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UV-radiation-induced formation of DNA bipyrimidine photoproducts in *Bacillus subtilis* endospores and their repair during germination

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Summary. The spore photoproduct (SP) is the main DNA lesion after UV-C irradiation, and its repair is crucial for the resistance of spores to UV. The aims of the present study were to assess the formation and repair of bipyrimidine photoproducts in spore DNA of various *Bacillus subtilis* strains using a sensitive HPLC tandem mass spectrometry assay. Strains deficient in nucleotide excision repair, spore photoproduct lyase, homologous recombination (*recA*), and with wild-type repair capability were investigated. Additionally, one strain deficient in the formation of major small, acid-soluble spore proteins (SASPs) was tested. In all SASP wild-type strains, UV-C irradiation generated almost exclusively SP (>95 %) but also a few by-photoproducts. In the major SASP-deficient strain, SP and by-photoproducts were generated in equal quantities. The status of the UV-induced bipyrimidine photoproducts was determined at different stages of spore germination. After a germination time of 60 min, >75% of the SP was repaired in wild-type strains and in the SASP-deficient strain, while half of the photo-induced SP was removed in the *recA*-deficient strain. SP-lyase-deficient spores repaired <20% of the SP produced. Thus, SP lyase, with respect to nucleotide excision repair, has a remarkable impact on the removal of SP upon spore germination. [**Int Microbiol** 2007; 10(1):39-46]

Key words: *Bacillus* endospores · UV-radiation · spore photoproduct · germination · DNA repair

Introduction

Endospores of gram-positive bacteria can persist over at least millennial time spans and survive extreme environments on Earth and even in space [10,17,19]. Solar ultraviolet radiation (UV) is a major factor limiting spore survival in both of these environments [19,20]. Most studies of spore UV resistance have been restricted to investigating the effects of monochromatic 254-nm UV-C in the model spore-former *Bacillus subtilis* [19,30]. From these studies, it has become clear that the resistance of *B. subtilis* spores to 254-nm UV can be accounted for by an interplay between mechanisms that: (i) alter DNA photochemistry within the dormant spore compared to the vegetative bacterium, and (ii) effectively repair UV-induced DNA damage in spores during subsequent germination [19,20,30].

The spectrum of bipyrimidine photoproducts formed in the DNA of UV-irradiated dormant spores is different from that of vegetative cells. The major DNA photoproduct in

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spores exposed to 254-nm UV-C radiation is 5,6-dihydro-5(α-thyminyl)thymine, known as the "spore photoproduct" (SP) [4,34]. Cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) are produced in small quantities at moderate UV fluences [5] whereas single- and double-stranded breaks are generated at very high UV fluences [31]. The preponderance of SP in the DNA of UV-irradiated spores can be explained by different factors, including the dehydrated state of the spore core, the presence of large amounts of dipicolinic acid, and the binding of small, acid-soluble spore proteins (SASPs) of the α/β-type to DNA. All of these key biochemical parameters result from developmental gene expression events that occur during the process of sporulation [19,20,30]. In particular, SASP-α and SASP-β in *B. subtilis* are encoded by the *sspA* and *sspB* genes, respectively, and their transcription is activated in stage III forespores by σ-G RNA polymerase [19,20,30]. Binding of α/β-type SASP to the DNA of spores, together with spore core dehydration, induces a change in the helical conformation of spore DNA from the B-form to an A-like form, which in turn alters its UV-C photochemistry to favor the production of SP [5,19,20,30]. Dipicolinic acid behaves as a photosensitizer that favors the formation of SP at the expense of the other bipyrimidine photoproducts [6,27].

Since dormant spores are metabolically inactive, repair of UV-C-induced DNA damage occurs during the process of germination, when spores reactivate and prepare to return to vegetative growth. Studies with mutants have identified two major DNA-repair systems involved at this stage: (i) the general nucleotide excision repair (NER) pathway, which excises SP and fills in the resulting single-stranded gap, and (ii) an SP-specific repair enzyme called SP lyase that cleaves SP back to the two original thymine residues in a monomerization reaction. The NER system is broadly conserved among prokaryotes [36]. Three subunits encode the Uvr excinuclease (*uvrABC*) of the *B. subtilis* NER mechanism [19]. SP lyase is encoded by *splB*, the second cistron of the *splAB* operon [8], and is widely conserved among endospore-forming bacteria [19]. The RecA-mediated pathway is also involved in DNA repair, albeit to a lesser extent [19,20,30]. Expression of each of these systems has been shown to be significantly regulated at the transcriptional level [19,20]. The NER system is under control of the SOS response mediated by LexA/RecA and is induced by DNA damage during vegetative growth [1–3,36] and germination [28], but is not developmentally activated during sporulation (PJ Riesenman and WL Nicholson, unpublished data). SP lyase is produced upon transcription of *splB* by σ-G RNA polymerase at morphological stage III in the developing forespore, where the enzyme is packaged into the dormant spore and activated during germination [7,21,22]. Transcription of *splB* is not observed during either vegetative growth or germination, nor is it induced by DNA damage during those processes [1,21] [R Moeller et al., unpublished data].

Earlier work on the repair of UV-induced photoproducts during spore germination relied on cumbersome techniques with limited sensitivity and ability to detect any of the UV photoproducts [15,16,33]. The development of a sensitive and rapid HPLC-MS/MS technique has allowed the separation, identification, and quantification of the majority of UV-induced DNA photoproducts in spores at a level of resolution previously unattained [5,6]. Here, we report on the formation and repair of all possible bipyrimidine photoproducts encountered within the DNA of UV-C-irradiated spores of strains deficient in different repair systems. The data are correlated with the UV radiation resistance of these strains, which is a component of their genetic makeup.

