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Alternative Excision Repair of Ultraviolet B- and C-Induced DNA Damage in Dormant and Developing Spores of *Bacillus subtilis*

Fernando H. Ramírez-Guadiana,[a] Marcelo Barraza-Salas,[a] Norma Ramírez-Ramírez,[a] Mayte Ortiz-Cortés,[a] Peter Setlow,[b] and Mario Pedraza-Reyes[a]

Department of Biology, Division of Natural and Exact Sciences, University of Guanajuato, Guanajuato, Mexico,[a] and Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut, USA[b]

The nucleotide excision repair (NER) and spore photoproduct lyase DNA repair pathways are major determinants of *Bacillus subtilis* spore resistance to UV radiation. We report here that a putative ultraviolet (UV) damage endonuclease encoded by *ywjD* confers protection to developing and dormant spores of *B. subtilis* against UV DNA damage. In agreement with its predicted function, a His_6-YwjD recombinant protein catalyzed the specific incision of UV-irradiated DNA *in vitro*. The maximum expression of a reporter gene fusion to the *ywjD* opening reading frame occurred late in sporulation, and this maximal expression was dependent on the forespore-specific RNA polymerase sigma factor, σ^G. Although the absence of YwjD and/or UvrA, an essential protein of the NER pathway, sensitized developing spores to UV-C, this effect was lower when these cells were treated with UV-B. In contrast, UV-B but not UV-C radiation dramatically decreased the survival of dormant spores deficient in both YwjD and UvrA. The distinct range of lesions generated by UV-C and UV-B and the different DNA photochemistry in developing and dormant spores may cause these differences. We postulate that in addition to the UvrABC repair system, developing and dormant spores of *B. subtilis* also rely on an alternative excision repair pathway involving YwjD to deal with the deleterious effects of various UV photoproducts.

The constant exposure of cells and spores of *Bacillus subtilis* to a number of environmental factors of chemical and physical origin may result in the production of number of types of DNA lesions, including strand breaks, apurinic/apyrimidinic (AP) sites, UV-induced pyrimidine dimers (PD) of various types and chemically altered bases (21, 25, 43). *B. subtilis* possesses an arsenal of preventive and repair mechanisms to counteract the mutagenic and deleterious effects of these insults (5, 10, 28, 29, 30, 31, 33, 38, 53, 55). The expression of genes that prevent and repair genetic insults in this bacterium is regulated in time and space by gene circuitries that respond to developmental and environmental conditions during growth and cell differentiation (6, 23, 24, 28, 34, 38, 49, 54, 55).

When nutritional and/or environmental conditions trigger sporulation, *B. subtilis* undergoes a final round of DNA replication, followed by polar septation and segregation of the chromosomal copies between two unequal cell-sized compartments that follow dissimilar programs of gene expression (reviewed in references 9 and 27). The end product of sporulation is a highly resistant endospore with no detectable metabolism that lacks most common high-energy compounds (39). This endospore can remain dormant for long periods of time, until it encounters the appropriate conditions to germinate and resume growth (42). It has been proposed that the formation of the spore may potentially be compromised by damaging the chromosomes of either cell compartment and that the sporulating cell presumably relies on mechanisms to sense, repair, or even tolerate DNA damage in order to generate the two cell types (31). In support of these notions, results have revealed that defects in chromosome partitioning or DNA damage can delay sporulation at the level of Spo0A activation (4, 18) and that DisA, a scanning checkpoint protein, temporarily blocks the initiation of sporulation when DNA damage is encountered (2). A recent report (31) further demonstrated that YqjH/YqjW-dependent translesion synthesis operates in sporulating *B. subtilis* cells and contributes to the processing of spontaneous and artificially induced genetic damage.

B. subtilis spores are 10 to 20 times more resistant to the killing effects of ultraviolet C (UV-C) radiation than are vegetative cells of the same organism. Two mechanisms are responsible for elevated spore UV resistance. First, upon interacting with a group of small-acid-soluble spore proteins (termed α/β-type SASP), the chromosomal DNA of *B. subtilis* spores acquires an A-type conformation with altered DNA photochemistry, such that UV-C irradiation of spores induces the formation of a special type of PD termed spore photoproduct (SP) between adjacent thymidine residues (40, 41, 43). Second, accumulated SP are processed during spore germination with participation of two repair routes: (i) the SplB dimer, a member of the radical-SAM family, specifically recognizes and splits SP back into two thymidine residues and (ii) incision and excision through the nucleotide excision repair (NER) pathway (21, 29, 30, 40, 41, 43).

