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Role of DNA Repair and Protective Components in *Bacillus subtilis* Spore Resistance to Inactivation by 400-nm-Wavelength Blue Light

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ABSTRACT The high intrinsic decontamination resistance of *Firmicutes* spores is important medically (disease) and commercially (food spoilage). Effective methods of spore eradication would be of considerable interest in the health care and medical product industries, particularly if the decontamination method effectively killed spores while remaining benign to both humans and sensitive equipment. Intense blue light at a ~400 nm wavelength is one such treatment that has drawn significant interest. This work has determined the resistance of spores to blue light in an extensive panel of *Bacillus subtilis* strains, including wild-type strains and mutants that (i) lack protective components such as the spore coat and its pigment(s) or the DNA protective α/β-type small, acid-soluble spore proteins (SASP); (ii) have an elevated spore core water content; or (iii) lack enzymes involved in DNA repair, including those for homologous recombination and nonhomologous end joining (HR and NHEJ), apurinic/apyrimidinic endonucleases, nucleotide and base excision repair (NER and BER), translesion synthesis (TLS) by Y-family DNA polymerases, and spore photoproduct (SP) removal by SP lyase (SPL). The most important factors in spore blue light resistance were determined to be spore coats/pigmentation, α/β-type SASP, NER, BER, TLS, and SP repair. A major conclusion from this work is that blue light kills spores by DNA damage, and the results in this work indicate at least some of the specific DNA damage. It appears that high-intensity blue light could be a significant addition to the agents used to kill bacterial spores in applied settings.

IMPORTANCE Effective methods of spore inactivation would be of considerable interest in the health care and medical products industries, particularly if the decontamination method effectively killed spores while remaining benign to both humans and sensitive equipment. Intense blue light radiation is one such treatment that has drawn significant interest. In this work, all known spore-protective features, as well as universal and spore-specific DNA repair mechanisms, were tested in a systematic fashion for their contribution to the resistance of spores to blue light radiation.

KEYWORDS *Bacillus subtilis*, spore resistance, blue light, DNA repair, decontamination, antimicrobial, endospores, protection

The development of new methods for bacterial inactivation has recently attracted increasing attention as a result of the increased prevalence of bacterial antibiotic resistance and the possible use of some bacteria as biological weapons, most notably

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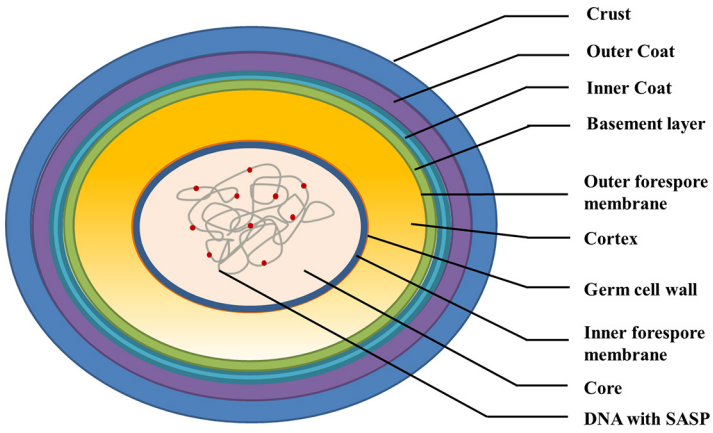

FIG 1 Schematic view of the multilayered *B. subtilis* spore coat structure (red dots displaying the SASP binding to the spore DNA).

spores of *Bacillus anthracis* (1–3). Spores of various species are also a major concern for the medical product, food, and health care industries, as spores cause much food spoilage and human disease and are very resistant to decontamination regimens that readily kill vegetative bacteria (4). Current methods for spore eradication include very high temperatures, UV radiation at 254 nm (UV-C), γ-radiation, and chemicals such as hydrogen peroxide, hypochlorite, chlorine dioxide, and peracetic acid (5). However, while these treatments can be effective, they also can be dangerous to bystanders, damage foodstuffs, and cause severe damage to sensitive equipment. As a consequence, there are continuing efforts to develop new, effective sporicidal regimens that are less damaging than ones currently in use.

Bacterial spores are orders of magnitude more resistant to sporicidal agents than are vegetative cells (6). This extreme resistance is due to the remarkable spore structure and its many unique protective components (Fig. 1) (7). The specific factors that contribute to spores' resistance include (i) the outer coat layers and associated pigments that can protect against UV radiation and many chemicals (8), (ii) the peptidoglycan cortex, which is involved in reducing the spore core water content essential for spore resistance to wet heat and some DNA-damaging agents, (iii) a relatively impermeable inner spore membrane important in resistance to DNA-damaging chemicals, (iv) saturation of DNA in the spore core with α/β-type small, acid-soluble spore proteins that alter DNA structure and result in protection against UV and γ-radiation, many genotoxic chemicals, and wet or dry heat, (v) the high level of Ca^{2+} with dipicolinic acid (CaDPA) in the spore core that lowers core water and alters spore UV radiation resistance (6, 7, 9); and (vi) a multitude of DNA repair enzymes in the spore core, including some that are spore specific, which repair spore DNA damage during germination and outgrowth (7, 9). Note that because of the minimal (if any) metabolic and enzymatic activity in the dormant spore core with its low water content, damage to essential spore macromolecules will accumulate until spores germinate, resume metabolism during outgrowth, and begin repairing macromolecular damage (10). Specific DNA damage repair in spore outgrowth includes dealing with a spore-specific UV photoproduct called the spore photoproduct (SP), which is repaired by an SP-specific repair enzyme called SP lyase (Spl) that monomerizes the SP dimer (11–13). SP and other DNA lesions can also be repaired during outgrowth by base excision repair (BER), which is the most commonly used DNA repair mechanism specific for base lesions. In BER, a glycosylase detects an altered base and hydrolyzes its *N*-glycosylic bond, producing a highly mutagenic apyrimidinic or abasic (AP) site (10). AP endonucleases, like ExoA and Nfo, then incise the DNA 5′ and 3′ to the AP sites, creating a gap in the DNA that is subsequently filled by DNA polymerase. Two other mechanisms are also relevant for spore DNA repair during outgrowth, nucleotide excision repair (NER)

