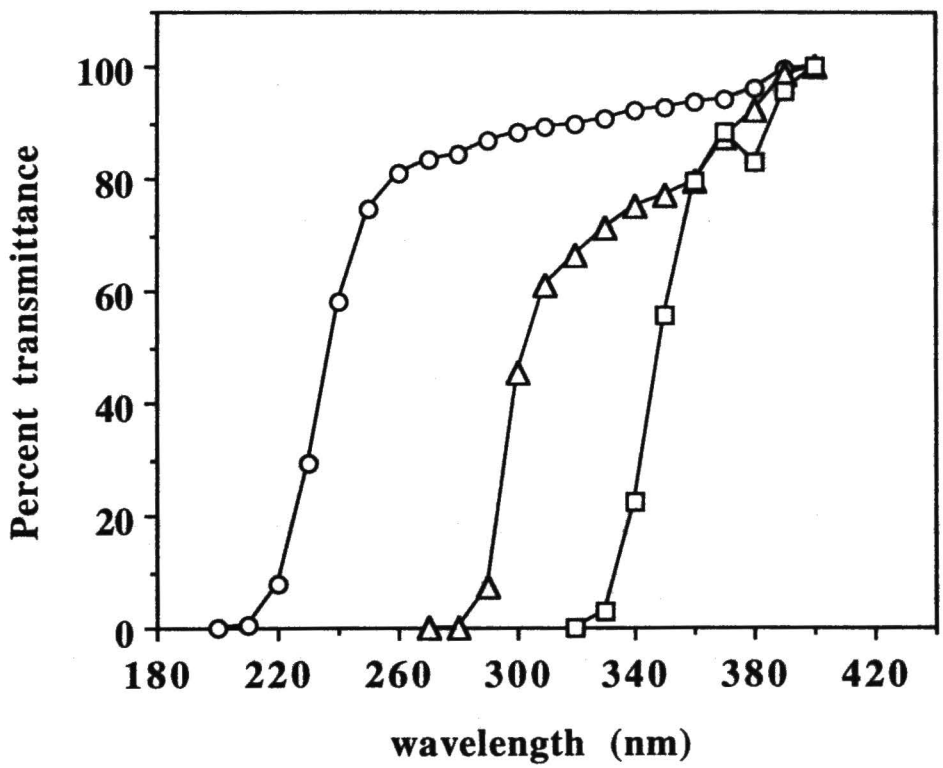
UV radiation sources and UV dosimetry

UV-C radiation was provided by a commercial low-pressure mercury arc lamp (model UVGL-25, UV Products, San Gabriel CA), which emits essentially monochromatic 254 nm radiation. The source of UV-B radiation was a commercial medium-pressure mercury arc lamp (model UVM-57, UV Products, San Gabriel CA) which emits a spectrum of UV wavelengths spanning 280 nm to 320 nm, with an emission maximum at approximately 302 nm. This lamp was further modified to limit the emission of shorter UV wavelengths by the placement of a polystyrene filter between the lamp and the sample; this polystyrene layer is opaque to UV wavelengths 280 nm or shorter (Fig. 1). The doses of UV produced by the artificial UV sources were measured using a UVX radiometer (UV Products, San Gabriel, CA) using the appropriate calibrated probe for UV-C or UV-B. Doses are reported in units of Joules per square meter. Biological UV dosimetry is described in a separate section (see below).

Sample preparation

Spores were mixed in pairwise combinations (strain WN171 with WN174, or strain WN170 with WN252) at a 1:1 ratio and diluted in water to a final concentration of approximately 10^8 CFU/ml before use. Three-10 µl aliquots of each resulting suspension (containing approx. 10^6 CFU per aliquot) were spotted onto a sterile microscope slide. The final diameter of each spot was approximately 1 cm. The slides were allowed to air-

Figure 1. **UV transmittance spectra of materials used as filters in this study** Values were normalized to their transmittance at 400 nm. Materials used were: Saran wrap, single layer (circles); polystyrene (triangles); 1/2" glass (squares).


Percent transmittance
100
80
60
40
20
0
180
220
260
300
340
380
420
wavelength (nm)

dry in the dark at 37°C and stored protected from light until use. Although slides were always used within a few days of preparation, a separate control experiment demonstrated no loss of spore viability upon prolonged storage at room temperature in the dark (approximately 3 months; data not shown). Each microscope slide contained triplicate spore spots. Strains which were used in these experiments carried different antibiotic resistance markers, so that the numbers of spores of each strain in any chosen pair could be quantitated either: (i) by plating on DSM medium containing the appropriate selective antibiotic, or (ii) in cases where one strain of the pair carried no antibiotic resistance marker, samples were plated onto DSM either lacking or containing the selective antibiotic, and the antibiotic-sensitive strain was quantitated by the difference in CFU on the two plates.

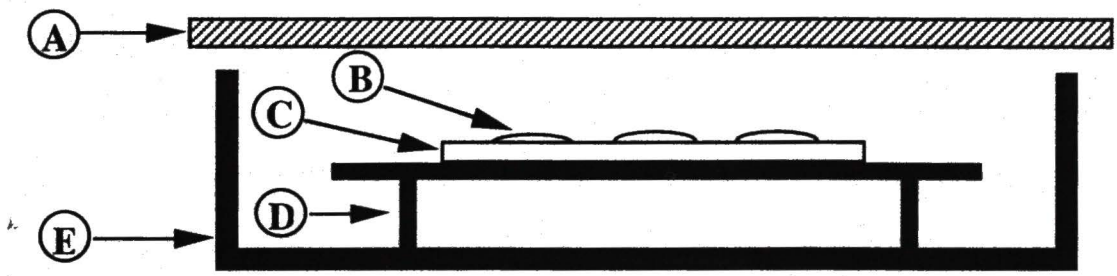
UV irradiation of samples

Sample slides were mounted on cardboard and were irradiated by different UV light sources. The time of exposure was determined empirically, and varied depending upon to the bacterial strain used and the source of UV light. Unirradiated control samples were treated identically to irradiated samples, but were kept either in an opaque box or wrapped with a layer of aluminum foil during exposure.

Artificial UV Light Exposure: Samples were placed on a rotating platform (30 rpm) perpendicular to the UV source. The UV-C lamp was set at a height of 30 cm above the sample, resulting in a dose rate of approximately 0.23 J/m^2 per sec. The UV-B lamp was placed 10 cm above the sample, resulting in a dose rate of approximately 5.2 J/m^2 per sec. Before each experiment, the lamps were warmed up for at least 15 min to stabilize their UV output.

Sunlight Exposure: For exposure to solar radiation, the experiments were conducted in two equal-sized cardboard boxes (31 cm X 24 cm X 4.5 cm); the top of each

Figure 2. **The box used for sample sunlight exposure.** Schematic diagram of the box used for exposure of spore samples to sunlight (not drawn to scale). A, cover (Saran wrap or 1/2" plate glass); B, spore sample; C, microscope slide; D, support; E, box.


A
B
C
D
E

box was equipped with a window (28 cm X 21 cm). Samples were affixed to a cardboard platform raised to 0.5 cm below window level (Fig. 2). When the samples were exposed to full spectrum sunlight, a single layer of Saran Wrap, which transmits all solar UV wavelengths (Fig. 1), was placed over the window. To filter out the UV-B portion of sunlight, the window was covered with 1/2 inch thick plate glass, which was opaque to UV wavelengths of 320 nm or shorter (Fig. 1).

The boxes were placed horizontally on the roof of the Medical Education Building No. 2 of the University of North Texas Health Science Center in Fort Worth, TX. Exposures started about at local 10:00 A.M. and ended at local 2:00 P.M., which corresponds to 10:17 A.M.-2:17 P.M. Central Standard Time, or 11:17 A.M.-3:17 P.M. Central Daylight Savings Time. All experiments described in this study were performed between February and September, 1995. Whenever possible, experiments were performed on cloudless or nearly-cloudless days to maximize sunlight intensity. Nevertheless, in order to obtain complete survival curves for particularly UV-resistant strains, or when exposing spores to the UV-A portion of sunlight only, it was necessary to perform a single experiment over the course of multiple (ranging from 2 to 8) days. In such cases, at the end of each daily exposure period, the sample boxes were covered with cardboard, moved to the laboratory, and stored in the dark until the next day when conditions were favorable for exposure to sunlight.