Material and methods

***Bacillus subtilis* strains, sporulation, and spore purification.** The following *Bacillus* sp. endospores were used: two wild-type strains, *B. subtilis* 168 DSM 402 [14] and *B. atrophaeus* DSM 675 [13] (both obtained from the DSMZ, Braunschweig, Germany); one strain deficient in NER, the spore photoproduct lyase (*ΔsplB*), and the homologous recombination pathway (*ΔrecA*), termed *ΔrecA ΔsplB ΔuvrB* (N Munakata, unpublished data); one NER- and SP-lyase-deficient strain (*ΔsplB ΔuvrB* [11]); one single homologous-recombination-deficient strain (*ΔrecA* [3]); and one strain lacking DNA saturation with SASPs, termed *ΔsspA ΔsspB* [5]. Unless otherwise stated, the indicated genes and products were of *B. subtilis* origin. Spores of each strain were obtained by cultivation under vigorous aeration in liquid Schaeffer sporulation medium [26], purified, and stored as described previously [13,14,18]. The mutations did not significantly affect sporulation efficiency. Spore preparations were free (<1%) of growing cells, germinating spores, and cell debris, as monitored by phase-contrast microscopy.

UV-C irradiation and extraction of spore DNA. Spores (5×10^8 spores/ml) in aqueous suspension (10 ml) were exposed to UV-C radiation from a low-pressure mercury lamp (Model NN 8/15, Heraeus, Berlin, Germany). All irradiations were carried out at 4°C with continuous stirring of the suspensions to ensure homogeneous exposures. The spectral irradiance of the mercury low-pressure lamp with a major emission line at 253.65 nm was measured with a double monochromator (Bentham model DM 300). A fluence rate of 90 μW/cm² was determined by using a UV-X radiometer (UVP Ultra-Violet Products, Cambridge, UK). After UV irradiation at defined fluences (0, 50, 125, 250, 375, 500 J/m^2), the following aliquots were taken for further analysis: 100 μl for survival studies, 1.5 ml for DNA photoproduct analysis, and separately 10 ml (exposed to a fluence of 125 J/m^2) for germination and repair studies. Survival was determined by the colony-forming assay [13]. For the analysis of DNA photoproducts, high-molecular-weight DNA was isolated from irradiated and germinating spores using the Wizard Genomic DNA Purification Kit (Promega, Mannheim, Germany) procedure for genomic DNA isolation from bacteria [23]. Prior to DNA isolation, the spore coats were chemically removed as described previously [14,25].

Spore germination. Ten ml of spore suspension exposed to 125 J/m^2 of UV-C radiation was transferred directly into 100 ml of 2×LB medium

with an addition of 4 mM L-alanine to stimulate initiation of spore germination [29] and incubated at 37°C for various times. Germination efficiency was determined as the loss of spore wet-heat resistance after heat treatment at 80°C for 10 min and following the decrease in OD at 600 nm, as described earlier [28]. After 0, 30, 60, 90, and 120 min of germination, 15-ml samples were taken for DNA extraction and subsequent photolesion analysis, and 100 µl to assay colony formation.

Quantitation of inter- (and intrastrand) bipyrimidine photoproducts. Isolated DNA samples from irradiated and germinating spores were enzymatically digested with nuclease P1 and phosphodiesterase I as described previously [5,23]. The digested samples were analyzed by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) to detect dimeric photoproducts involving adjacent pyrimidines released as modified dinucleoside monophosphates [5,6]. The following photoproducts were quantitated as dinucleoside monophosphates using analytical methods described previously [5]: SP; cyclobutane pyrimidine dimers (CPD) of thymine-thymine (CPD TT), thymine-cytosine (CPD TC), cytosine-thymine (CPD CT), and cytosine-cytosine (CPD CC); as well as the pyrimidine (6-4) and pyrimidone photoproducts (6-4 PP) of thymine-thymine (6-4 TT) and thymine-cytosine (6-4 TC). The dimeric pyrimidine photoproducts were quantified in the multiple reaction-monitoring (MRM) mode using transitions reported previously [5,6]. Additional experiments were carried out in the product ion scan mode. For this purpose, the full fragmentation spectra (mass range 200–450) of pseudomolecular ions at 545, 530, and 531 m/z were recorded during HPLC analysis.

Numerical and statistical analysis. Survival graphs were constructed by plotting the logarithm of the fraction of surviving spores vs. UV fluence [13]. To determine the graph parameters, the following relationship was used: $\ln (N/N_0) = -k_i \times F + n$, with N = CFU/ml after irradiation, N_0 = CFU/ml without irradiation, k_i = inactivation constant (m^2/J), F = UV fluence (J/m^2), and n = extrapolation number, i.e., the intercept with the ordinate of the extrapolated semi-log straight line. The constants k_i and n were determined by linear regression. The ratio of the k_i values of the repair-deficient strains to those of wild-type strain 168 was called the "repair factor with regard to survival" (RF_S). It reflected the contribution of the involved repair processes to overall survival. Further, the fluence resulting in 10% survival (F_{10}) was determined. Photoproduct induction s were obtained by plotting the number of photolesions per 10^4 bases vs. fluence. The efficiency of photoproduct induction was determined from: $PP = k_{PP} \times F$, with PP = photoproducts/10^4 bases, k_{PP} = photoproduct induction constant (m^2/J), and k_{PP} determined by linear regression of the induction s. For quantifying the distribution of the UV-induced DNA bipyrimidine photoproducts in the irradiated spores, the amounts at each fluence were summarized, normalized, and expressed as the number of photolesions/10^4 bases per Jm^{-2} according to a previously described method [23]. All experiments were repeated at least three times. The data shown are mean values with standard deviations and were compared statistically using Student's *t* test. Values were analyzed in multigroup pair-wise combinations. Differences with *P* values ≤0.05 were considered statistically significant. To compare the resistance of spores with different genetic backgrounds, all of the spores were prepared at the same time and tested together [13,14].