and DNA mismatch repair (MMR) (7). The NER process recognizes and reduces radiation- and chemical-induced DNA base damage, while MMR specifically repairs base-base mismatches and insertion/deletion mispairs caused by errors in DNA replication and recombination (14–16). Finally, DNA can also be repaired by either homologous recombination (HR) or nonhomologous end joining (NHEJ), designed to ameliorate DNA double-strand breaks.

Photodynamic inactivation of vegetative bacteria and spores by high-intensity visible light within the blue range of the spectrum at wavelengths of ~400 nm has recently garnered significant interest due to the intrinsic antimicrobial characteristics of blue light (17–19). Significant activity has been demonstrated against *Bacillus* and *Clostridium* species; however, spores require a significantly larger dose of blue light to mediate killing than do vegetative cells (20). The sporicidal activity of blue light in vegetative bacteria was suggested to be due to the photoexcitation of endogenous porphyrins (photosensitizers) present in the bacterial cell wall, which results in the accumulation of cytotoxic reactive oxygen species (ROS), such as singlet oxygen and hydroxyl radicals (21–23). Indeed, blue light killing of *Helicobacter pylori* and *Propionibacterium acnes* is oxygen dependent (20). Notably, high-intensity blue light treatment is now an accepted method for spore inactivation and one that may find applied uses. However, very little is known of how blue light actually kills spores. Therefore, in the current work, we have examined possible factors involved in spore resistance to blue light, with the goal of learning how blue light actually mediates spore killing.

RESULTS AND DISCUSSION

Blue light is known to have intrinsic antimicrobial effects without the addition of exogenous photosensitizers (21, 22). This property has promise for applications in areas such as medical product sterilization, surface decontamination, or food preservation. The effects of blue light have been tested relatively extensively for vegetative bacteria. However, less is known about the effects of blue light on dormant spores, including how blue light kills spores and what spore factors are important in blue light resistance. Consequently, spores of *B. subtilis* were used as a model system to examine the roles of various DNA repair pathways and spore protective components on spore resistance to blue light with a peak output at 400 nm. In total, spores of 25 isogenic mutants and their respective wild-type strains were exposed to blue light doses of up to 21,000 kJ/m^2, and spore survival was assessed at various fluences (Fig. 2A through D and 3A and B; Tables 3 and 4).

Role of protective structural components in spore inactivation by blue light. Spore structural components (Fig. 1), in particular pigmentation in the spore coat and components in the spore core such as α/β-type SASP, have previously been shown to provide a cumulative protective shield against inactivation by a variety of agents, including genotoxic chemicals, some oxidizing agents, and UV radiation (5, 24). Strikingly, spores of many, but not all, mutants with defects in spore-protective components exhibited decreased blue light resistance (Fig. 2A and 3A; Table 3). The proteins, loss of which decreased spore blue light resistance, in order from having the largest to the least effects, were (i) DNA protective α/β-type SASP (25, 26); (ii) the entire spore coat, most but not all of which is absent in a *cotE gerE* mutant (27) (although a *cotE* mutation which affects only the outer spore coat [28] had a minimal but not significant effect); (iii) CotA, responsible for most outer spore coat pigment production (29, 30); and DacB, as *dacB* spores have an ~75% increased level of core water (25, 31). Overall, these results show that α/β-type SASP in the spore core and, to a lesser extent, spore pigmentation are important in protecting spores against killing by blue-light. The major effect of the absence of most α/β-type SASP on spore blue light resistance also indicates that DNA is the major target for blue light damage leading to spore killing.