Recovery of Spores and Survival Assay

After exposure of spores to UV or sunlight, the samples were stored in an opaque slide box. Recovery of spores from the microscope slide was carried out by the method of Lindberg and Horneck (1991). One hundred microliters of sterile 10% (w/v) polyvinyl alcohol (PVA; Mol. Wt. 30,000-70,000, Sigma Chemical Co., St. Louis, MO) prepared in water, were pipetted onto the surface of sample spots and air dried at 37°C for 1.0-1.5 hour. Then, using a sterile scalpel and forceps, the PVA film containing the spores was

peeled from the slide. The PVA film was dissolved in 1.0 ml of buffer (10 mM potassium phosphate, pH 7.4, 150 mM NaCl) and diluted serially tenfold in the same buffer. Dilutions were plated on solid DSM plates containing the appropriate selective antibiotic and the surviving fraction of the spores was determined by counting the number of colonies after 20 to 40 of hours incubation at 37°C (Nicholson and Setlow, 1990a). The survival percentage of spores (P) was calculated by the following relationship:

$$P = (N_t / N_0) \times 100\% \qquad \text{(Equation 1)}$$

Where N_0 and N_t stand for the spores' CFU at the exposure time 0 and time t, respectively.

Biological dosimetry

The biologically effective dosage of UV radiation from natural or artificial sources was measured using the following test system, modified from Wang (1991). Samples were prepared as described above using a mixture of spores of *Bacillus subtilis* strains WN170 (wild-type; Cm^R) and WN252 (*uvrA42, Δspl::ermC*; MLS^R). Spores of strain WN170 served as an internal control to eliminate fluctuations in the recovery of dry spores from the glass, since there is essentially no killing of WN170 spores during the short periods of exposure used for dosimetry. WN252 spores are very sensitive to UV (L. Chooback, P. Fajardo-Cavazos, and W. Nicholson, unpublished results), and their UV inactivation kinetics are semilogarithmic (Wang, 1991). The percent survival (S) of WN252 spores was obtained from the relationship:

$$S = (R_t / R_o) \times 100\% \qquad \text{(Equation 2)}$$

Where R_t and R_o stand for the ratio of WN252 CFU to WN170 CFU at time t and at time 0

exposure, respectively. The UV dosage or exposure time yielding 10% survival of WN252 spores (i.e., the 90% lethal dose, or LD_{90}) is defined as one "Sporocidal Dosimeter Unit" (SDU).

Statistical analysis

LD_{90} values obtained from experiments testing spore resistance to artificial UV or sunlight were analyzed using Analysis of Variance, either by hand calculation or by computer program. Values were analyzed in multi-group pairwise combination (Tukey), and differences with a *P* value of less than 0.01 were considered statistically significant.

CHAPTER III

RESULTS

Control experiments

Several control experiments were performed to test the reliability and accuracy of both the spore dosimetry system and the protocols for recovering and quantitating survivors from the samples.

Effect of 10% PVA on spore viability

The possibility was explored that the 10% PVA used to resuspend the spores from microscope slides affects spore survival. A mixture of spores of strains WN170 and WN252 were suspended in water (Treatment 1) or in 10% PVA (Treatments 2 and 3) and incubated at 37°C for 1.5 hr, either in liquid (Treatments 1 and 2) or subjected to air-drying as is done normally in an experiment (Treatment 3). Spores were then recovered, diluted, and plated on DSM to quantitate survivors. The results of two separate experiments (6 samples) indicated that over the time period tested, incubation of spores in 10% PVA did not reduce spore viability (Table 2).

Test for concentration-dependent shielding of spores

Previously it was reported that more concentrated spore samples will produce a shielding effect, i.e. over 5 x 10^6 CFU/3 mm diameter spot on a filter, will exhibit a tailing in the survival curves upon UV irradiation (Munakata, et al.,1986). Since there was some day-to-day variation in the concentration of spores applied to samples in our testing system (ranging between 6.0 x10^5 - 1.4 x10^6 CFU/10 mm diameter spot on a slide), we tested

Table 2 **Effect of incubation in 10% PVA on spore viability**

TREATMENT*	PERCENT RECOVERY (± S.D.)	RANGE(%)
(1)	100.0 ± 13.14	86.01-112.08
(2)	110.0 ± 5.15	105.28-115.48
(3)	107.5 ± 7.49	99.04-113.35

*TREATMENT (1): spores + water (37°C x 1.5 hr)

TREATMENT (2): spores + 10% PVA liquid (37°C x 1.5 hr)

TREATMENT (3): spores + 10% PVA (37°C x 1.5 hr air dry)

five different spore concentrations for shielding effects, over a range of 5.0 x 10^4 to 5.0 x 10^6 spores per sample spot. Spores of strain WN170 were exposed to different doses of UV-C, and survivors quantitated. The results of this control experiment indicated that over the range of concentrations of spores used there was no significant shielding that might affect test results (Fig. 3).

Test of long-term heat resistance of spores

During the course of sunlight exposure experiments, it was observed that sample temperatures could approach 73°C at mid-day during the summer noontime. Control experiments were therefore performed in order to assess the effect of heat upon spore survival in the test system. Fig. 4 shows that after keeping spores at 60°C dark incubator for 20 hours, spore survival was still at the lever of 70% or higher. In addition, no significant difference in heat resistance was observed among the four strains used in the experiments (Fig. 4).

Test of aluminum foil shielding effect

To ensure that the sporocidal effects observed during sunlight exposure were in fact only due to exposure to solar radiation, a parallel set of spore samples were wrapped in one layer of aluminum foil in each experiment to reflect any sunlight and exposed in exactly the same manner as the test system. For every experiment and for spores of all strains tested, survival values at the end of the exposure period were above 70% for aluminum foil shielded samples (data not shown). Fig. 5 shows the typical survival curves of one experiment. It should also be noted that sample temperatures during this field experiment were including 73°C, similar to those used in the long term heat resistance test (Fig. 4). This field experiment also proves that spores were stable under the high temperature during sunlights encountered exposure.

Figure 3. **Effect of spore concentration on survival to UV-C exposure** Spores of strain WN170 were applied to sample spots at the indicated number of spores per spot, subjected to UV-C, and spore survival quantitated as described in Materials and Methods.

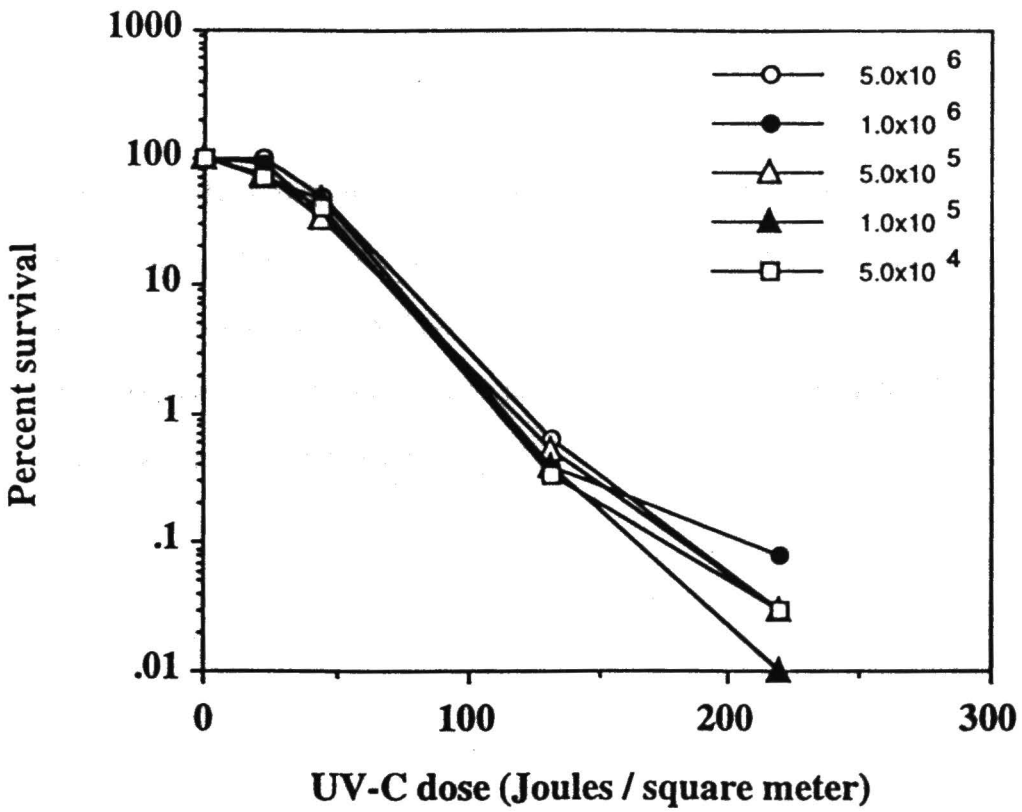

1000
100
10
1
.1
.01
0
100
200
300
Percent survival
UV-C dose (Joules / square meter)
5.0x10 6
1.0x10 6
5.0x10 5
1.0x10 5
5.0x10 4

Figure 4 **Heat tolerance of spores from the strains used in this study** Dried films containing spores of strains WN170 (triangles), WN171 (open circles), WN174 (closed circles), and WN252 (squares) were exposed to 60°C for the indicated times and survival quantitated as described in Materials and Methods.