Results

Survival and induction of DNA bipyrimidine photoproducts upon UV-C irradiation. Spores of wild-type *B. atrophaeus* and *B. subtilis* strains, as well as *B. subtilis* strains carrying either deletions of the genes encoding α/β-SASP (*ΔsspA ΔsspB*), or deletions in genes encoding components of the major DNA repair pathways (SP lyase: *ΔsplB*, NER: *ΔuvrB*, Rec: *ΔrecA*) were exposed to 254-nm UV-C radiation. Spore UV inactivation kinetics and photoproduct production were assessed in the same samples (Fig. 1). The UV survival graphs of the spores were either uni-exponential

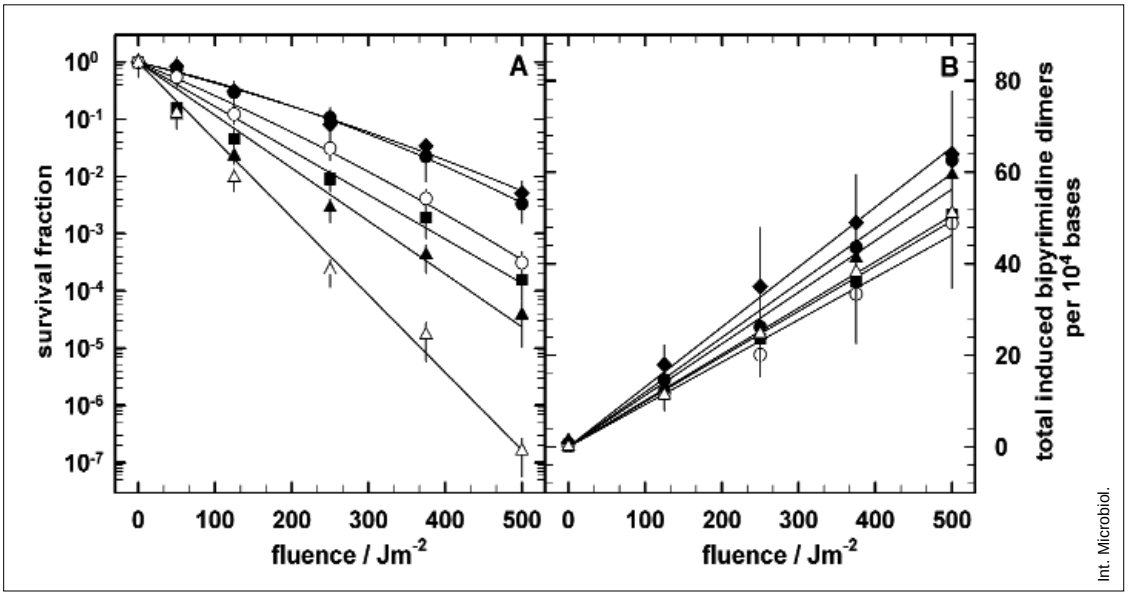

Fig. 1. Fluence-survival (**A**) and induction of bipyrimidine photoproducts (**B**) of *Bacillus atrophaeus* (closed diamonds), *B. subtilis* 168 (closed circles), *ΔsspA ΔsspB* (closed squares), *ΔrecA* (open circles), *ΔsplB ΔuvrB* (closed triangles), and *ΔrecA ΔsplB ΔuvrB* (open triangles) after exposure to 254-nm UV-C. Data are averages ± standard deviations (*n* = 4).

Table 1. Survival graph characteristics after UV-C irradiation (data obtained from Fig. 1 A, B)

Strain	F_{10}	k_i	n	RF_s	k_{PP}
Bacillus atrophaeus	247.3 ± 29.2	$(1.3 \pm 0.2)\times10^{-2}$	1.7 ± 0.2	0.93	0.12 ± 0.02
B. subtilis 168	258.7 ± 18.5	$(1.4 \pm 0.4)\times10^{-2}$	2.0 ± 0.3	1.00	0.13 ± 0.01
ΔsspAΔsspB	112.3 ± 19.4	$(1.6 \pm 0.3)\times10^{-2}$	0.7 ± 0.2	1.19	0.10 ± 0.03
ΔrecA	191.8 ± 32.1	$(1.8 \pm 0.4)\times10^{-2}$	1.6 ± 0.4	1.35	0.09 ± 0.02
ΔsplBΔuvrB	67.4 ± 26.4	$(2.3 \pm 0.3)\times10^{-2}$	0.7 ± 0.3	1.69	0.11 ± 0.04
ΔrecAΔsplBΔuvrB	60.5 ± 11.2	$(2.9 \pm 0.5)\times10^{-2}$	0.6 ± 0.3	2.09	0.10 ± 0.01

F_{10}, Fluence (J/m^2) of UV-C irradiation reducing the survival of the spore population to 10% [1]; k_i, inactivation constant (m^2/J); n, extrapolation number, i.e., the intercept with the ordinate of the extrapolated semi-log straight line [24]; RF_s, repair factor with regard to survival of *B. subtilis* 168; k_{PP}, photoproduct induction constant (m^2/J).