Role of different DNA repair mechanisms in spore resistance to 400-nm-wavelength blue light. Since there is minimal, if any, metabolism within the dormant spore, any DNA damage accumulated by spores cannot be repaired in the dormant spores themselves. However, there are multiple DNA repair enzymes in dormant *B. subtilis* spores that can potentially repair spore DNA damage early in spore outgrowth

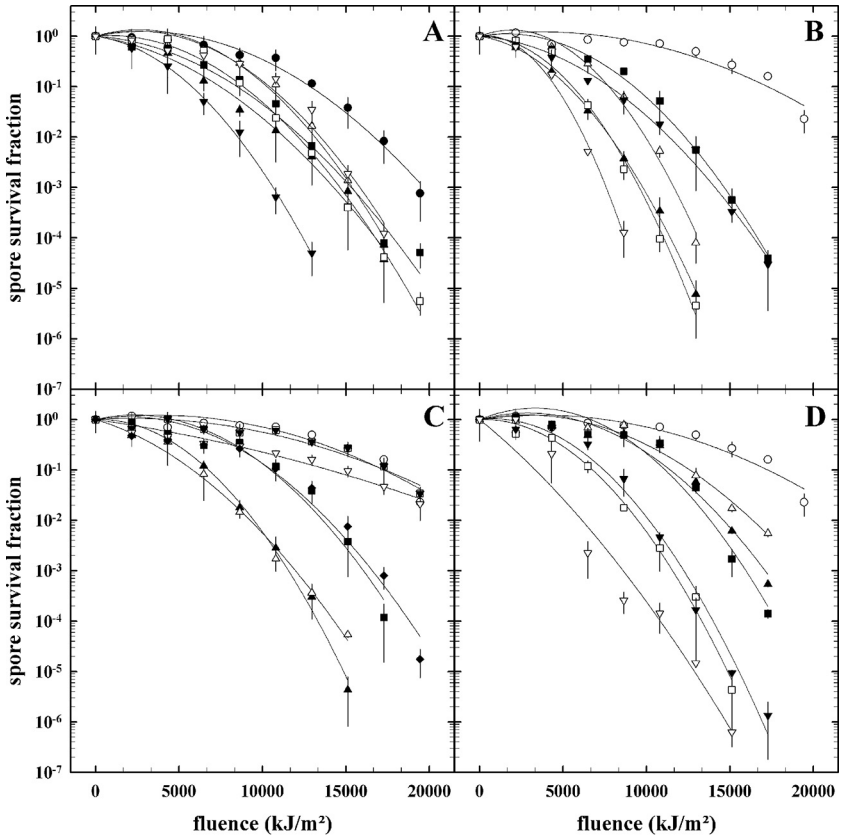

FIG 2 Survival of *B. subtilis* spores in response to 400-nm-wavelength blue light. Spores were prepared and irradiated with blue light and spore killing was measured and calculated as described in Materials and Methods. Spores of various strains are as follows. (A) Protection; wild-type (strain PS832; filled circles), *sspA sspB* (filled upward triangles), *cotE* (open upward triangles), *cotE gerE* (filled squares), *cotA* (open squares), *sspA sspB cotE* (filled downward triangles), and *dacB* (open downward triangles). (B) Repair; wild-type (strain 168; open circles), *exoA* (filled upward triangles), *nfo* (open upward triangles), *exoA nfo* (filled squares), *polY1* (open squares), *polY2* (filled downward triangles), and *polY1 polY2* (open downward triangles). (C) Repair; wild-type (strain 168; open circles), *splB* (filled upward triangles), *uvrAB* (open upward triangles), *splB uvrAB* (filled squares), *ligD ku* (open squares), *recA* (filled downward triangles), *ligD ku recA* (open downward triangles) and *ligD ku splB* (filled diamonds). (D) Repair; wild-type (strain 168; open circles), *mutSL* (filled upward triangles), *disA* (open upward triangles), *sbcDC* (filled squares), *splB* (open squares), *mfd* (filled downward triangles), and *splB mfd* (open downward triangles). Data are shown as means and standard deviations ($n = 3$). Error bars for survival data that are not visible are smaller than the symbol. Illustrative lines of best fit were added to each data set on the graphs to aid interpretation.

when active metabolism returns (9, 32). Consequently, we examined the contribution that various DNA repair systems might make to spore blue light resistance. Indeed, a large number of mutant spores lacking one DNA repair gene exhibited significantly decreased blue light resistance (Fig. 2B through D and 3B; Table 4). Genes in which mutations resulted in 2- to 3-fold decreases in D_{10} (dose in kJ/m² that is necessary to reduce survival to 10%) values included (i) *uvrAB*, important for NER, especially after UV damage (33, 34), (ii) *splB*, essential for repair of SP formed maximally by UV-C irradiation of spores and also by UV-A and UV-B irradiation (35), (iii) *exoA* and *nfo*, important in BER (34), (iv) *mfd*, responsible for repair of genes undergoing transcription (34), and (v) *polY1* and *polY2*, which can carry out TLS over DNA lesions that would otherwise cause cell death (36, 37). Double mutations in any of the genes described above (i to v) either gave similar effects as the single mutations or gave larger effects. In contrast to the genes noted above, mutations in which had large effects on spore blue light resistance, mutations in a number of other DNA repair genes had much smaller to minimal effects on spore blue light resistance. These included *sbcDC*, involved in repair of DNA crosslinks (34), *mutSL*, important in DNA mismatch repair (34), and *disA*, involved in a DNA damage-dependent checkpoint formation in outgrowing spores (37). This last