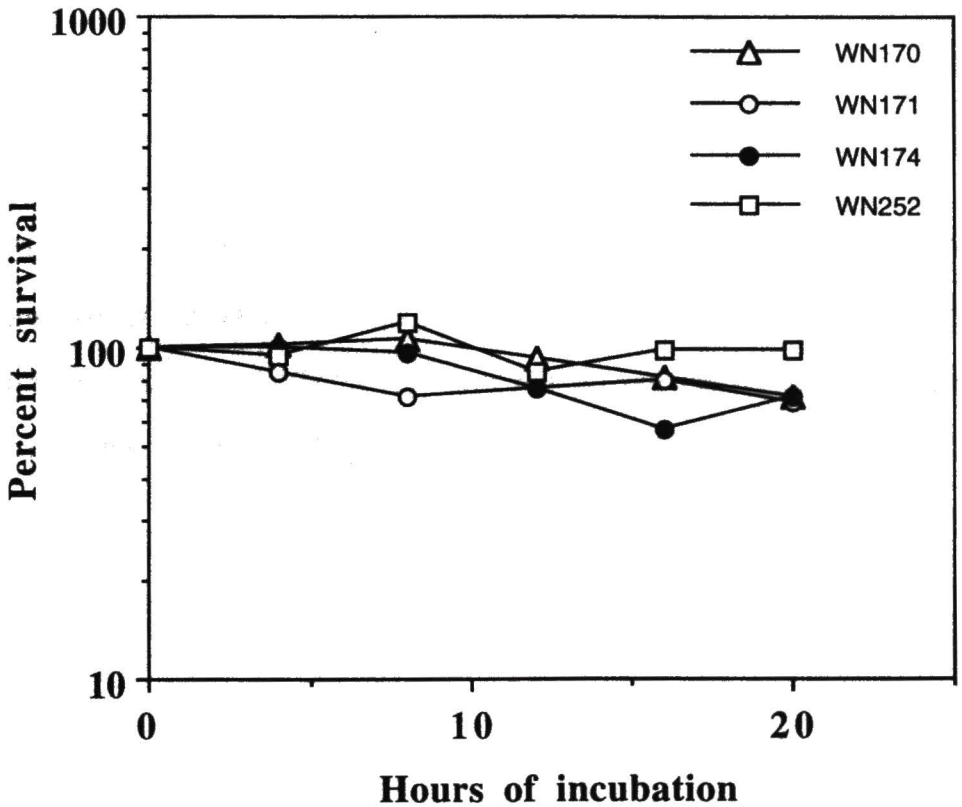
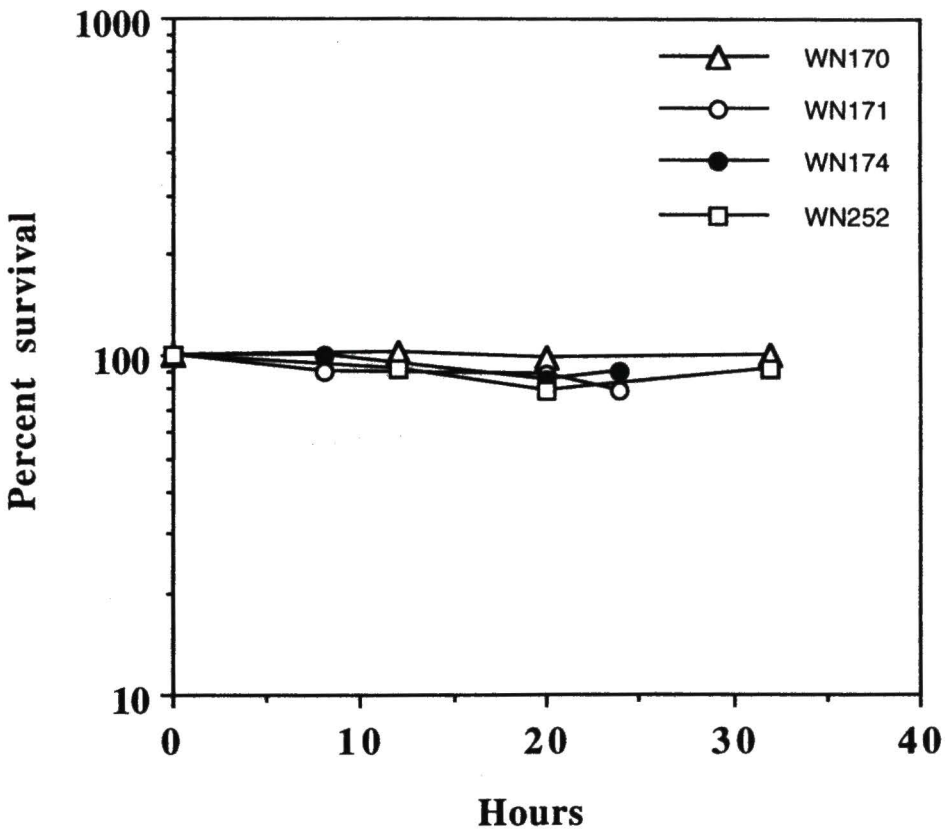

1000
100
10
Percent survival
0
10
20
Hours of incubation
WN170
WN171
WN174
WN252

Figure 5. **Survival of aluminum foil-covered spores to the UV-A portion of sunlight** WN170 (triangles), WN171 (open circles), WN174 (closed circles) and WN252 (squares). Experiment was performed on sunny days from June 20, 1995 to July 3, 1995.