(*ΔsspA ΔsspB* strain and all repair-deficient strains except *ΔrecA*) or showed a slight shoulder, as indicated by n > 1 (*B. subtilis* wild-type and *ΔrecA* strain, and *B. atrophaeus*; Fig. 1A and Table 1). The F_{10} values of the wild-type spores and the *recA* mutant (Table 1) were in good agreement with published data (compiled as LD_{90} by Nicholson et al. [19]), whereas the F_{10} values of the *ΔsspA ΔsspB*, *ΔsplB ΔuvrB* and *ΔrecA ΔsplB ΔuvrB* strains were substantially higher, by a factor of 4–10, than published values [12,19]. It is well-established that the sporulation conditions influence the UV responses of spores [9,18]. In our work, however, spores of all strains were produced by the same method, a condition that allowed comparison of their UV resistance, whereas the literature data were compiled from different studies. Our data showed that spore UV resistance decreased in the order wild-type > *ΔrecA* > *ΔsspAΔsspB* > *ΔsplBΔuvrB* > *ΔrecAΔsplB ΔuvrB*, which is in agreement with previously published results [19]. Examination of the RF_S values (Table 1) showed that, among the repair mechanisms, the *recA* system was the least efficient for repairing UV-C damage induced in spores, whereas the combined bipyrimidine photoproducts repair mechanisms *splB* and *uvrB* had a higher repair efficiency, which was further ameliorated by the presence of the *recA* system.

The fluence-dependent formation of bipyrimidine photoproducts induced in the DNA of UV-C-irradiated spores is shown in Fig. 1B. The total amount of photoproducts/10^4 bases increased linearly with the applied fluence. It is interesting to note that the induction constant k_{PP} was slightly lower for the *recA*, *splB uvrB*, *recA splB uvrB*, and *sspA sspB* mutants than for the wild-type strains (Table 1). A similar effect was observed by Douki et al. [5] for the latter strain. However, the differences in k_{PP} were not statistically significant; thus, none of the mutations tested was found to significantly alter the overall quantum yield of total DNA photoproduct formation with respect to the wild-type strains.

Using HPLC-MS/MS, it was possible to determine not only the total amount of bipyrimidine photoproducts, but also to identify and quantify each type of photoproduct formed in the UV-irradiated spores. The results are compiled in Table 2. SP was the predominant DNA photoproduct (93 ± 5%) in all spores with wild-type SASP formation. CPD TC, CPD TT, and CPD CT as well as 6-4 TC and 6-4 TT were also detected, albeit in small amounts (5–7% of the total bipyrimidine photoproducts). Whereas the amount of total DNA photoproducts was similar in spores of the *ΔsspA ΔsspB* strain and in those possessing SASP, the spectrum of induced photoproducts was significantly altered compared to the pattern

Table 2. Yields of photolesions in spores exposed to UV-C radiation

Strain	SP	CPD TC	CPD TT	6-4 TC	CPD CT	6-4 TT	Total
B. atrophaeus	$(1.4 \pm 0.4)\times10^{-1}$	$(3.9 \pm 0.3)\times10^{-3}$	$(2.8 \pm 0.7)\times10^{-3}$	$(3.0 \pm 0.4)\times10^{-3}$	$(8.7 \pm 2.1)\times10^{-4}$	$(5.2 \pm 1.8)\times10^{-4}$	$(1.4 \pm 0.5)\times10^{-1}$
B. subtilis 168	$(1.1 \pm 0.2)\times10^{-1}$	$(3.2 \pm 0.3)\times10^{-3}$	$(3.0 \pm 0.6)\times10^{-3}$	$(2.5 \pm 0.3)\times10^{-3}$	$(7.2 \pm 1.7)\times10^{-4}$	$(4.8 \pm 1.4)\times10^{-4}$	$(1.2 \pm 0.2)\times10^{-1}$
ΔsspAΔsspB	$(0.5 \pm 0.1)\times10^{-1}$	$(1.2 \pm 0.1)\times10^{-2}$	$(2.3 \pm 0.2)\times10^{-2}$	$(1.3 \pm 0.3)\times10^{-2}$	$(1.5 \pm 0.1)\times10^{-3}$	$(1.8 \pm 0.4)\times10^{-3}$	$(1.0 \pm 0.3)\times10^{-1}$
ΔrecA	$(0.9 \pm 0.3)\times10^{-1}$	$(4.2 \pm 0.3)\times10^{-3}$	$(2.4 \pm 0.5)\times10^{-3}$	$(3.3 \pm 0.4)\times10^{-3}$	$(8.2 \pm 2.3)\times10^{-4}$	$(5.5 \pm 2.0)\times10^{-4}$	$(1.0 \pm 0.3)\times10^{-1}$
ΔsplBΔuvrB	$(1.0 \pm 0.2)\times10^{-1}$	$(2.2 \pm 0.3)\times10^{-3}$	$(2.8 \pm 0.7)\times10^{-3}$	$(2.6 \pm 0.6)\times10^{-3}$	$(7.0 \pm 1.9)\times10^{-4}$	$(3.0 \pm 0.8)\times10^{-4}$	$(1.1 \pm 0.2)\times10^{-1}$
ΔrecAΔsplBΔuvrB	$(0.9 \pm 0.3)\times10^{-1}$	$(3.1 \pm 0.3)\times10^{-3}$	$(4.0 \pm 1.1)\times10^{-3}$	$(2.8 \pm 0.4)\times10^{-3}$	$(6.7 \pm 3.1)\times10^{-4}$	$(7.1 \pm 2.6)\times10^{-4}$	$(1.1 \pm 0.3)\times10^{-1}$

Data are expressed as lesions/10^4 bases per Jm^{-2}. Data are averages and standard deviations $n = 4$.