B. subtilis spores irradiated with solar or artificial UV-B light accumulate a wider spectrum of PDs than do spores irradiated with UV-C in which SP is by far the major photoproduct, since UV-B photoproducts in spores include not only SP but also cyclobutane pyrimidine dimers (CPDs) (44). *B. subtilis* does not contain a DNA photolyase that splits CPDs using a visible light-dependent mechanism (43). Therefore, germinating *B. subtilis* spores eliminate UV-B-induced CPDs and other UV pyrimidine

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Address correspondence to Mario Pedraza-Reyes, pedrama@ugto.mx.
F.H.R.-G. and M.B.-S. contributed equally to this article.
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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Genotype and description[b]	Source or reference[c]
Strains		
E. coli		
XL10-Gold Kan	Tet^r Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r) Tn*5* (Kan^r) Amy]	Stratagene, La Jolla, CA
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA argF*)*U169 deoR recA endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96*	Laboratory stock
PERM555	DH5α harboring pPERM555; Amp^r	This study
PERM635	DH5α harboring pPERM635; Amp^r	This study
PERM653	DH5α harboring pPERM653; Amp^r	This study
PERM661	XL10 Gold harboring pPERM661; Amp^r Kan^r	This study
PERM972	XL10 Gold harboring pPERM972; Amp^r Kan^r	This study
B. subtilis		
168	*trpC2*	Laboratory stock
WN118*	*sigG*Δ*1 trpC2*	46
PERM639*	Δ*ywjD*::*lacZ*; Er^r	pPERM635→168†
PERM985*	Δ*uvrA*::Cm; Cm^r	pPERM972→168†
PERM1009*	Δ*ywjD*::*lacZ* Δ*uvrA*::Cm Er^r Cm^r	pPERM972→639†
PERM557*	*trpC2 ywjD-lacZ*; Cm^r	pPERM555→168†
PERM755*	*sigG*Δ*1 trpC2 ywjD-lacZ*; Cm^r	pPERM555→WN118†
Plasmids		
pCR-Blunt-II-TOPO	Cloning vector	Invitrogen Life Technologies, Grand Island, NY
pJF751	Integrational *lacZ* fusion vector; Amp^r Cm^r	12
pCP115	Integrational vector; Amp^r Cm^r	BGSC[d]
pQE30	Vector that contains a T_5 promoter that enables N-terminal His$_6$-tagged protein expression; Amp^r	Qiagen, Inc., Valencia, CA
pMUTIN4	Integrational vector; Amp^r Em^r	50
pPERM555	712-bp EcoRI/BamHI fragment of *ywjD* cloned in pJF751; Amp^r Cm^r	This study
pPERM635	615-bp HindIII/SacI fragment (internal region of ORF) of *ywjD* cloned in pMUTIN4; Amp^r Er^r	This study
pPERM653	960-bp fragment of the *ywjD* ORF cloned in pCR-Blunt II TOPO; Kan^r	This study
pPERM661	960-bp of the *ywjD* ORF cloned between the BamHI-PstI restriction sites of pQE30; Amp^r	This study
pPERM972	867-bp EcoRI-BamHI (internal region of ORF) of *uvrA* cloned in pCP115; Amp^r Cm^r	This study

[a] *, The background for this strain is 168.
[b] Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Er^r, erythromycin resistance; Kan^r, kanamycin resistance.
[c] †, DNA of the plasmid to the left of the arrow was used to transform the strain to the right of the arrow.
[d] BGSC, *Bacillus* Genetic Stock Center.

adducts from spore DNA primarily through the NER pathway involving the UvrA protein and to a minor extent via repair that is dependent on the RecA protein (21, 38, 44, 51). We report here the existence of an alternative DNA repair mechanism encoded by *ywjD* that, together with the NER system, is involved in processing UV-induced DNA damage in dormant spores and developing spores of *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and spore preparation. The strains used in the present study are listed in Table 1. All *B. subtilis* strains are isogenic with and derived from a laboratory 168 strain. The growth medium used routinely was Luria-Bertani (LB) medium (20), and sporulation was induced in Difco sporulation medium (DSM) (37). When appropriate, ampicillin (Amp; 100 μg/ml), chloramphenicol (Cm; 5 μg/ml), kanamycin (Kan; 10 μg/ml), erythromycin (Er; 1 μg/ml), or IPTG (isopropyl-β-D-thiogalactopyranoside; 0.5 mM) was added to the media. Liquid cultures were incubated at 37°C with vigorous aeration. Spores of all strains were prepared at 37°C on 2× SG medium (2× DSM supplemented with 0.1% glucose) agar plates without antibiotics, and spores were harvested, cleaned, and stored as described previously (22). All dormant spore preparations used in the present study were free (≥98%) of growing cells, germinated spores, and cell debris as determined by phase-contrast microscopy. The optical density of the liquid cultures was monitored at 600 nm using an Ultrospec Pharmacia spectrophotometer.