1000
100
10
Percent survival
0
10
20
30
40
Hours
WN170
WN171
WN174
WN252

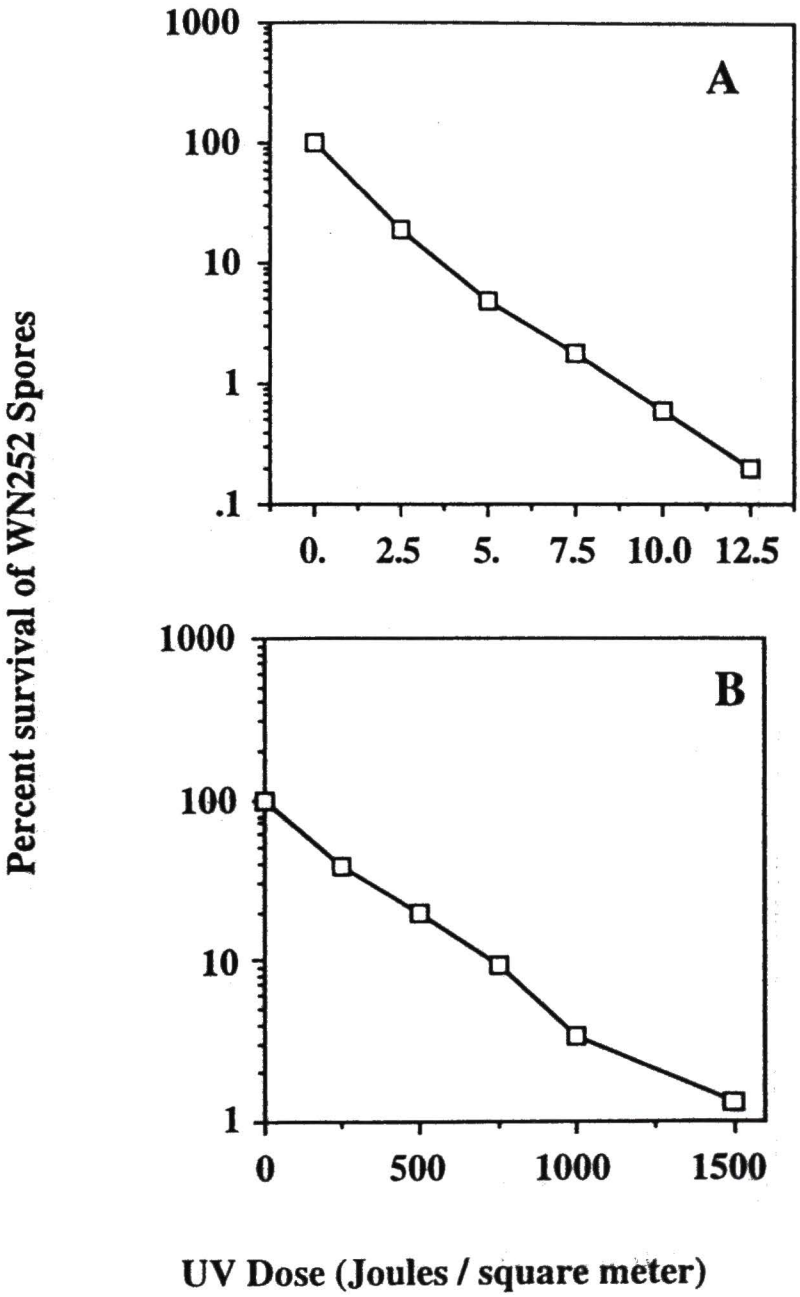
Dosimeter Measurement

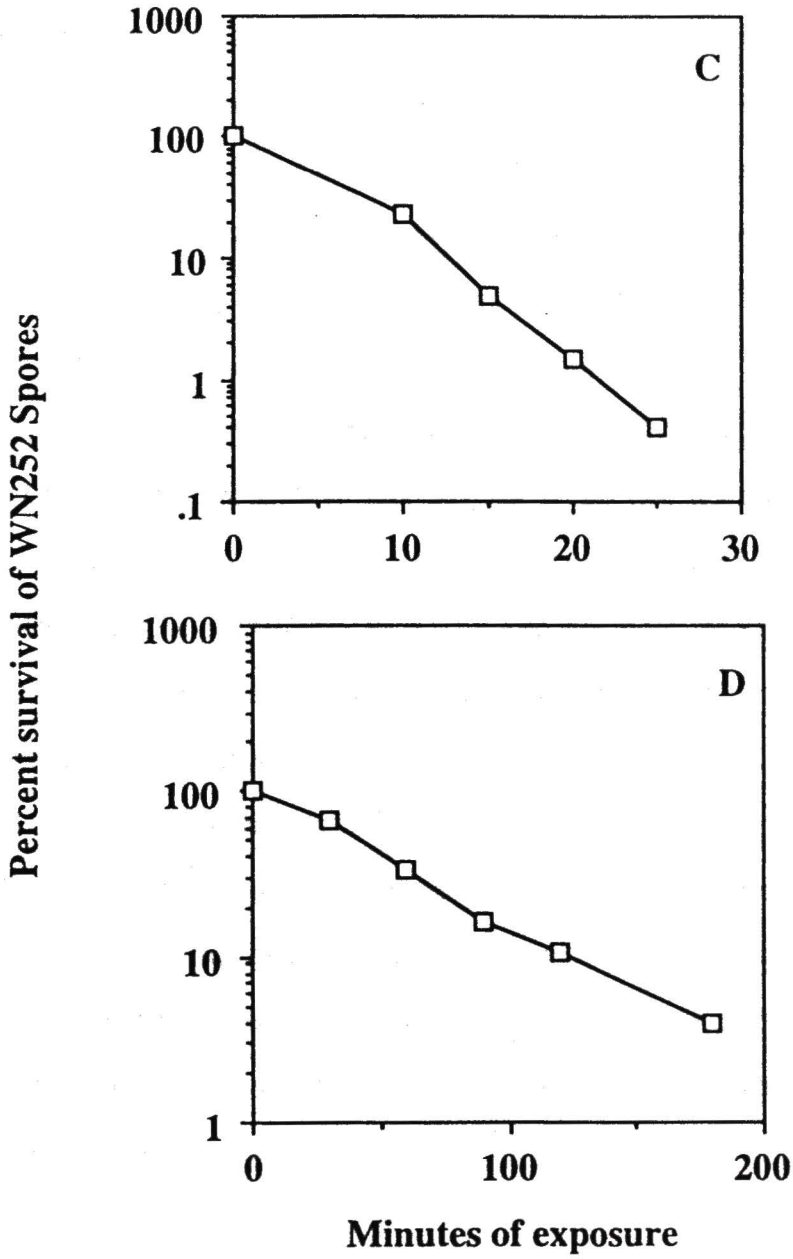
To obtain a quantitative understanding of the relationship between sunlight exposure and photobiological effects caused by exposure demands accurate and reliable dosimetry for solar UV radiation. Tyrrell (1978) and Munakata (1981) developed a biological dosimetric system, in which they employed UV sensitive spores of *Bacillus subtilis* strain UVSSP-42-1 (lacking both *uvr* and *spl* repair systems). The UV inactivation of these spores is largely an exponential process, so that the dose per lethal event can be readily calculated.

In our dosimeter system, we adopted Wang's (1991) method. Two *Bacillus subtilis* strains, WN252 (uvr^-, spl^-) and WN170 (uvr^+, spl^+) spores were mixed together at a ratio of about 5 : 1. The total spore concentration was kept near 1.0×10^6, which was determined to be free of shielding effects (Fig. 2), and which was previously used by Wang (1991). To test the response of the dosimeter to UV, it was exposed to different sources of UV light for varying periods of time, then spores were recovered and the percent survival was determined. Under artificially produced UV-C or UV-B radiation, WN252 spores were killed exponentially without an indication of a shoulder, while WN170 spores were kept relatively constantly at more than 70% survival (data not shown). A slight shoulder was observed in the survival curves of WN252 exposed to full-spectrum sunlight or to the UV-A portion of sunlight. Using Equation 2 to calculate the survival of WN252, Fig. 6 shows relative survival curves under different sources of UV radiation. Based on these curves, we can define a "Sporocidal Dosimeter Unit" (SDU) as the LD_{90} values determined by the exposure of WN252 to different UV treatments. Successive short exposure periods were added to calculate the cumulative dose of UV radiation received by spore samples during long exposure times.

To compare the biological dose with the physical UV dose, we measured the physical dose of artificial UV light at same time. One SDU corresponded to 3.45 J/m^2 for

Figure 6. **Response of the biological dosimeter to UV from artificial or natural sources for SDU calculation** Survival curves of WN252 in response to exposure to UV-C (Panel A), UV-B (Panel B), full spectrum sunlight (Panel C), and the UV-A part of sunlight (Panel D). The sunlight exposure curves were the averages of two separate experiments performed on August 4th, 1995 and September 3rd,1995. Survival percentages were calculated using Equation 2 as described in Materials and Methods.


1000
100
10
1
.1
A
0.
2.5
5.
7.5
10.0
12.5
1000
100
10
1
B
0
500
1000
1500
Percent survival of WN252 Spores
UV Dose (Joules / square meter)


1000
100
10
1
.1
C
0
10
20
30
1000
100
10
1
D
0
100
200
Percent survival of WN252 Spores
Minutes of exposure

UV-C, and 707.5 J/m^2 for that of UV-B radiation, respectively.

Constant variation in solar radiation is received at any given point on the Earth's surface, due to atmospheric conditions and the continually-changing solar angle. Therefore, in field experiments the biologically-relevant UV dose is usually defined as the time in minutes for the dosimeter to accumulate one SDU during a specified time interval (e.g., 30 min starting at local noon) (Munakata, 1981; Wang, 1991). For example, when exposed to full-spectrum sunlight on August 4, 1995, the dosimeter measured one SDU in 12.6 min, and when exposed to the UV-A portion of sunlight on September 3, 1995, the dosimeter measured one SDU after 118.3 min of exposure (data not shown).

Fig. 7 shows the monthly-change (seasonal changes) of total SDU measured between local 10:00 A.M. and 2:00 P.M. on several days between January 27 and May 23, 1995. Though there are day-to-day variations (due to weather conditions), a rise in biologically effective dose in term of SDU can be seen due to the increasing noontime solar angle during this part of the year. The data are consistent with previous observations from long-term monitoring studies (Munakata, 1989).

Fig. 8 shows the variation in UV intensity of sunlight between local 10:00 A.M. and 2:00 P.M. during one typical day of exposure (Apr. 7th, 1995). The UV intensity was expressed as total SDU during each certain time period. The experiment shows that the dosimeter detects short-term variations in solar UV flux, and that the strongest solar radiation occurred during local midday (Fig. 8), which is also consistent with previous results (Munakata, 1981; Wang, 1991).

Resistance of spores to artificial UV-C and UV-B

Fig. 9 shows the resistance of spores of strains WN170, WN171, and WN174 to artificially produced UV-C and UV-B radiation. Upon UV-C irradiation (Fig. 9a), WN170 spores showed the highest resistance, followed by WN171 and then WN174 spores.