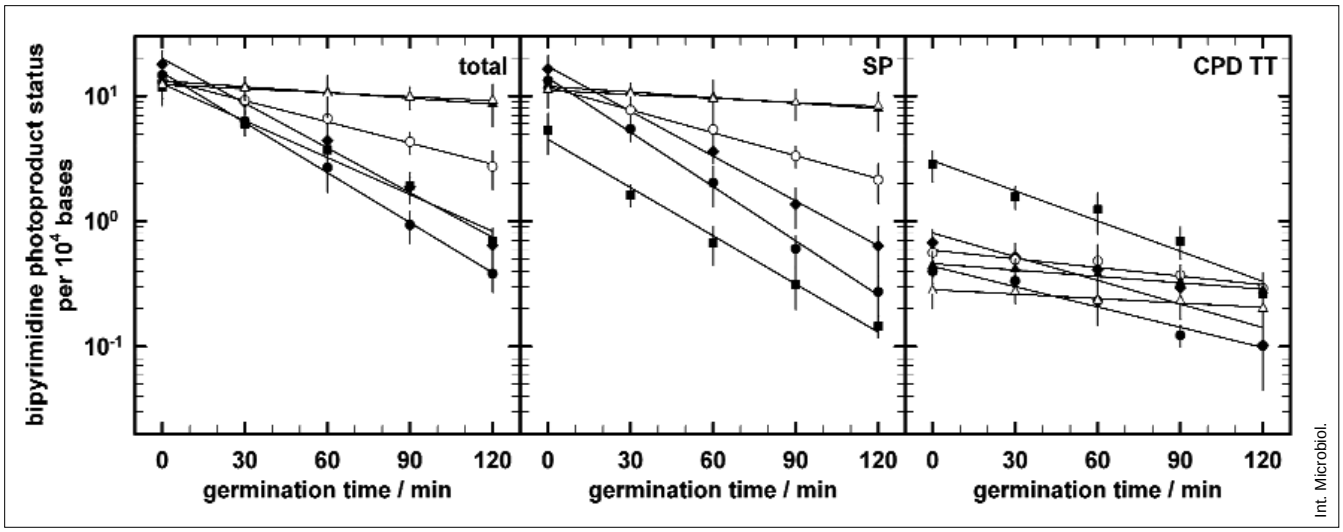

Fig. 2. Repair kinetics of total bipyrimidine photoproducts, the spore photoproduct (SP), and CPD TT during germination of *B. atrophaeus* (closed diamonds), *B. subtilis* 168 (closed circles), *ΔsspA ΔsspB* (closed squares), *ΔrecA* (open circles), *ΔsplB ΔuvrB* (closed triangles) and *ΔrecA ΔsplB ΔuvrB* (open triangles) spores exposed to 125 J/m² of UV-C radiation. Data are reported as averages and standard deviations (n = 6).

observed in the wild-type. In *ΔsspA ΔsspB* spores, SP was still the main DNA lesion, with 48 ± 6% of the overall photoproducts, and the residual photoproducts were distributed among CPDs (CPD TT 24 ± 5%, CPD TC 13 ± 8%, CPD CT 2 ± 1%) and 6-4 PPs (6-4 TT 2 ± 1%, 6-4 TC 13 ± 7%).

Photoproduct repair during spore germination. In order to monitor the ability of wild-type and mutant spores to repair UV-induced DNA damage during germination, spores of each strain were irradiated with 254-nm UV-C to a dose of 125 J/m². The spores were then germinated in 2×LB medium with L-alanine, and the kinetics of photoproduct repair during germination were assessed. To monitor germination, samples were taken in parallel for the determination of optical density and germination-associated loss of heat resistance. Each strain showed normal spore germination kinetics as monitored by typical optical density measurements, loss of heat resistance, and low cell-replication activity during the first 2 h of germination (data not shown). In short, spores of all strains germinated at approximately the same rate and extent, in good agreement with published data [14].

The repair kinetics of the total bipyrimidine photoproducts of SP and of CPD TT showed a first-order exponential decrease with germination time (Fig. 2). *B. atrophaeus*, *B. subtilis* 168, and the *ΔsspA*, *ΔsspB* spores showed nearly equally efficient rates of overall repair of all bipyrimidine dimers, whereas there was little or nearly no repair of the total photoproducts during germination of *ΔsplB ΔuvrB* spores and *ΔrecA ΔsplB ΔuvrB* mutants. After 1 h of germination, a high percentage of the total induced photolesions was repaired in *B. atrophaeus* (75 ± 15%) and *B. subtilis* 168 (81 ± 10%), and in the α/β-type SASP deficient strain (69 ± 18%) (Table 3). The removal of total photoproducts from the DNA of the *ΔrecA* strain was 49 ± 15%, i.e., significantly

Table 3. Table 3. Repair of bipyrimidine photoproducts in spore DNA of wild-type and mutant strains