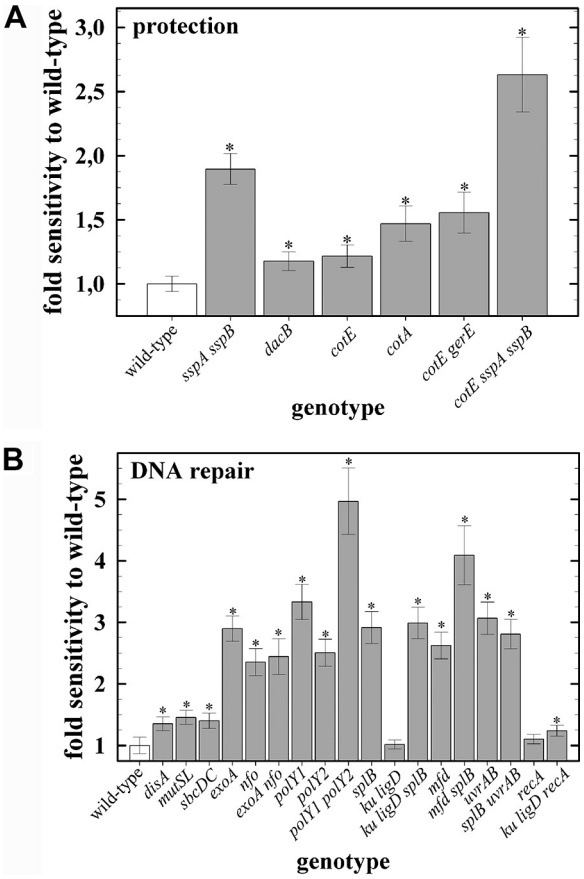

FIG 3 Mutant spores' sensitization to blue light relative to the respective wild-type spores. (A) Mutants lacking proteins involved in spore protection and (B) mutants in genes involved in different DNA repair mechanisms. Data obtained in Fig. 2A through D was used to calculate spores' fold sensitivity to blue light relative to that of wild-type spores, as described in Materials and Methods.

checkpoint allows time for repair of at least some types of DNA damage before outgrowing spores initiate DNA replication (37). In addition, a deletion in the *recA* gene, important in DNA repair by HR (38), or in both the *ku* and *ligD* genes, important in DNA repair by NHEJ, had no effects on spore resistance to blue light (39). Overall, the effects of these mutations in DNA repair genes on spore blue light resistance further suggest that spore DNA is the lethal target in spores for blue light. The results also give some indications of which repair pathways deal with blue light lesions and thus indicate lesions in spore DNA caused by blue light.

Conclusions. The work in this communication strongly indicates that DNA is the spore component that is the major target for lethal damage caused by blue light. This is consistent with the increased blue light sensitivity of spores lacking most DNA protective α/β-type SASP or the DNA repair proteins Spl, ExoA, Nfo, and Mfd. In addition, that *polY1* and/or *polY2* mutations caused the biggest decreases in spore blue light resistance is consistent with lethal blue light damage being to DNA, as the products of these two genes are important in replication over DNA lesions that would block replication by replicative DNA polymerases. It is, of course, next to impossible to rule out the possibility that blue light also causes some damage to other spore components—for example, to spore proteins or lipids—perhaps by oxidative damage caused by ROS generated by blue light.

Given that DNA is the major blue light target, an important question is whether this damage is a direct effect of blue light or an indirect effect, perhaps caused by generation of ROS. Indeed, blue light killing of growing bacteria has been suggested to be due to ROS generation, as ROS scavengers or anoxic conditions are reported to

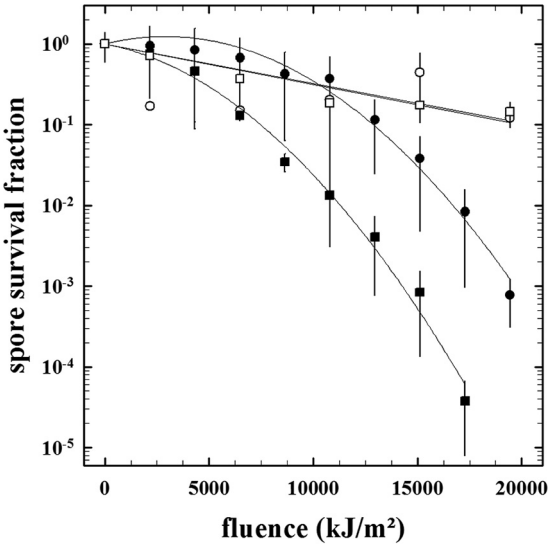

FIG 4 Survival of *B. subtilis* spores in response to 400-nm-wavelength blue light under anoxic conditions (H_2/N_2). Spores were prepared to a concentration of 1×10^7/ml and irradiated with blue light within an anaerobic chamber, and spore killing was measured and calculated as described in Materials and Methods. Spores of various strains are as follows: wild-type (strain PS832) oxic (filled circles), wild-type (strain PS832) anoxic (open circles), *sspA sspB* (strain PS356) oxic (filled squares), and *sspA sspB* (strain PS356) anoxic (open squares). Data are shown as means and standard deviations ($n = 3$). Error bars for survival data that are not visible are smaller than the symbol. Illustrative lines of best fit were added to each data set on the graphs to aid interpretation.