Figure 7. **The monthly change in UV intensity of sunlight (SDU)** Measurement of SDU during the first half of 1995, using the biological dosimeter. Each circle represents the SDU of that day from local 10:00 A.M. to 2:00 P.M., determined from a set of triplicate samples.

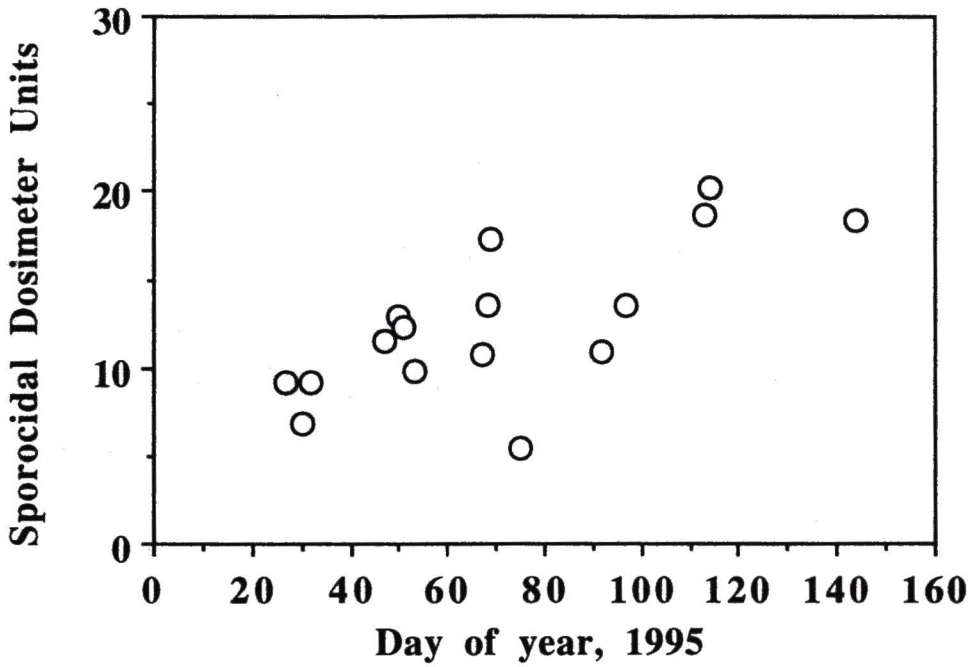

Sporocidal Dosimeter Units
30
20
10
0
0
20
40
60
80
100
120
140
160
Day of year, 1995

Figure 8. **The variation in UV intensity of sunlight (SDU) in one day of exposure** Measurement of SDU from biological dosimeter response to full-spectrum sunlight on a typical day (April 7th, 1995). Each bar was determined from triplicate samples during a 40-min exposure period.

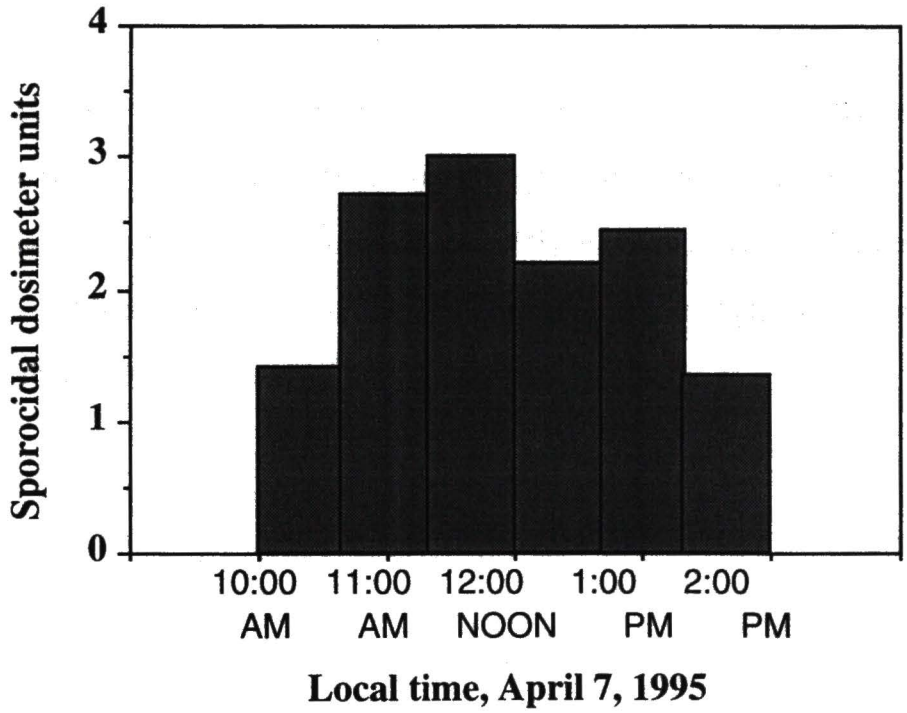
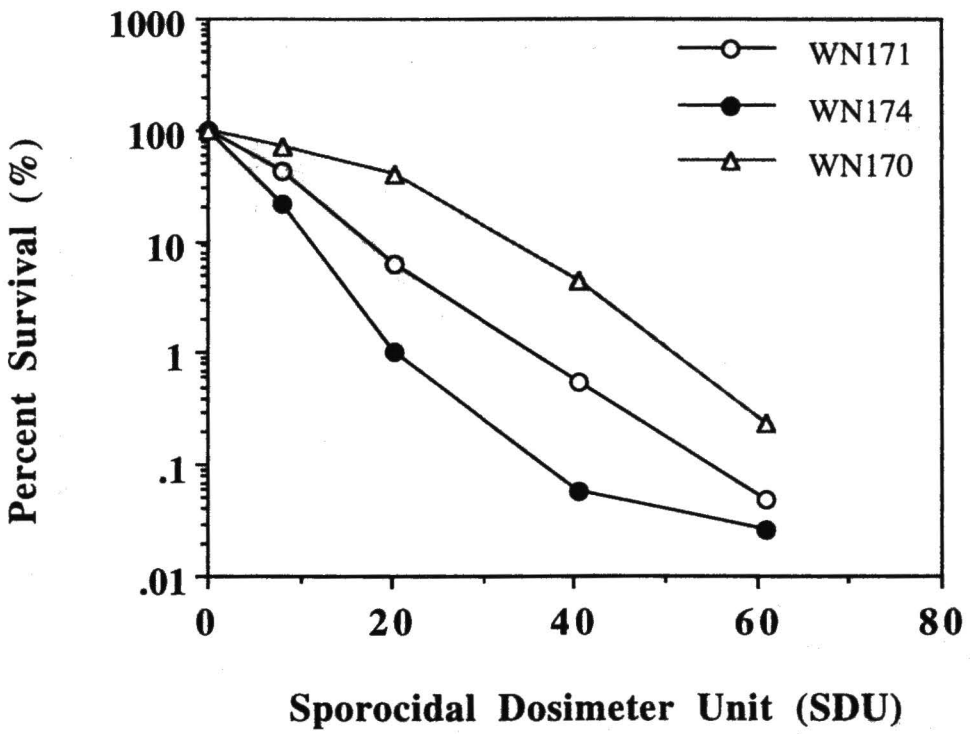
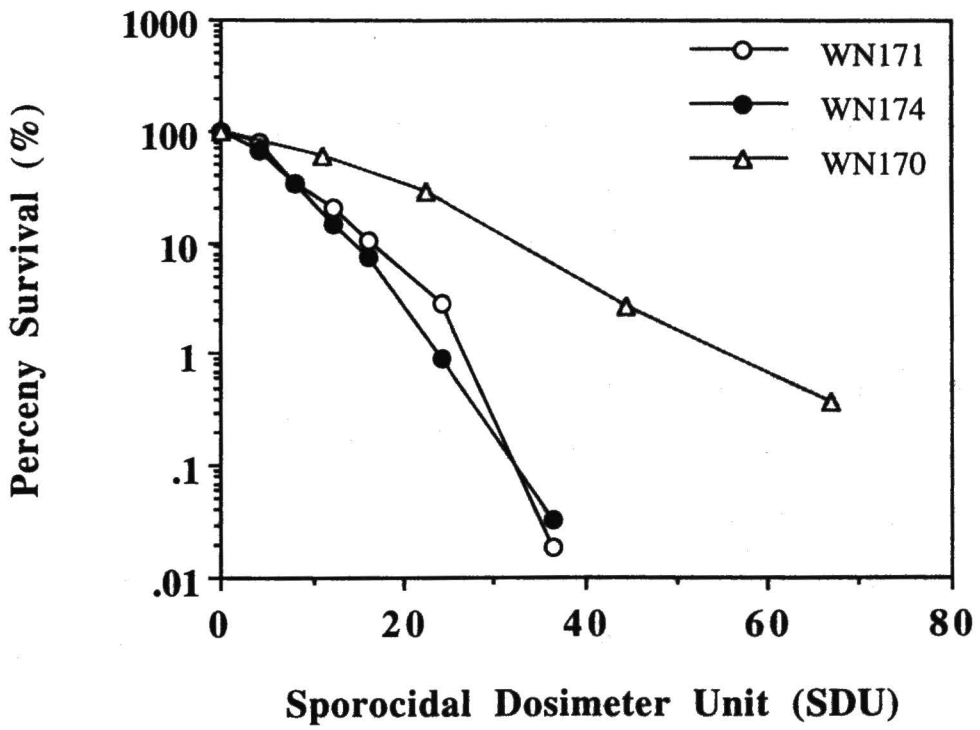