Strain	SP	CPD TC	CPD TT	6-4 TC	CPD CT	6-4 TT	Total
B. atrophaeus	78.1 ± 12.1	69.1 ± 13.0	39.1 ± 7.5	40.4 ± 7.7	43.1 ± 15.6	46.9 ± 8.7	75.5 ± 14.6
B. subtilis 168	84.7 ± 13.8	76.0 ± 7.1	41.9 ± 12.2	26.4 ± 9.1	62.4 ± 20.1	83.4 ± 16.6	81.2 ± 10.4
ΔsspAΔsspB	83.1 ± 11.4	55.8 ± 18.3	56.8 ± 9.5	47.7 ± 10.6	39.2 ± 8.7	47.8 ± 9.8	69.1 ± 18.2
ΔrecA	53.1 ± 7.6	34.2 ± 9.5	14.2 ± 7.1	28.5 ± 9.3	28.2 ± 10.3	45.6 ± 14.9	49.4 ± 15.2
ΔsplBΔuvrB	18.8 ± 10.5	29.2 ± 12.3	14.9 ± 5.6	25.2 ± 14.2	23.7 ± 12.7	27.6 ± 10.6	19.2 ± 13.1
ΔrecAΔsplBΔuvrB	15.3 ± 8.3	8.2 ± 5.3	15.5 ± 10.1	13.1 ± 8.3	19.8 ± 9.5	19.2 ± 9.8	15.1 ± 9.6

Measurements were made at 60 min of germination and are expressed as percent of photoproduct repaired relative to time zero of germination. Data are averages and standard deviations (n = 6).

lower during the same period of germination (Table 3). Negligible, but still measurable, photoproduct removal was achieved in germinating *ΔsplB ΔuvrB* spores (19 ± 8%) or *ΔrecA ΔsplB ΔuvrB* spores (15 ± 9%) after 1 h of germination. These data further support the supposition that SP lyase and NER enzymes play major roles in the repair of UV damage induced in the DNA of bacterial endospores [1], whereas homologous recombination has only a minor contribution. Note that these repair studies were done with spores exposed to a relatively low UV fluence (125 J/m²), well below the F_{10} values of the wild-type strains. The data for RecA-mediated repair might differ if higher doses are applied. It is also interesting to note that the germinating *ΔrecA ΔsplB ΔuvrB* spores lacking SP lyase, NER, and RecA repair pathways still exhibited some residual DNA repair activity, perhaps due to additional, uncharacterized minor DNA repair pathway(s) that become active during germination.

The high resolution and sensitivity of the HPLC-MS/MS system facilitated the measurement of each bipyrimidine photoproduct as residual lesions after 60 min of germination (Table 3). This afforded an opportunity to assess the relative efficiency of SP lyase, NER, and Rec repair for the removal of each photoproduct. Based on the irradiation conditions used, it could be inferred that SP lyase and NER were the major repair systems involved in the removal of SP, whereas homologous recombination had a minor contribution (Table 3). Since SP lyase has been shown to be specific for SP and inactive towards other DNA photoproducts [32], the relative contributions of NER and RecA in coping with CPDs and 6-4 PPs could be assessed. The results showed that the NER and RecA systems have a similar repair capacity for all CPDs (CPD TT, CPD CT, and CPD TC) and the 6-4 TC photoproduct. NER was slightly more efficient than RecA in repairing 6-4 TT (Table 3); however, this difference was not statistically significant.

Discussion

DNA photoproduct repair during spore germination. As mentioned, wild-type and repair-deficient spores as well as spores deficient in major SASP formation were tested. Note that the pattern and kinetics of germination were identical for the spores of all strains tested. Therefore, the differences observed in photoproduct repair among the different strains must have been due to their genetic makeup and not to unequal germination rates. *B. subtilis* 168 and *B. atrophaeus*, which are closely related according to their 16S rDNA sequence [13] and both have wild-type repair capacity, repaired over 80% of the induced initial SP after 60 min of germination. This efficient repair of DNA photoproducts was mainly performed by SP lyase, which is expressed during sporulation in the forespore [21,22], packaged into the dormant spore, and activated during germination to ensure the early DNA-photoproduct removal of potentially induced SP [24]. SP lyase was also very effective during germination of UV-irradiated spores deficient in α/β-type SASP, in which SP was induced to a lesser amount but still remained the main photolesion and the major repair target during spore germination. The repair of all other bipyrimidine photoproducts was slower (at least 50–75%) than that of SP. This observation is in agreement with the concept of an evolutionary advantage provided by the specific formation of SP in spores, a lesion that is more efficiently repaired than the other photoproducts formed in other cell types and in vegetative bacteria [19,20]. Due to the lack of SP lyase, SP repair was significantly reduced in the two *splB*-deficient strains; however, they also showed a slower repair of the other photoproducts, CPDs and 6-4PPs, probably due to the additional deficiency in NER (*ΔuvrB*). Since in the *recA*-deficient strain the gene product of *splB* is available, SP repair was maintained; however, the deficiency in *ΔrecA* led to a reduced rate of CPD and 64PP repair. RecA, as a multifunctional protein mainly involved in homologous recombination, ensures genome integrity. After UV radiation, RecA participates in the overall repair and closing of DNA strand breaks. It might also support homologous recombination during NER by gap filling after photoproduct excision. A deficiency in both pyrimidine-dimer excision mechanisms, NER (*ΔuvrB*) and SP lyase, led to a deficiency in overall DNA-photoproduct repair. Combined NER and RecA deficiency yielded the lowest amount of DNA-photoproduct repair. These results show that NER and homologous recombination (RecA) can at least partly substitute for SP lyase and thereby prevent deleterious UV effects—at least to a certain extent—in spores lacking this enzyme.