greatly reduce blue light killing of growing cells or spores (40). Importantly, killing of wild-type and α^-/β^- spores by blue light was minimal under anoxic conditions (Fig. 4). This indicates that with at least these spores, ROS generated in spores by blue light are what kills spores, presumably some of the damage is to DNA, and α/β-type SASP are known to protect spore DNA extremely well against ROS (9) (Fig. 4). That DNA is a major target of ROS generated by blue light is also consistent with the increased blue light sensitivity of spores lacking Spl, UvrAB, ExoA, Nfo, Mfd, PolY1, and/or PolY2. It is also possible that with wild-type spores, in which DNA is well protected against blue light, ROS could generate lethal damage to one or more essential spore core proteins. Indeed, this is thought to be how hydrogen peroxide kills dormant spores (37). However, since *uvrAB* and, in particular, *spl* spores exhibit decreased blue light resistance, and Spl only repairs SP, it seems most likely that SP is generated directly by blue light. Indeed, SP is generated by irradiation with UV-A alone (41–43). It is also possible that the increased killing of *uvrAB* spores is due to decreased SP repair, as this is carried out by both Spl and NER using UvrAB (44, 45). It has to be kept in mind that ROS detoxification by catalase or superoxide dismutase appears to play a very minor role, if any, in spore resistance to ionizing radiation or oxidizing agents (46, 47). These enzymes play no role in dormant spore resistance to oxidizing agents, most likely because enzymes in the spore core are inactive due to the core's low water content.

A second question is why *cotE*, *cotA*, and *cotE gerE* spores that have spore coat defects are more blue light sensitive than wild-type spores. There appear to be two possible explanations for this finding, as follows. (i) These coat-defective spores lack pigment in the coats that can absorb 400-nm-wavelength radiation. Thus, the effects of the *cot* mutations may simply be to reduce the spore coat absorption of blue light such that ROS generation in the spore core, where spore DNA is located, is reduced. (ii) Alternatively, blue light may also generate ROS in spore coat layers, but these reactive species would normally be neutralized by reacting with the large amount of spore coat protein. However, ROS generated in the outer layers of *cot* spores would have much less adjacent coat protein to react with and might then damage more inner spore layers, such as the inner membrane. Indeed, inner membrane damage has been shown to be the mechanism whereby oxidizing

TABLE 1 DNA-repair deficient *B. subtilis* strains used in this study

Strain no.	Genotype[a]	Repair mechanism absent	Source or reference
168	Wild-type	None	Laboratory strain
BP130	*trpC2* Δ*splB*::*spc*^{r}	Spore photoproduct (SP) lyase monomerization of SP	This study[b]
GP1175	*trpC2* Δ*uvrAB*::*erm*^{r}	Nucleotide excision repair (NER)/repair of UV-induced damage	34
BD010	*trpC2* Δ*splB*::*spc*^{r} Δ*uvrAB*::*erm*^{r}	NER and SP lyase	Laboratory strain (transformation of GP1175 into BP130)
WN1087	*trpC2 ku ligD*::*erm*^{r}	Nonhomologous end joining (NHEJ)	39
YB3000	*YB886 recA*::pREC260::*erm*^{r}	Multifunctional protein involved in homologous recombination (HR) and DNA repair	38
WN1141	*trpC2 ku ligD*::*erm*^{r} *recA*::pREC260	HR and NHEJ	58
BD011	*trpC2 ku ligD*::*erm*^{r} Δ*splB*::*spc*^{r}	NHEJ and SP lyase	Laboratory strain (transformation of BP130 into BSF1846)
GP1167	*trpC2 mfd*::*ermC*^{r}	Transcription-repair coupling factor (Mfd)	34
BD012	*trpC2 mfd*::*ermC*^{r} Δ*splB*::*spc*	Mfd & SP lyase	Laboratory strain (transformation of BP130 into GP1167)
GP987	*trpC2* Δ*disA*::*tet*^{r}	DNA integrity scanning protein DisA	59
GP1190	*trpC2* Δ*mutSL*::*aphA3*	DNA mismatch repair MMR	34
GP898	*trpC2* Δ*exoA*::*aphA3*^{r}	Apurinic/apyrimidinic (AP) endonuclease	34
GP1502	*trpC2* Δ*nfo*::*cat*^{r}	AP endonuclease	34
GP1503	*trpC2* Δ*exoA*::*aphA3*^{r} Δ*nfo*::*cat*^{r}	Base excision repair pathway (BER), AP endonucleases ExoA and Nfo/repair of oxidative DNA damage	34
GP894	*trpC2* Δ*sbcDC*::*aphA3*^{r}	DNA exonuclease; DNA interstrand cross-link repair	34
PERM646	*trpC2 polY1*::*erm*^{r}	Translesion synthesis (TLS) DNA polymerase Y1	37
PERM647	*trpC2 polY2*::*kan*^{r}	TLS DNA polymerase Y2	37
PERM715	*trpC2 pMUTIN4*::*polY polY2*::*ermC*^{r}	TLS DNA polymerases Y1 and Y2	37

[a]Strain descriptions are according to SubtiWiki (55). Antibiotic resistance is designated as follows: *cat*^{r}, resistant to chloramphenicol (5 μg/ml); *ermC*, resistant to erythromycin (2 μg/ml); *erm*^{r}, resistant to erythromycin-lincomycin (2 and 25 μg/ml, respectively); *spc*^{r}, resistant to spectinomycin (100 μg/ml); *tet*^{r}, resistant to tetracycline (10 μg/ml); and *aphA3*^{r}/*kan*^{r}, resistant to kanamycin (10 μg/ml).