Genetic and molecular biology techniques. Preparation of competent *Escherichia coli* or *B. subtilis* cells and their transformation with plasmid DNA were performed as described previously (3, 35). Chromosomal DNA from *B. subtilis* was purified as described by Cutting and Vander Horn (7). Large-scale preparation and purification of plasmid DNA was accomplished using commercial ion-exchange columns, according to the instructions of the supplier (Qiagen, Inc., Valencia, CA). Small-scale preparation of plasmid DNA from *E. coli* cells, enzymatic manipulations, and agarose gel electrophoresis were performed by standard techniques (20, 35). PCR products were obtained with homologous oligonucleotides and Vent DNA polymerase (New England BioLabs, Ipswich, MA).

Design of a plasmid to overexpress *ywjD*. The open reading frame (ORF) of *ywjD* lacking the first and stop codons was amplified by PCR utilizing Vent DNA polymerase and the oligonucleotide primers 5′-GGATCCATTTTCAGATTCGGGTTCGTT-3′ (forward) and 5-CTGCAGTGACTTCCATTGCAGCGCA-3′ (reverse), that inserted BamHI and PstI restriction sites, respectively, into the cloned DNA (the restriction site sequences are underlined). The PCR fragment was first ligated into PCR-Blunt-II-TOPO (Invitrogen Life Technologies, Grand Island, NY) and transformed into *E. coli* DH5α. The resulting construct (pPERM653) was cut with BamHI and PstI, and the 960-bp *ywjD* insert was purified and ligated into BamHI/PstI-treated pQE30 (Qiagen). This expression vector provided the start and stop codons, as well as an IPTG-inducible T5-promoter to direct the expression of the *ywjD* ORF. The resulting construct was transformed into *E. coli* XL-10 Gold (Stratagene, La Jolla, CA),

generating *E. coli* strain PERM661. The plasmid in this strain was subjected to both restriction analysis and DNA sequencing to ensure the proper insertion of *ywjD* into pQE30, in which a His_6 tag was fused in frame at the N-terminal coding region of *ywjD*.

Purification of His_6-YwjD. *E. coli* strain PERM661 was grown at 37°C in 50 ml of LB medium to an optical density at 600 nm (OD_{600}) of 0.5. At this point, the culture was supplemented with IPTG to a final concentration of 0.5 mM, and the expression of *ywjD* was induced for 2 h. Cells were collected by centrifugation and washed twice with 10 ml of 50 mM Tris-HCl (pH 6.0)–150 mM NaCl (buffer A) and then disrupted in 10 ml of buffer A containing lysozyme (5 mg/ml) for 1 h at 37°C. The cell lysate was subjected to centrifugation (27,200 $\times$ *g*) to eliminate undisrupted cells and cell debris, and the supernatant was applied to 5-ml of Ni-NTA-agarose (Qiagen) column equilibrated with buffer A. The column was washed with 50 ml of buffer A alone and then with 50 ml of buffer A containing 10 mM imidazole, and then the protein bound to the resin was eluted with 6 ml of buffer A containing 200 mM imidazole; 1.5-ml fractions were collected during this last step. Aliquots (15 μl) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (17).

Determination of His_6-YwjD enzyme activity. UV-endonuclease activity of His_6-YwjD was assayed against a sample of closed circular plasmid pBR322 that was exposed to UV (450 J/m^2) according to a previously described protocol (14). A typical reaction mixture in a volume of 25 μl contained 500 ng of purified His_6-YwjD, 200 ng of purified unirradiated or irradiated plasmid pBR322 in 50 mM Tris–HCl (pH 7.9), 50 mM KCl, 15 mM $MgCl_2$ and 1 mM dithiothreitol. The reactions were incubated at 37°C for 1 h and analyzed by electrophoresis on a 1% agarose gel that was stained with ethidium bromide. Since the enzymatic activity of the recombinant protein is identical to that reported in the extract of *Schizosaccharomyces pombe* cells, a reaction mixture containing 100 U (0.25 μg) of *S. pombe* UV-damage-endonuclease (Sp-Uvde1) (New England BioLabs) instead of His_6-YwjD was used as a positive control.