Sporocidal dosimeter units
4
3
2
1
0
10:00 AM
11:00 AM
12:00 NOON
1:00 PM
2:00 PM
Local time, April 7, 1995

Figure 9. **Survival curves of strains exposed to artificially produced UV light** Survival curves of spores of *Bacillus subtilis* strain WN170 (triangles), WN171 (open circles), and WN174 (closed circles) after exposure to UV-C (Panel A) or UV-B (Panel B). The curves are the average of two (WN170), or five (WN171 and WN174) experiments.


1000
100
10
1
.1
.01
Percent Survival (%)
0
20
40
60
80
Sporocidal Dosimeter Unit (SDU)
WN171
WN174
WN170


1000
100
10
1
.1
.01
Perceny Survival (%)
0
20
40
60
80
Sporocidal Dosimeter Unit (SDU)
WN171
WN174
WN170

This order of resistance was consistently obtained in each separate experiment. Looking at the shape of average survival curves of three strains, only WN170 exhibited a "shoulder" before its exponential decline. The other two strains showed strictly exponential curves. A "shoulder" survival curve on a semi-logarithmic plot signifies a retention of repair capacity to radiation dose, i.e. cells have an intrinsic damage repair capacity operating over a limited dose range (quasi-threshold), since it lessens with increasing dose. As this capacity is finally depleted, the curve will show exponential decrease (Alper and Cramp, 1989). Although strain WN171 and WN174 spores did not showed "shoulder" on their survival curves, it did not mean that these two strains' spores did not have any repair capacity, but that the experiment initial dose already surpassed the limited dose range of their repair capacity. As a matter of fact, they were more than an order of magnitude more resistant than the double mutant strain WN252. Certainly, however, their repair capacity were much lower than WN170 spores (2 to 3 times lower). Their LD_{90} values were 33.0 SDU (equivalent to 113.7 J/m^2) for WN170, 17.0 SDU (58.6 J/m^2) for WN171 and 11.1 SDU (38.2 J/m^2) for WN174 spores, respectively (Table 3). Statistical analysis of the LD_{90} values obtained for WN170, WN171, and WN174 with UV-C indicated that the differences among the LD_{90} values are significant at the 1% confidence level (Table 4).

On the other hand, upon UV-B radiation (Fig. 9b), WN170 spores had the highest resistance, but the resistance of WN171 and WN174 spores was quite similar, since upon several repetitions of the experiment neither strain consistently demonstrated higher resistance to the other. The shape of survival curves was similar to the exposure of artificially-produced UV-C light, i.e., WN170 spores showed a small "shoulder" on its survival curve. Their LD_{90} values were 31.4 SDU (equivalent to 2.2×10^4 J/m^2) for WN170, 15.4 SDU (equivalent to 1.1×10^4 J/m^2) for WN171 and 13.8 SDU (equivalent to 9.7×10^3 J/m^2) for WN174 spores, respectively (Table 3). Statistical analysis of the LD_{90} values showed that there is no significant difference between WN171 and WN174 at

Table 3. **Summary of LD_{90} values**[a]

STRAIN	UV-C	UV-B	FULL SUN	UV-A SUN
WN170	33.0 ± 2.8	31.4 ± 5.2	14.2 ± 0.8	6.6 ± 0.5
WN171	17.0 ± 2.6	15.4 ± 3.8	8.9 ± 1.7	4.6 ± 0.9
WN174	11.1 ± 1.0	13.8 ± 2.6	9.3 ± 1.8	3.2 ± 0.6
WN252	1.0	1.0	1.0	1.0

[a]LD_{90} values expressed in SDU ± standard deviation.

Table 4. **Statistical Analysis of Differences in LD_{90} (p values)**[a]

TREATMENT	STRAIN	WN170	WN171	WN174
UV-C	WN170		< 0.01	< 0.01
	WN171	< 0.01		< 0.01
	WN174	< 0.01	< 0.01	
UV-B	WN170		< 0.01	< 0.01
	WN171	< 0.01		NSD[b]
	WN174	< 0.01	NSD[b]	
FULL	WN170		< 0.01	< 0.01
SPECTRUM	WN171	< 0.01		NSD[b]
SUNLIGHT	WN174	< 0.01	NSD[b]	
UV-A	WN170		< 0.01	< 0.01
SUNLIGHT	WN171	< 0.01		< 0.01
	WN174	< 0.01	< 0.01	

[a]LD_{90} values from Table 3 were analyzed in pairwise combination within a single exposure regimen, using Analysis of Variance, and the p value reported.

[b]NSD=No statistical differance (p>0.05).

the 1% confidence level, although strain WN170 is significantly more UV-B resistant than either WN171 or WN174 (Table 4).

Resistance of spores to full spectrum sunlight

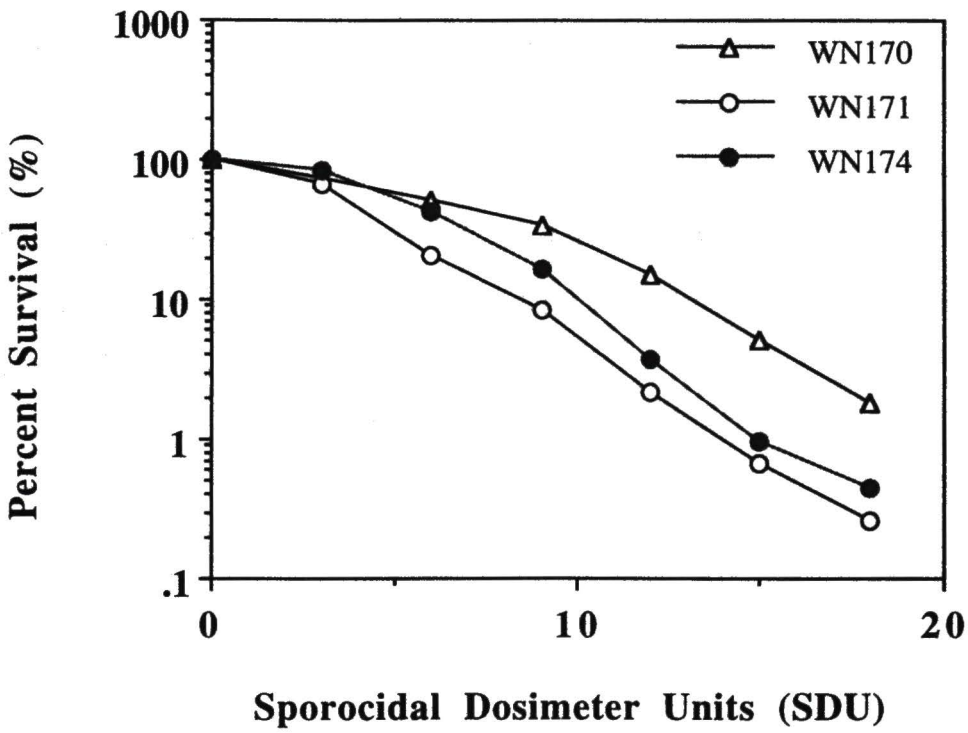
Natural sunlight is considerably more complex than artificially-produced UV light. In order to see if the laboratory model of spore UV resistance relates to what happens to spores in the environment, we conducted experiments in which spores were exposed to natural solar light.