[b]Deletion of the *splB* gene was achieved by transformation with a PCR product consisting of the flanking regions of the target gene and an intervening spectinomycin resistance cassette (spec), as described previously (60). The flanking regions and the spec cassette were amplified using the oligonucleotide pairs FC216/FC217 (5′-GCAAGTACATACGGAAACTTGAAAATCATGTTCCCTATGA/5′CCAGTCCCTATCACCTCAAATGGTTCGCTGCGGATATTCCAGCGCCCTCGGTTCTATATACAC), FC218/FC219 (5′CGCCGAGCGCCTACGAGGAATTTGTATCGGGAACCGATAATTCTGCCATTGATGAGCG/5′-CCTTGATTTGACGGATGGGCTAAAAGATTGCC), and spc-fwd-kan/spc-rev-kan (5′-CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACGTAACGTGACTGGCAAGAG/5′-CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGTTTATTGTTTTCTAAAATCTG), respectively. The fragments were purified and fused by PCR. The resulting deletion cassette was used to transform *B. subtilis* according to the protocol by Kunst and Rapoport (61). Transformants were selected on LB agar plates supplemented with 100 μg/ml spectinomycin. Deletion of the *splB* gene was confirmed by DNA sequencing.

agents, such as ozone and hypochlorite, kill spores, and this damage is much more severe in *cotE* spores (48). We favor the first explanation, since *cotA* spores lack only CotA and not many other coat proteins, whereas *cotE* and *cotE gerE* spores lack many spore coat proteins in addition to CotA (30, 49, 50).

Finally, whether all spore damage by blue light, in particular to DNA, is a direct effect of this irradiation or an indirect effect is not yet completely clear. However, we can draw some conclusions as to the identities of the DNA damage caused by blue light. First, it appears clear that DNA double-strand breaks are not responsible for spore killing by blue light, as loss of proteins involved in HR or NHEJ had at most minimal effects on blue light killing of spores. In contrast, agents such as γ-radiation and vacuum UV radiation do generate double-strand breaks in DNA, and HR and NHEJ proteins are important in spore resistance to these agents (51). The one DNA lesion that is clearly generated in spore DNA by blue light is SP, as an *spl* mutation causes a large decrease in spore blue light resistance, and Spl only repairs SP, which can also be repaired by the NER pathway (45). While ExoA and Nfo do not participate in SP repair by Spl, they are important in repair of other types of DNA damage, including abasic sites and oxidized bases, both of which can be generated directly or indirectly by ROS (52–54). Notably, UV-A has been shown to generate SP, but it does not cause significant cyclobutane-type pyrimidine dimer formation between adjacent pyrimidines in DNA (44, 45). However, there are additional DNA photoproducts that could be generated by blue light, including 6 to 4 photoproducts between adjacent pyrimidines (55, 56). Thus, direct analysis of all DNA photoproducts generated by blue light irradiation of spores seems warranted.

TABLE 2 *B. subtilis* strains giving spores with alterations in protective components

Strain no.	Genotype[a]	Protective factor(s) altered	Source or reference(s)
PS832	Wild-type 168	None (40.4% ± 2.3% core water)	Laboratory strain
PS3328	Δ*cotE*::*tet*^{r}	Outer spore coat morphogenetic protein; assembly of outer spore coat layer	62
PS4424	Δ*cotA*	Outer spore coat pigmentation; more abundant at the mother cell-distal pole of the forespore	Adam Driks strain AD749
PS4150	Δ*cotE* Δ*gerE*::*tet*^{r} *spc*^{r}	Outer and inner spore coat	63
PS578	Δ*sspA* Δ*sspB*::*kan*^{r}	α- and β-type SASP[b]	64
PS3395	Δ*sspA* Δ*sspB* Δ*cotE*::*kan*^{r}	Outer coat and α- and β-type SASP[b]	65
PS1899	Δ*dacB*::*cat*^{r}	Core hydration (64.5% ± 5.5% core water)	25, 31

[a]Strain descriptions are according to SubtiWiki (66). Antibiotic resistance is indicated as follows: *cat*^{r}, resistant to chloramphenicol (5 μg/ml); *spc*^{r}, resistant to spectinomycin (100 μg/ml); *tet*^{r}, resistant to tetracycline (10 μg/ml); and *kan*^{r}, resistant to kanamycin (10 μg/ml).
[b]Δ*sspA* Δ*sspB* spores lack ~80% of their α-/β-type SASP pool.

MATERIALS AND METHODS

Spore production and purification. Endospores from two sets of *B. subtilis* strains were used in this study and are listed in Tables 1 and 2. All mutants are isogenic with their respective wild-type strains, which in this study were either 168 or PS832, the latter being a laboratory derivative of the former strain. The first set of strains were comprised of a panel of mutants that generated spores deficient in various DNA repair activities (Table 1); the second set comprised mutants generating spores altered in various protective factors or structures, such as the spore coat, spore coat pigment, core hydration, or α/β-type SASP (Table 2; Fig. 1). Spores were prepared by cultivation of growing cells in double-strength liquid Schaeffer sporulation medium (SSM) (57) with vigorous aeration at 37°C for 72 h. Spores were harvested and purified by repeated washing steps using sterile water, followed by lysozyme and DNase I treatment for removal of remaining vegetative cells (5). An additional heat inactivation step at 80°C for 10 min was conducted to ensure inactivation of remaining vegetative cells or germinated spores. Final spore preparations were free (>99%) from vegetative cells, germinated spores, and cell debris, as determined by phase-contrast microscopy. Spores were stored at 4°C until used for experiments.