Construction of a *B. subtilis* strain containing a *ywjD-lacZ* gene fusion. Construction of an in-frame translational fusion between the *ywjD* gene and the *E. coli lacZ* gene was carried out in the integrative plasmid pJF751 (12) as follows. A 712-bp EcoRI/BamHI fragment extending from 661 bp upstream of the translation start codon of *ywjD* to 51 bp downstream of this point was amplified with Vent DNA polymerase (New England BioLabs), using chromosomal DNA from *B. subtilis* 168. The oligonucleotide primers used in the PCR were 5′-GAATTCGCGCATGATCGGAGAAGGCG-3′ (forward) and 5′-GGATCCCGCATCCCAAAGGCTCATCGC-3′ (reverse) (the restriction sites are underlined). The 712-bp PCR fragment was inserted between the EcoRI and BamHI sites of pJF751, and the resulting construct (pPERM555) was transformed into *E. coli* DH5α. Plasmid pPERM555 was introduced by transformation into competent cells of *B. subtilis* 168 (wild type), as well as into a *B. subtilis* Δ*sigG* strain (a generous gift from W. Nicholson, University of Florida), generating strains PERM557 and PERM755, respectively (Table 1).

β-Galactosidase assays. *B. subtilis* strains carrying the *ywjD-lacZ* fusion were grown and sporulated at 37°C in liquid DSM. Cells from 1-ml samples were harvested by centrifugation during vegetative growth (Veg) and throughout sporulation. Cell pellets were frozen and stored at −20°C until the determination of β-galactosidase activity (12, 22). Briefly, washed cell samples were first disrupted with lysozyme and subjected to centrifugation. The β-galactosidase activity present in the supernatant fraction was measured and assigned to the mother cell fraction (which actually consisted of mother cells plus lysozyme-sensitive forespores). The pellets collected 4 to 8 h after T_0 (the time when the slopes of the logarithmic and stationary phases of growth intersected), which consisted of lysozyme-resistant forespores containing spore coats, were subjected to spore coat removal (22) and washed in 50 mM Tris-HCl (pH 7.5) buffer, and a second round of lysozyme treatment was applied to the forespore fraction for determination of β-galactosidase activities (19, 22). The β-galactosidase activity was determined using *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate and expressed in Miller units as described previously (22). The endogenous ONPGase specific activities expressed by the wild-type and *sigG B. subtilis* strains without a *lacZ* fusion during growth and sporulation were determined in parallel and subtracted from the data obtained for strains carrying the *ywjD-lacZ* fusion. The ONPGase activities in both strains lacking the *ywjD-lacZ* fusion gave similar values and fluctuated between 40% (Veg, $T_0 - T_3$) and 30% ($T_4 - T_8$) of the values determined in the Δ*sigG* strain carrying the *ywjD-lacZ* fusion.

RT-PCR. Total RNA from vegetative and sporulating *B. subtilis* 168 cells was isolated using the TRI Reagent (RNA/DNA/protein isolation reagent; Molecular Research Center, Inc., Cincinnati, OH). Reverse transcription-PCRs (RT-PCRs) were performed for 29 cycles with the RNA samples and a Master Amp RT-PCR kit (Epicentre Technologies, Madison WI) according to the instructions of the provider. The primers used for RT-PCRs were 5′-CTGCGAACAAGCTTTCATC-3′ (forward) and 5′-GTTTGACACCGCGGATG-3′ (reverse) to generate a 555-bp RT-PCR product extending from 216 to 771 bp downstream from the start codon of *ywjD*. The absence of chromosomal DNA in the RNA samples was assessed by PCRs with Vent DNA polymerase (New England BioLabs) and the primer set described above.