The experiments were carried out from mid-February to mid-May, 1995. Several experiments were done, each accompanied by dosimeter measurement and dark controls, which were wrapped with a layer of aluminum foil during exposure. The results are shown in Fig. 10. The survival pattern resembled that observed using artificially-produced UV-B radiation: WN170 spores had the highest resistance and the resistance of WN171 and WN174 spores was similar, since upon several repetitions of the experiment, neither strain consistently demonstrated higher resistance to the other. However, the shape of survival curves (Fig. 10) were slightly different than the shapes obtained from artificially-produced UV light (Fig. 9). All three strains exhibited a "shoulder" on their survival curves before their exponential decline, the "shoulder" for WN170 spores being the biggest. The average LD_{90} values of strains WN170, WN171 and WN174 were 14.2 SDU, 8.9 SDU, and 9.3 SDU, respectively (Table 3). Statistical analysis of their LD_{90} values indicated that there is no significant difference between WN171 and WN174 at the 1% confidence level, and the strain WN170 was significantly more resistant to full-spectrum sunlight than either WN171 or WN174 (Table 4).

Resistance of spores to the UV-A Part of Sunlight

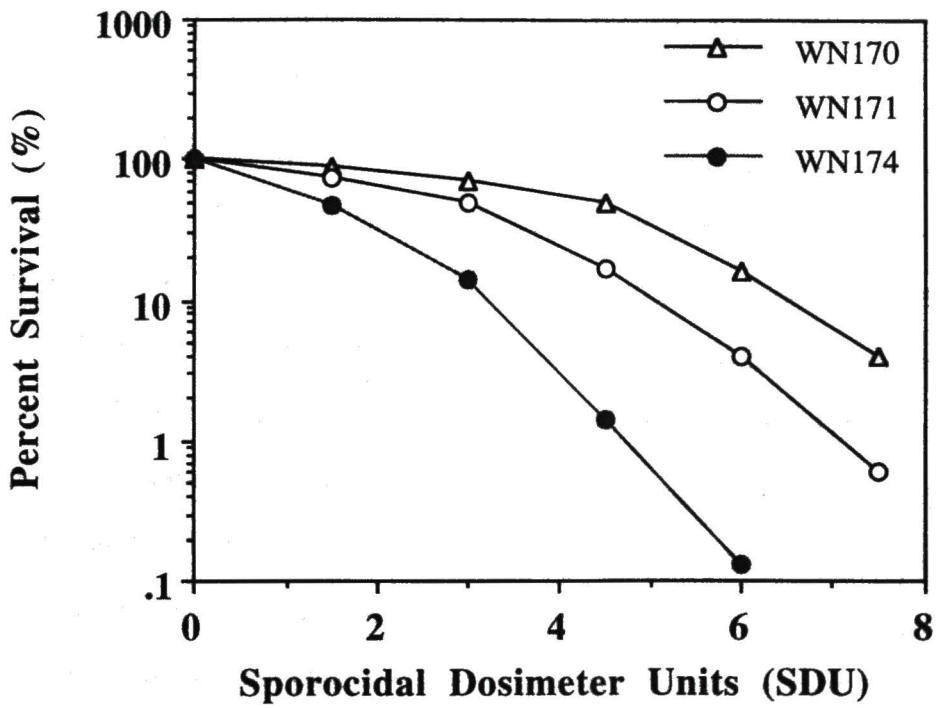
We next tested the effects on spores of exposure to the portion of sunlight containing only wavelengths of UV-A and longer. The experiments were carried out from

Figure 10. **Survival curves of strains exposed to full spectrum of sunlight** Survival curves of spores of *Bacillus subtilis* strain WN170 (triangles), WN171 (open circles), and WN174 (closed circles) after exposure to full spectrum sunlight. The curves were the average of two (WN170) or five experiments (WN171 and WN174), performed from mid-February to mid-May, 1995.


1000
100
10
1
.1
0
10
20
Percent Survival (%)
Sporocidal Dosimeter Units (SDU)
WN170
WN171
WN174

mid-June to mid-July,1995, and were performed in exactly the same way as full-spectrum sunlight exposure experiments, except that the UV-B portion of sunlight was removed by using 1/2 inch plate glass as a filter (see Fig. 1). Because the samples were only exposed to the relatively low-energy UV-A part of sunlight, it was empirically determined that the exposure times used must be much longer. Strain WN170 spores again had the highest resistance, followed by WN171 and then by WN174 spores; the order of resistance was consistent over several experimental repetitions (Fig. 11). The shape of the survival curves obtained resembled that observed on exposure of full spectrum of sunlight, except the curves exhibited a more obvious " shoulder". The LD_{90} values for spores of strains WN170, WN171, and WN174 were 6.6 SDU, 4.6 SDU, and 3.2 SDU, respectively (Table 3). Statistical analysis indicated that all three LD_{90} values are significantly different at the 1% confidence level (Table 4).

Fig. 11. **Survival curves of strains exposed to UV-A portion of sunlight** Survival curves of spores of *Bacillus subtilis* strain WN170 (triangles), WN171 (open circles), and WN174 (closed circles) after exposure to UV-A portion of sunlight. The curves were the average of three experiments, performed between mid-June to mid-July, 1995.


1000
100
10
1
.1
Percent Survival (%)
0
2
4
6
8
Sporocidal Dosimeter Units (SDU)
WN170
WN171
WN174

CHAPTER IV

DISCUSSION

Bacterial spores must rely on the expression of specific mechanisms to correct damage to their DNA induced as a result of exposure to UV during dormancy. To date, it is well established that SP which accumulates in the DNA of dormant spores is corrected very specifically and efficiently during early germination through the combined activities of the *uvr* and *spl* pathways (Munakata, 1969; Munakata and Rupert, 1974). In the *uvr* repair pathway, a variety of bulky lesions (SP, TT, etc.) are recognized and endonucleolytic cleavage of the DNA is initiated, both 3' and 5' to the lesion, by the activities of the UvrA , UvrB and UvrC proteins. The UvrD helicase, DNA polymerase I and DNA ligase then further process lesions and resynthesize DNA (Yasbin et al., 1993). The *spl* repair pathway is dedicated to the *in situ* monomerization of SP during spore germination, and is specific to the spore stage of the *Bacillus subtilis* life cycle only. The exact mechanism of this error-free pathway is not clear (Setlow, 1988b). By cloning the wild-type *spl* gene from *Bacillus subtilis* 168, and then computer-assisted comparisons between the deduced amino acid sequence of SP lyase and other DNA repair proteins, it was found that a region of the carboxyl half of SP lyase shares sequence homology with the DNA photolyases from a number of prokaryotes and lower eukaryotes, suggesting that these two classes of enzymes may have descended from a common ancestral protein (Fajardo-Cavazos et al., 1993). The important differences between them are (i) their distinct substrate specificities, and (ii) the fact that SP lyase is not dependent on visible light for activity (Fajardo-Cavazos, et al., 1993), instead is dependent on some other kind of energy source

(Munakata and Rupert, 1974; Wang and Rupert, 1977).

In this series of experiments, we have examined the survival of four types of *Bacillus subtilis* spores upon irradiation with artificially produced UV-C and UV-B light, as well as full-spectrum sunlight and the UV-A portion of sunlight. These four strains represent four different possible combinations of mutations in *uvr* and/or *spl* repair pathways. The results of these experiments are summarized in Fig. 12 and Table 3.

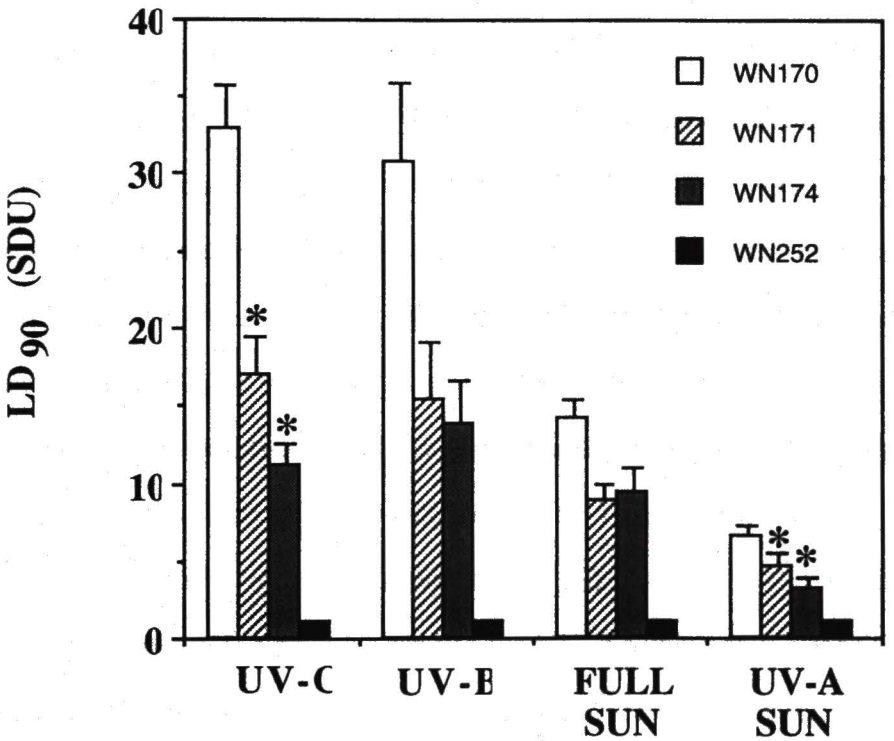
As shows in Fig.12 and on Tables 3 and 4, under the artificially produced UV-C irradiation, there was a significant difference in sensitivity to the killing effects of this kind of UV light among WN170, WN171 and WN174 spores. WN170 had the highest resistance, and strain WN171 had significantly higher resistance than WN174 did (Table 4). This suggests that at short UV-C wavelengths, the *spl* repair system was more efficient in correcting this damage than *uvr* pathway.