Assay of spore resistance to blue light radiation. High-intensity blue light at a wavelength of 400 nm was generated using a light-emitting diode (LED) flood array (Henkel-Loctite, Hemel Hempstead, United Kingdom) (see Fig. S1 in the supplemental material). The LED array emission peaked at 400 nm at a bandwidth of ±8.5 nm at a full-width half maximum (Fig. 5), as determined using a USB2000 spectrophotometer (Ocean Optics, Oxford, United Kingdom). The device is provided with 144 reflectorized LEDs, which produce a homogeneous illuminated area of 10 × 10 cm. The array produces a uniform light irradiance of 600 J/m^2/s^{-1} at 15.5 cm from the test area and was calibrated at the Defense Science and Technology Laboratory (Dstl), Salisbury, United Kingdom, using a PM100D radiant power meter (Thorlabs, Newton, NJ). The fluence rate was calculated accordingly. The spores were diluted in 2 ml phosphate-buffered saline (PBS; 0.7% Na_2HPO_4 ×2 H_2O, 0.3% KH_2PO_4, 0.4% NaCl; pH 7.5) with a spore concentration of 1 × 10^6 and placed in wells of 12-well microtiter plates before exposure to blue light. Duplicate plates were used; one was exposed to high-intensity blue light and sealed with an Absolute quantitative PCR (qPCR) plate sealer (Thermo Fisher Scientific, Paisley, Scotland) to prevent evaporation, while nontreated control samples were placed next to the exposure plate in the blue light cabinet and wrapped in aluminum foil to account for the heating effect during the treatment.

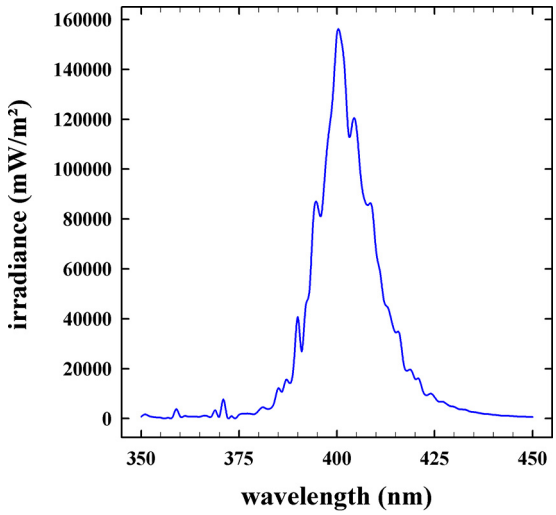

FIG 5 Spectral output of the LED array (Henkel-Locite) used for spore exposure.

TABLE 3 Survival parameters of blue light-exposed *B. subtilis* spores with alterations in protective components[a]

Strain	D_{10} (kJ/m²)	D_0 (kJ/m²)	D_Q (kJ/m²)	IC (m²/kJ)
Wild-type	13,529 ± 1408	11,821 ± 1007	9,514 ± 892	3.66 (±0.41) × 10^{-4}
Δ*cotE*	11,127 ± 981	9,973 ± 942	9,095 ± 772	6.53 (±0.59) × 10^{-4}
Δ*cotA*	9,210 ± 768*	7,826 ± 691*	6,274 ± 587*	9.54 (±0.88) × 10^{-4}*
Δ*cotE* Δ*gerE*	8,702 ± 874*	6,598 ± 667*	5,326 ± 493*	9.87 (±0.90) × 10^{-4}*
Δ*sspA* Δ*sspB* Δ*cotE*	5,142 ± 601*	3,468 ± 289*	2,512 ± 188*	8.75 (±0.09) × 10^{-4}*
Δ*sspA* Δ*sspB*	7,037 ± 841*	5,071 ± 464*	3,580 ± 291*	6.70 (±0.78) × 10^{-4}*
Δ*dacB*	11,493 ± 765*	10,322 ± 1525*	9,431 ± 981*	1.12 (±0.27) × 10^{-3}*

[a]Values were calculated from curves in Fig. 2A and are averages and standard deviations ($n = 3$). D_{10}, dose in kJ/m² that is necessary to reduce survival to 10%; D_0, required dose to reduce relative survival to 37%; D_Q, last dose with 100% spore survival rate. Asterisks indicate survival values that were significantly different from those for wild-type spores ($P \leq 0.05$).

Temperature measurement. The temperature of treated spore suspensions was measured in 12-well plates, in which only 9 wells were used to ensure a consistent exposure to all wells. A submersible aquarium thermometer (ETI, United Kingdom) was used for temperature measurements and was recorded and plotted constantly throughout the treatment at 5 min intervals. In a subsequent test, this temperature measurement was repeated over a period of 3 months on 3 separate occasions, with an extremely high data consistency on each occasion.