Construction of mutant strains. A construct to disrupt *ywjD* was generated in the integrative vector pMUTIN4 (50). To this end, a 615-bp HindIII-SacI internal fragment of the *ywjD* gene (from bp 313 to 928 downstream of the *ywjD* translational start codon) was amplified by PCR with Vent DNA polymerase (New England BioLabs), using chromosomal DNA from *B. subtilis* 168. The oligonucleotide primers for amplifying the *ywjD* fragment were 5′-AAGCTTCTGCGAACAAGCTTTCAT-3′ (forward) and 5′-GAGCTCGTTTGACACCGCGGATG-3′ (reverse) (the restriction sites are underlined). The *ywjD* PCR fragment was inserted between the HindIII and SacI restriction sites of pMUTIN4 (50). The resulting construct designated pPERM635 was generated in *E. coli* DH5α. Plasmid pPERM635 was used to transform wild-type *B. subtilis* generating strain PERM639 (Table 1). The presence of an IPTG-inducible Pspac promoter in pMUTIN4 allowed expression of genes located downstream of the disrupted *ywjD* gene avoiding polar effects in the resulting mutant. To inactivate the *uvrA* gene, an 849-bp internal DNA fragment of *uvrA*, extending from 867 to 1716-bp from the translational start site, was amplified by PCR using genomic DNA of *B. subtilis* 168 as a template and the oligonucleotide primers 5′-CGGAATTCGCCGATCTTGTCATCCCCA-3′ (forward) and 5′-GCGGATCCTATCACCTGTCCGCCGTGA-3′ (reverse) containing EcoRI or BamHI sites, respectively (underlined). PCR amplification of the target sequence was performed with Vent DNA polymerase (New England BioLabs). The *uvrA* fragment was recovered and cloned between the EcoRI-BamHI sites of integrative vector pCP115 (*Bacillus* Genetic Stock Center), generating plasmid pPERM972, which was transformed into *E. coli* DH5α. This plasmid was used to transform *B. subtilis* wild-type and PERM639 strains, generating strains PERM985 and PERM1009, respectively (Table 1).

UV irradiation of *B. subtilis* spores and sporulating cells. Spores of the different strains were obtained and purified as described previously (22). To obtain sporulating cells, strains were grown overnight at 37°C in LB medium and diluted 50-fold into DSM, and growth at 37°C was monitored by determining the OD_{600}. Samples for UV exposure were collected by centrifugation (5,000 $\times$ *g*) 5 h after the cessation of exponential growth (T_5) and washed once with phosphate-buffered saline (PBS; 0.7% Na_2HPO_4, 0.3% KH_2PO_4, 0.4% NaCl [pH 7.5]).

Sporulating cells or spores (5 ml at an OD_{600} of 0.5 [1×10^6 to 3×10^6 viable count]) in PBS were stirred continuously and UV irradiated at room temperature. Artificial UV-C radiation was provided by a commercial low-pressure mercury arc lamp (model UVG-11; UV Products, Upland, CA) that emitted essentially monochromatic 254-nm UV radiation. Artificial UV-B radiation was provided by a commercial medium-pressure mercury arc lamp (model UVM-57; UV Products, Upland, CA) that emitted a spectrum of UV wavelengths from 280 to 320 nm, with peak

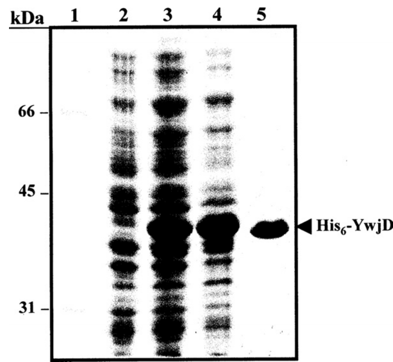

FIG 1 SDS-PAGE analysis of His_6-YwjD induction and purification through a Ni-NTA-agarose column. *E. coli* strain PERM661 (XL10 Gold harboring pPERM661; Amp^r Kan^r) was grown at 37°C in 50 ml of LB medium to an OD_{600} of 0.5. IPTG induction of *ywjD* expression and purification of His_6-YwjD was performed as described in Materials and Methods. Aliquots (15 μl) of cell lysates (lanes 2 to 4) or from the peak protein fraction eluted from the Ni-NTA agarose column with 200 mM imidazole (lane 5) were electrophoresed on an SDS-polyacrylamide (12%) gel that was stained with Coomassie blue. Lane 1 shows the molecular mass standards.