Role of SASPs in spore UV resistance. Spores deficient in α/β-type SASP formation were less resistant against the effects of UV-C irradiation than wild-type SASP spores. This was concomitant with a significant change in the overall DNA photochemistry of spores exposed to UV-C. Since PS356 spores, used in these studies, contain only ~20% of the amount of α/β-type SASPs [5] found in wild-type spores, we were able to monitor a pronounced shift in the DNA photolesion distribution upon UV-C irradiation, with an increased proportion of CPDs and 64PPs at the expense of SP with respect to SASP-containing spores. These data are in agreement with a similar observation reported in a previous publication [5]. The repair kinetics of DNA photoproducts

during germination of wild-type spores (*B. subtilis* 168 and *B. atrophaeus*) and *ΔsspA ΔsspB* spores were compared. The decreasing order of repair efficiency of UV-induced DNA photoproducts was the same in the three cases: SP >>> CPD TC >> CPD TT >> 6-4 TC >> CPD CT > 6-4 TT. In spores with and without deletions in SASP formation, SP was the primary target for DNA repair during spore germination. This is due to the fact the SplB plays a major role in SP removal and *splB*-formation was not affected. The repair of other DNA bipyrimidine photoproducts was delayed because proteins of the NER pathway have to be synthesized de novo during spore germination.

DNA repair and survival. The present work shows that DNA repair plays an important role in spore germination, ensuring the return to vegetative life, i.e., the replication of repaired DNA [28]. A comparison of the capacities to repair the various photoproducts present in the different strains indicated that the removal of SP is required for maximum colony-forming ability during spore germination. The focus of this study was bipyrimidine photoproduct repair in germinating spores after UV-C irradiation. Beside the DNA bipyrimidine photoproducts, other (minor) types of spore damage were induced by UV radiation, such as DNA strand breaks, DNA-protein cross-links, and oxidatively generated modifications. Therefore, in minor damage-repair pathways, such as homologous recombination, SP lyase does not play a large role, i.e., *ΔrecA* and *ΔrecA ΔsplB ΔuvrB* spores should be less able to repair DNA strand breaks produced in minor quantities by UV radiation [31] than *ΔsplB ΔuvrB* spores. Recently, Weller et al. [35] identified in *B. subtilis* a Ku homolog (encoded by the *ykoV* gene) that retains the biochemical characteristics of the eukaryotic Ku heterodimer. The bacterial Ku specifically recruits a DNA ligase (encoded by *ykoU*) to DNA ends which then stimulates DNA ligation. The loss of these proteins leads to hypersensitivity of stationary-phase *B. subtilis* cells to ionizing radiation [35]. From the observation that the Ku system is conserved in spore-forming bacterial species (e.g., *Bacillus* and *Streptomyces*), Weller et al. [35] speculated that nonhomologous end-joining (NHEJ) via the prokaryotic Ku system functions during subsequent spore germination to repair double-stranded breaks induced in dormant bacterial spores. It will be essential to study the role of NHEJ in spore resistance to environmental UV radiation (≥ 290 nm), which produces strand breaks such as single- and double-stranded breaks as minor photoproducts [31]. Further work on microbial UV photobiology at the genetic level is in progress (Moeller et al., unpublished data).

In conclusion, our work shows that DNA repair efficiency in germinating spores is inherent to their genetic makeup. This finding improves our understanding of the repair pathways necessary for photoproduct repair and germination, as well as the molecular basis for the unique resistance of spores to extreme environmental stressors such as UV radiation.

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References

1. Au N, Kuester-Schoeck E, Mandava V, et al. (2005) Genetic composition of the *Bacillus subtilis* SOS system. J Bacteriol 187:7655-7666
2. Cheo DL, Bayles KW, Yasbin RE (1991) Cloning and characterization of DNA damage-inducible promoter regions from *Bacillus subtilis*. J Bacteriol 173:1696-1703
3. Cheo DL, Bayles KW, Yasbin RE (1992) Molecular characterization of regulatory elements controlling expression of the *Bacillus subtilis recA*1 gene. Biochimie 74:755-762
4. Donnellan Jr. JE, Setlow RB (1965) Thymine photoproducts but not thymine dimers are found in ultraviolet irradiated bacterial spores. Science 149:308-310
5. Douki T, Setlow B, Setlow P (2005a) Effects of the binding of α/β-type small, acid-soluble spore proteins on the photochemistry of DNA in spores of *Bacillus subtilis* and in vitro. Photochem Photobiol 81:163-169
6. Douki T, Setlow B, Setlow P (2005b) Photosensitization of DNA by dipicolinic acid, a major component of spores of *Bacillus* species. Photochem Photobiol Sci 4:951-957
7. Fajardo-Cavazos P, Nicholson WL (2000) The TRAP-like SplA protein is a trans-acting negative regulator of spore photoproduct lyase synthesis during *Bacillus subtilis* sporulation. J Bacteriol 182:555-560
8. Fajardo-Cavazos P, Salazar C, Nicholson WL (1993) Molecular cloning and characterization of the *Bacillus subtilis* spore photoproduct lyase (*spl*) gene, which is involved in repair of ultraviolet radiation-induced DNA damage during spore germination. J Bacteriol 175:1735-1744
9. Gould GW (1983) Mechanisms of resistance and dormancy. In: Hurst A, Gould GW (eds) The bacteria spore, vol.2. Academic Press, London, pp 173-209
10. Horneck G, Mileikowsky C, Melosh HJ, Wilson JW, Cucinotta FA, Gladman B (2002) Viable transfer of microorganisms in the solar system and beyond. In: Horneck G, Baumstark-Khan C (eds) Astrobiology: the quest for the conditions of life. Springer, Berlin, pp 55-76
11. Makino F, Munakata N (1978) Deoxyuridine residues in DNA of thymine-requiring *Bacillus subtilis* strains with defective N-glycosidase activity for uracil-containing DNA. J Bacteriol 134:24-29
12. Mason JM, Setlow P (1986) Essential role of small, acid-soluble spore proteins in resistance of *Bacillus subtilis* spores to UV light. J Bacteriol 167:174-178
13. Moeller R, Horneck G, Facius R, Stackebrandt E (2005) Role of pigmentation in protecting *Bacillus* sp. endospores against environmental UV radiation. FEMS Microbiol Ecol 51:231-236
14. Moeller R, Horneck G, Rettberg P, Mollenkopf HJ, Stackebrandt E, Nicholson WL (2006) A method for extracting RNA from dormant and germinating *Bacillus subtilis* strain 168 endospores. Curr Microbiol 53:227-231
15. Munakata N, Rupert CS (1972) Genetically controlled removal of "spore photoproduct" from deoxyribonucleic acid of ultraviolet-irradiated *Bacillus subtilis* spores. J Bacteriol 111:192-198
16. Munakata N, Rupert CS (1974) Dark repair of DNA containing "spore photoproduct" in *Bacillus subtilis*. Mol Gen Genet 130:239-250