As the UV wavelength shifts to the longer artificially produced UV-B region , the difference in sensitivity between wild-type strain WN170 spores with either WN171 or WN174 spores is still significant (Fig. 12; Table 4). But the UV sensitivity of strain WN171 and WN174 spores become almost identical (Fig. 9b). This same pattern can also be seen for the full spectrum sunlight exposure experiment (Fig. 10). The results taken together suggest that in response to artificially produced UV-B, or to sunlight containing UV-B, both the *spl* and *uvr* pathways contribute roughly equally to spore resistance (Fig. 12).

Upon exposure to the portion of sunlight containing wavelengths of UV-A or longer, the survival curve patterns again became similar to the UV-C pattern (Fig. 12). That is, the differences in spore UV resistance among each pair of these strains are significant (Table 4), and the resistance order from high to low was WN170 > WN171 > WN174 > WN252 (Fig. 12).

The exact explanation in the above observations is not known, but may relate to the

Figure 12. **UV resistance of *Bacillus subtilis* spores to various treatments** The LD_{90} of each strain in SDU is shown. Error bars denote standard deviations. Wild type (WN170) was statistically different from mutants under all treatments (see table 4). Asterisks indicate statistically significant differences between the mutants (1% confidence level).


40
30
20
10
0
LD 90 (SDU)
WN170
WN171
WN174
WN252
*
*
*
*
UV-C
UV-B
FULL SUN
UV-A SUN

DNA UV photochemistry. Upon the artificially produced UV-C radiation, the spore photoproduct is likely to be the major DNA damage, since the UV-C region of UV light is corresponding to the DNA absorption peak (260 nm). The spore resistance patterns observed are probably similar upon either UV-B or the full spectrum of sunlight exposure, since the most effective solar wavelengths for the sporocidal action are in the UV-B region (305-325 nm) (Munakata, 1989). In this region of UV light, many researches found that spore photoproduct was still a major DNA damage (Donnellan and Setlow, 1965; Munakata and Rupert, 1974: Tyrell, 1978). The present experiments results showed however that *uvr* and *spl* pathways have almost identical repair capacity (Fig. 12). This indicates that in response to UV-B, spore DNA accumulates a types of damage in addition to SP, which can be repaired by the *uvr* pathway but not by the *spl* pathway. Finally, when the spore resistance to the UV-A portion of sunlight is tested, it appears that the spore photoproduct again comprises the major photoproduct among the DNA damages that can be repaired either by *uvr* and *spl* pathways, since WN171 (*uvr*-, *spl*+) spores have higher resistance than WN174 (*uvr*+, *spl*-) spores (Fig. 12).

Much attention has been focused on the UV-B portion of sunlight since it is a major cause of DNA damage, and it has been proposed that the ozone depletion due to human activity will increase the flux of UV-B which reaches the earth's surface. On the other hand, UV-A is usually considered innocuous both because of its relatively lower energy and because critical biological targets (nucleic acid and proteins) have low or no extinction coefficients at these wavelengths. Moreover, UV-A can be used by DNA photolyase (mainly 300- 500 nm), which has the ability to catalyize photoreactivation that restores the damaged part of a DNA molecule (mainly cyclobutane pyrimidine dimers) *in situ*, without breaking the sugar phosphate DNA backbone (Sancar. 1994). However, recent research has clearly demonstrated that UV-A, especially wavelengths less then 365 nm, can induce production of pyrimidine dimers, besides producing other kinds of cellular and DNA

damages (Tyrell, 1978; Urbach, 1992; Kohen et al., 1995). A hypothesis has been postulated that nonspecific DNA damage may be mediated indirectly by absorption of UV-A by molecules other than DNA and the resulting generation of active oxygen intermediates, such as peroxides, superoxide anion or hydroxyl radical. These activated oxygen species not only can damage the DNA molecule, but can induce damage to other cellular components as well (Urbach, 1992; Kohen et al., 1995).

In a preliminary series of experiments, we tested the contribution of the two major DNA repair systems, *uvr* and *spl*, to spore resistance to artificially-produced UV-A radiation, using up to over 2 x 10^4 J/m^2 (nearly 8.0 hours exposure) under a UV-A lamp. The UV-A lamp used was of insufficient power for these experiments, as under these conditions only a 50% decrease in survival could be seen for spores from strain WN252 (*uvr*$^-$, *spl*$^-$), and spores of the other three strains remained almost unaffected (data not shown). So the experiments were shifted to the UV-A portion of sunlight, using a filter to block the UV-B portion of sunlight (Fig. 1). It was shown that UV-A can also induce DNA damages that can be repaired either by *uvr* or *spl* repair systems (Fig. 11, Fig. 12). Surprisingly, strain WN171 spores had higher resistance than strain WN174 spores (Fig. 12), suggesting that among the DNA damages that can be repaired by either *uvr* or *spl* pathways, a major portion was still spore photoproduct.

However, the results of Fig.12 taken as a whole, show an interesting trend; that is, when the UV light shifted from artificially-produced UV-C to the UV-A portion of sunlight (i.e., as the UV wavelength gets longer), the SDU values of LD_{90} among all strains relative to WN252 get lower. The SDU ratio of LD_{90} values of WN170 : WN171 : WN174 : WN252 decreased from 33 : 17 : 11 : 1 to 6.6 : 4.6 : 3.2 : 1 (Table 3, Fig. 12). The most obvious decreases were between artificially-produced UV-B to full spectrum of sunlight and between full spectrum of sunlight to UV-A portion of sunlight. The data suggests that at wavelengths longer than UV-B, spore viability is decreased due to radiation

damage being produced that is repairable neither by the *uvr* nor the *spl* pathways. Target sites for such damage are not necessarily restricted to DNA. On the other hand, the *uvr* and *spl* pathways are the two major repair systems responsible for spore resistance to UV-C or UV-B radiation; hence DNA damage is the major cause of spore inactivation at shorter UV wavelengths (Fig. 12).

As we know, there are many other repair systems which exist in *Bacillus subtilis* besides *uvr* and *spl*, such as base excision, recombinational repair, and SOS-mediated repair systems (Yasbin et al., 1993). At present it is unclear what contribution these repair systems make to spore resistance properties; however, this could be tested, as mutants lacking some of these additional repair systems are available in *B subtilis.* Another interesting avenue to explore further would be to determine the nature of other cellular damages which lead to spore inactivation at biologically-relevant wavelengths of radiation.

CHAPTER V

SUMMARY

In these series of experiments, we have studied the resistance of spores of four different strains of *Bacillus subtilis* varying in the two major DNA repair systems responsible for spore UV resistance. Upon artificially produced UV-C, UV-B, full spectrum sunlight, and UV-A portion of sunlight, the relative contributions of the two major DNA repair pathways, *uvr* and *spl*, to the survival of spores have been found as follows:

1) Both *uvr* and *spl* repair systems played important roles for the survival of *Bacillus subtilis* spores to UV-C and UV-B irradiation. Among them, *spl* was more efficiency in repair of lethal spore damage caused by the artificially-produced UV-C, presumably spore photoproduct. In repairing the damage produced by artificially produced UV-B, both repair systems appeared to play roughly equivalent roles, suggesting that other type(s) of DNA damage in addition to spore photoproduct is (are) produced by UV-B.

2) In response to either full-spectrum sunlight or the UV-A portion of sunlight, the differences of LD_{90} values for all strains were similar, suggesting that sunlight causes a significant amount of lethal damage to the spore that is not reparable by either the *uvr* or the *spl* DNA repair systems. Such damage may be occurring on cellular components other than DNA.

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