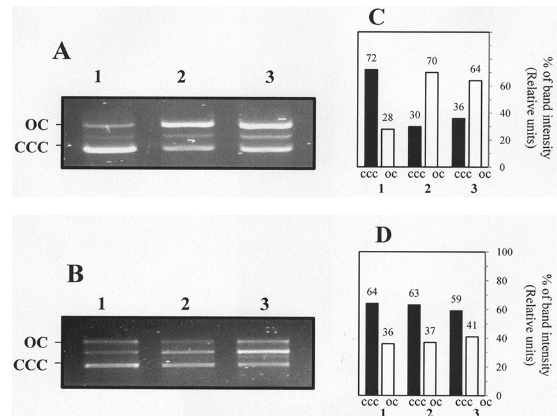

FIG 2 (A to D) Endonuclease activity of His_6-YwjD. (A) CPD-pBR322 (0.2 μg) was incubated without (lane 1) or with either His_6-YwjD (0.5 μg; lane 2) or Sp-Uvde1 (0.25 μg; lane 3). (B) Unirradiated pBR322 (0.2 μg) was incubated without (lane 1) or with either His_6-YwjD (1 μg; lane 2) or Sp-Uvde1 (0.25 μg; lane 3). The reactions were incubated for 1 h at 37°C and analyzed by electrophoresis on a 1% agarose gel, which was stained with ethidium bromide. (C and D) Densitometry of the experiments described in panels A and B, respectively. Quantification of the band intensity was accomplished by densitometry using Quantity One 1-D software from Bio-Rad Laboratories (Hercules, CA). The abbreviations used in panels A, B, C, and D denote the migration positions of covalently closed circular plasmid (CCC) and nicked circular plasmid (OC). The results shown in panels C and D are representative of two experiments that yielded essentially similar (±20%) results.

emission at 302 nm. The UV dose produced by the lamps was measured using a model UVX radiometer (UV Products, Upland, CA). The survival of spores and sporulating cells during these treatments was measured by plating aliquots of serial dilutions in PBS on LB medium agar plates; colonies were counted after 24 to 48 h of incubation at 37°C. The experiments were repeated three times, and values were plotted as averages of triplicate determinations ± the standard deviations (SD). In all cases, killing curves were performed with different spore or sporulating cell preparations, and these yielded essentially similar (±20%) results.

Statistical analyses. The 90% lethal dose (LD_{90}) values (the UV dose in J/m^2) to give 90% killing from experiments testing spore or sporulating cell resistance to UV-B and UV-C were evaluated by analysis of variance using commercial software (Kaleidagraph version 3.6.2; Synergy Software, Reading, PA). Differences with *P* values of ≤0.01 were considered statistically significant.

RESULTS

Expression and purification of YwjD from *E. coli*. Analysis of the *ywjD* coding sequence revealed an ORF of 960 bp that encodes a 36,740-Da protein. Amino acid sequence alignments (1) showed that YwjD exhibits homologies of 51, 52, and 47% with the UV-damage endonucleases from *S. pombe* Uvde1 (Sp-Uvde1) (47), *Neurospora crassa* Nc-Uvde (52), and *Deinococcus radiodurans* Uvs (48), respectively. It has been demonstrated that the enzymes belonging to the Uve/Uvs/Uvde protein family catalyze the incision of PD dimer containing DNA (13). However, to our knowledge, the biochemical function of the product encoded by *ywjD* has not been elucidated. Therefore, we produced and purified a recombinant YwjD protein. The *ywjD* ORF (960 bp) was produced in *E. coli* as an His_6 N-terminal tagged protein, with expression of the fusion protein induced by IPTG. Initial expression attempts using ≥1 mM IPTG resulted in the production of inclusion bodies in the *E. coli* host cells (data not shown). However, induction with 0.5 mM IPTG for 1 or 2 h at 37°C generated significant amounts of a soluble protein with the expected ~37-kDa molecular mass of YwjD (Fig. 1, lanes 3 and 4). This protein was purified to homogeneity by nickel chelate affinity chromatography (Fig. 1, lane 5).

YwjD induces the specific nicking of a CPD-containing DNA substrate. To investigate whether *ywjD* encodes a protein capable of acting on a DNA substrate containing CPDs, we first prepared a plasmid containing this type of lesion. An *E. coli* strain harboring plasmid pBR322 was exposed to a UV-C dose of 450 J/m^2, since this treatment induces the formation of three to four CPDs per plasmid molecule (14), and the plasmid was purified from both irradiated and unirradiated cells. In agreement with its predicted function, the purified His_6-YwjD protein specifically promoted the incision of the CPD-containing plasmid (Fig. 2A, lane 2, and Fig. 2C). Importantly, the recombinant enzyme was unable to nick unirradiated pBR322 (Fig. 2B, lane 2, and Fig. 2D). As a positive control, we utilized commercially available Sp-Uvde1 that was incubated with samples of pBR322 with or without CPDs. As in the case of YwjD, Sp-Uvde1 catalyzed the incision of the CPD-containing plasmid DNA (Fig. 2A, lane 3, and Fig. 2C) and left the unirradiated plasmid intact (Fig. 2B, lane 3, and Fig. 2D).