Recovery and evaluation of spore survival. Spore survival was determined using appropriate serial dilutions in sterile distilled water. Aliquots of spore dilutions were plated on nutrient broth agar plates and incubated overnight at 37°C to determine the CFU from macroscopically visible colonies.

Numerical and statistical analysis. In all experiments, the *B. subtilis* spore survival fraction was determined from the quotient N/N_0, with N as the mean CFU of blue light-treated samples and N_0 as the mean CFU of untreated controls. Spore inactivation curves were obtained by plotting the logarithm of N/N_0 as a function of dose in kJ/m² for blue light fluency. The inactivation constant (IC) in J/m² was determined from the slope of the dose-effect curves for each sample. In order to determine the slope of the curve, only the linear part of the curve was taken for calculation; the shoulder or nonlinear part of the curve (D_Q value) was neglected. The data in Tables 3 and 4 provide radiation-relevant parameters of the spore inactivation curves. D_{10} demonstrates the dose in kJ/m² that is necessary to reduce survival to 10%, whereas D_0 shows the required dose to reduce relative survival to 37% and best characterizes the sensitivity of the cellular system. The D_Q value, which is also called the quasithreshold (i.e., the intercept of the regression line), is the last dose with 100% spore survival rate. The inactivation constant (IC) was determined from the slope of the dose-effect curves and gives an insight into the "speed" of spore inactivation. All values of N and N_0 were averages of triplicate measurements in each of three independent blue light exposures. All data are expressed as means ± standard deviations ($n = 3$). Significances of differences in the inactivation rates were investigated by analysis of variance (multigroup one-way analysis of variance [ANOVA]), using SigmaPlot Software Version 13.0 (Systat Software GmbH, Erkrath, Germany). Differences with P values of ≤0.05 were considered statistically significant (25, 26).

TABLE 4 Survival parameters of blue light-exposed spores of DNA repair-deficient mutant spores[a]

Strain	D_{10} (kJ/m²)	D_0 (kJ/m²)	D_Q (kJ/m²)	IC (m²/kJ)
Wild-type	16,775 ± 1,215	13,652 ± 1,055	11,980 ± 982	(3.52 ± 0.29) × 10^{-4}
Δ*splB*	5,753 ± 514*	4,270 ± 361*	3,143 ± 320*	(8.83 ± 1.05) × 10^{-4}*
Δ*uvrAB*	5,473 ± 470*	3,712 ± 308*	2,374 ± 264*	(7.43 ± 0.84) × 10^{-4}*
Δ*splB* Δ*uvrAB*	5,974 ± 507*	4,177 ± 380*	2,688 ± 302*	(5.01 ± 0.55) × 10^{-4}*
Δ*ku ligD*	16,525 ± 1179	11,353 ± 1,044	6,671 ± 620	(3.02 ± 0.33) × 10^{-4}
Δ*recA*	15,223 ± 1068	9,748 ± 963	3,028 ± 1055	(1.32 ± 0.19) × 10^{-4}
Δ*ku ligD* Δ*recA*	13,556 ± 980*	6,068 ± 577*	586 ± 86*	(1.75 ± 0.23) × 10^{-4}*
Δ*ku ligD* Δ*splB*	5,611 ± 480*	2,406 ± 211*	28 ± 4*	(6.08 ± 0.72) × 10^{-4}*
Δ*mfd*	6,395 ± 529*	5,023 ± 489*	3,881 ± 364*	(9.53 ± 1.05) × 10^{-4}*
Δ*mfd* Δ*splB*	4,097 ± 362*	2,784 ± 230*	1,493 ± 125*	(1.07 ± 0.12) × 10^{-3}*
Δ*disA*	12,411 ± 1,005*	9,812 ± 887*	7,566 ± 790*	(5.96 ± 0.62) × 10^{-4}*
Δ*mutSL*	11,535 ± 897*	9,517 ± 851*	7,987 ± 738*	(8.25 ± 0.95) × 10^{-4}
Δ*exoA*	5,792 ± 414*	3,918 ± 355*	2,751 ± 269*	(1.03 ± 0.15) × 10^{-3}*
Δ*nfo*	7,125 ± 663*	5,822 ± 607*	4,751 ± 459*	(1.01 ± 0.09) × 10^{-3}*
Δ*exoA* Δ*nfo*	6,868 ± 538*	5,535 ± 512*	4,488 ± 391*	(8.95 ± 1.06) × 10^{-4}*
Δ*sbcDC*	11,970 ± 1,049*	9,908 ± 982*	8,602 ± 766*	(1.23 ± 0.24) × 10^{-3}*
Δ*polY1*	5,036 ± 430*	3,926 ± 350*	2,883 ± 264*	(1.18 ± 0.11) × 10^{-3}*
Δ*polY2*	6,697 ± 589*	4,651 ± 410*	3,186 ± 295*	(6.39 ± 0.71) × 10^{-4}*
Δ*polY1* Δ*polY2*	3,378 ± 367*	2,134 ± 244*	1,189 ± 147*	(1.05 ± 0.09) × 10^{-3}*

[a]Values were calculated from data in Fig. 2B, C, and D, and are averages and standard deviations ($n = 3$). Asterisks indicate survival values that were significantly different from those for wild-type spores ($P \leq 0.05$).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01604-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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The results of this study will be included in the master's thesis of the first author (B.D.).

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