17. Nicholson WL (2004) Ubiquity, longevity, and ecological roles of Bacillus spores. In: Ricca E, Henriques AO, Cutting SM (eds) Bacterial spore formers: probiotics and emerging applications. Horizon Scientific Press, Norfolk, UK, pp 1-15
18. Nicholson WL, Law JF (1999) Method for purification of bacterial endospores from soils: UV resistance of natural Sonoran desert soil populations of *Bacillus* spp. with reference to *B. subtilis* strain 168. J Microbiol Methods 35:13-21
19. Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P (2000) Resistance of bacterial endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev 64:548-572
20. Nicholson WL, Schuerger AC, Setlow P (2005) The solar UV environment and bacterial spore UV resistance: considerations for Earth-to-Mars transport by natural processes and human spaceflight. Mutat Res 571:249-264
21. Pedraza-Reyes M, Gutierrez-Corona F, Nicholson WL (1994) Temporal regulation and forespore-specific expression of the spore photoproduct lyase gene by sigma-G RNA polymerase during *Bacillus subtilis* sporulation. J Bacteriol 176:3983-3991
22. Pedraza-Reyes M, Gutierrez-Corona F, Nicholson WL (1997) Spore photoproduct lyase operon (*splAB*) regulation during *Bacillus subtilis* sporulation: modulation of *splB-lacZ* fusion expression by P1 promoter mutations and by an in-frame deletion of *splA*. Curr Microbiol 34:133-137
23. Pogoda de la Vega U, Rettberg P, Douki T, Cadet J, Horneck G (2005) Sensitivity to polychromatic UV-radiation of strains of *Deinococcus radiodurans* differing in their DNA repair capacity. Int J Radiat Biol 81:601-611
24. Rebeil R, Nicholson WL (2001) The subunit structure and catalytic mechanism of the *Bacillus subtilis* DNA repair enzyme spore photoproduct lyase. Proc Natl Acad Sci USA 98:9038-9043
25. Riesenman P, Nicholson WL (2000) Role of spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. Appl Environ Microbiol 66:620-626
26. Schaeffer P, Millet J, Aubert JP (1965) Catabolic repression of bacterial sporulation. Proc Natl Acad Sci USA 45:704-711
27. Setlow B, Setlow P (1993) Dipicolinic acid greatly enhances the production of spore photoproduct in bacterial spores upon ultraviolet irradiation. Appl Environ Microbiol 59:640-643
28. Setlow B, Setlow P (1996) Role of DNA repair in *Bacillus subtilis* spore resistance. J Bacteriol 178:3486-3495
29. Setlow B, Melly E, Setlow P (2001) Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. J Bacteriol 183:4894-4899
30. Setlow P (2001) Resistance of spores of *Bacillus* species to ultraviolet light. Environ Mol Mutagen 38:97-104
31. Slieman TA, Nicholson WL (2000) Artificial and solar UV radiation induces strand breaks and cyclobutane dimers in *Bacillus subtilis* spore DNA. Appl Environ Microbiol 66:199-205
32. Slieman TA, Rebeil R, Nicholson WL (2000) Spore photoproduct (SP) lyase from *Bacillus subtilis* specifically binds to and cleaves SP (5-thyminyl-5,6-dihydrothymine) but not cyclobutane pyrimidine dimers in UV-irradiated DNA. J Bacteriol 182:6412-6417
33. Spizizen J (1958) Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc Natl Acad Sci USA 44:1072-1078
34. Varghese AJ (1970) 5-thyminyl-5,6-dihydrothymine from DNA irradiated with ultraviolet light. Biochem Biophys Res Commun 38:484-490
35. Weller GR, Kysela B, Roy R, et al. (2002) Identification of a DNA nonhomologous end-joining complex in bacteria. Science 297:1686-1689
36. Yasbin RE, Cheo D, Bol D (1993) DNA repair systems. In: Sonenshein AL, Hoch JA, Losick R (eds) *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. ASM Press, Washington, DC, pp 529-537