Analysis of *ywjD* expression during growth and sporulation. To determine when in the *B. subtilis* life cycle YwjD is expressed, the temporal pattern of *ywjD* expression was determined using two approaches. First, a *B. subtilis* strain harboring a translational *ywjD-lacZ* gene fusion was grown and sporulated in liquid DSM, and samples were harvested at various times and assayed for β-galactosidase. The results indicated that low levels of the reporter gene fusion protein were present during logarithmic growth and early in sporulation, with maximal levels reached at 5 h after sporulation (Fig. 3A, T_5). To further investigate whether *ywjD* is transcribed throughout the *B. subtilis* life cycle, total RNA samples were isolated from a wild-type *B. subtilis* culture during growth and sporulation and processed for RT-PCR. The RT-PCR results showed the presence of mRNAs of *ywjD* not only in exponentially growing cells but also both early and late in sporulation (Fig. 3C).

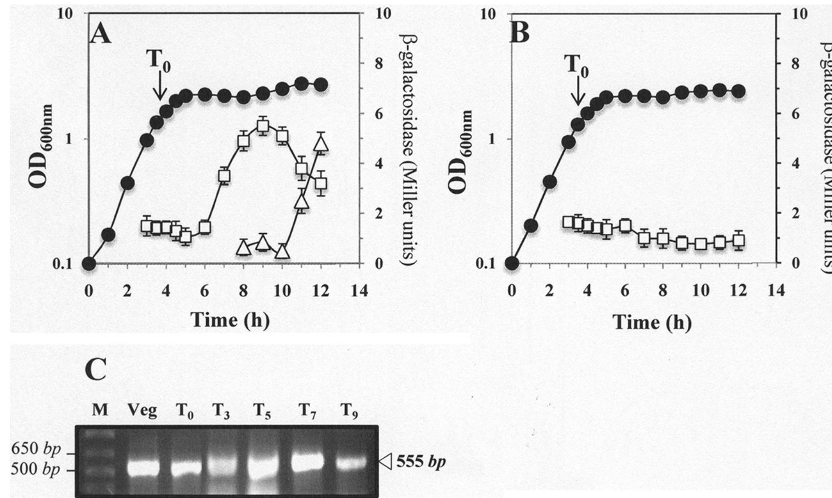

FIG 3 (A to C) Levels of β-galactosidase from *B. subtilis* wild-type (A) and $\Delta\sigma^G$ (B) strains containing a *ywjD-lacZ* fusion and RT-PCR analysis of *ywjD* transcription (C). (A and B) *B. subtilis* strains PERM557 (*ywjD-lacZ*) (A) and PERM755 (*sigGΔ1 ywjD-lacZ*) (B) were grown at 37°C in DSM (●). Cell samples were collected in vegetative growth (Veg) and at various times during sporulation and treated with lysozyme, and the extracts were assayed for β-galactosidase as described in Materials and Methods (□). In panel A, the β-galactosidase activity inside lysozyme-resistant forespores (△) was assayed as described in Materials and Methods. The values shown are averages of duplicate independent experiments ± the SD of the β-galactosidase specific activity expressed as Miller units (22). (C) RNA samples (~1 μg) isolated from a *B. subtilis* 168 DSM culture at the times indicated were processed for RT-PCR analysis as described in Materials and Methods. The arrowhead shows the size of the expected RT-PCR products. Lanes: M, DNA markers, 1-kb Plus ladder; Veg, logarithmic growth; T_0, the time when the slopes of the logarithmic and stationary phases of growth intersected; T_1 to T_9, times in hours after T_0.

The maximum level of β-galactosidase from the *ywjD-lacZ* fusion was achieved late in sporulation, and this level then fell as sporulation proceeded. This behavior is similar to that of genes expressed primarily if not exclusively in the forespore compartment of the sporulating cell, and often under the control of the RNA polymerase forespore-specific sigma factor, σ^G (15). Indeed, further analysis showed that the *ywjD*-directed β-galactosidase activity accumulated inside lysozyme-resistant forespores beginning at T_6 and continued to accumulate until at least T_8, confirming that expression of the reporter gene occurred inside the developing spore (Fig. 3A). Since many genes expressed specifically in developing forespores are transcribed by RNA polymerase with the forespore-specific sigma factor σ^G ($E\sigma^G$), we examined the levels of β-galactosidase from the *ywjD-lacZ* fusion in a *sigG* strain (Fig. 3B). Strikingly, *ywjD-lacZ* expression did not increase during sporulation of the *sigG* strain, although expression was similar to that given by the *lacZ* fusion in the wild-type strain during logarithmic growth (Fig. 3A).

Role of YwjD in resistance of *B. subtilis* spores to UV radiation. The expression pattern of *ywjD* during late sporulation, and its σ^G dependence at this time is reminiscent of genes whose transcription is confined to the forespore compartment, such as *splB*, *sspA*, and *sspB*, genes encoding proteins important in spore UV resistance (11, 19, 23, 24). This is also consistent with the rapid apparent decrease in β-galactosidase specific activity following T_6 that may be due to the inability to assay β-galactosidase when it becomes packaged into the maturing spore (19). Since it is likely that there are significant levels of YwjD in dormant spores (Fig. 3A), it is possible that this YwjD is involved in protecting *B. subtilis* spores against UV-induced DNA damage. However, disruption of either *ywjD* or *uvrA* alone did not sensitize dormant spores to UV-B or UV-C (Fig. 4). Thus, the LD_{90}s to UV-C radiation of wild-type spores (210 ± 8.1 J/m^2) and those calculated for the single *ywjD* (217.5 ± 12.5 J/m^2) and *uvrA* (223 ± 6.2 J/m^2) spores

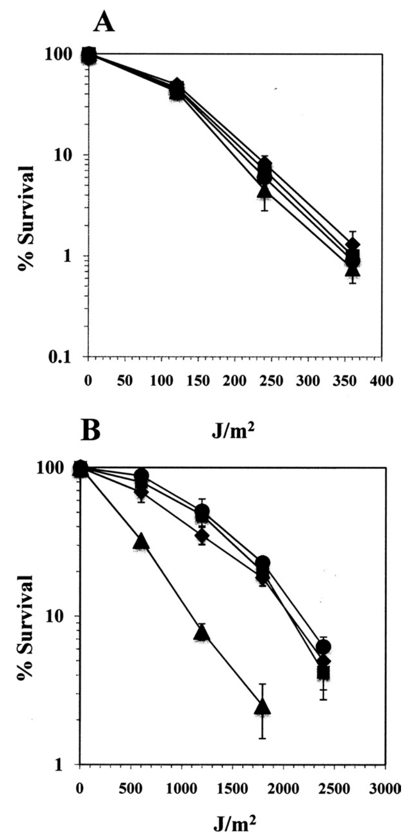

FIG 4 (A and B) Resistance of wild-type, *ywjD*, *uvrA*, and *ywjD uvrA* spores to UV-C (A) and UV-B (B). The spores obtained from *B. subtilis* wild-type (●), PERM639 (*ywjD* [■]), PERM985 (*uvrA* [◆]), and PERM1009 (*ywjD uvrA* [▲]) strains were assayed for UV-C (A) or UV-B (B) resistance as described in Materials and Methods. The results are expressed as averages ± the SD of three independent experiments.

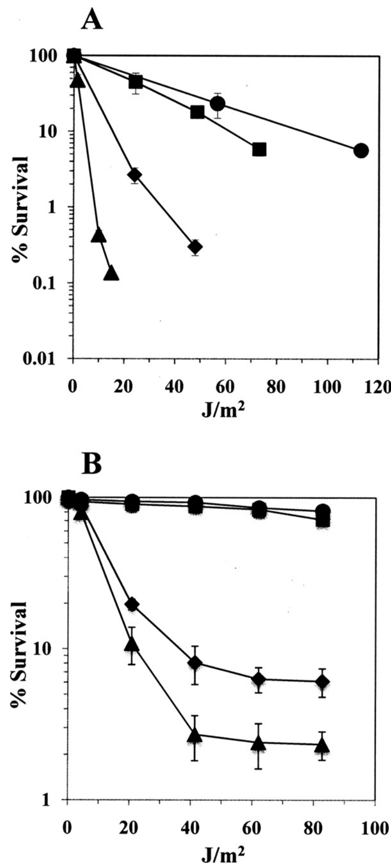

FIG 5 (A and B) Resistance of developing wild type, *ywjD*, *uvrA*, and *ywjD uvrA* T_5 sporangia to UV-C (A) and UV-B (B). *B. subtilis* wild-type (●), PERM639 (*ywjD* [■]), PERM985 (*uvrA* [◆]), and PERM1009 (*ywjD uvrA* [▲]) strains were sporulated in DSM. The sporulating cells were isolated 5 h after the end of logarithmic growth and treated with different doses of UV-C or UV-B, and the cell viability was determined as described in Materials and Methods. The results are expressed as averages ± the SD of at least three independent experiments.

were not significantly different (Fig. 4A). In contrast, dormant spores carrying mutations in both of these DNA repair genes were significantly more sensitive to UV-B (Fig. 4B), and the LD_{90} values were 2,125 ± 30 J/m^2 for wild-type spores and 1,075 ± 25 J/m^2 for the *ywjD uvrA* spores. However, the spores of the double knockout strain were not more susceptible to UV-C than were the wild-type spores (Fig. 4A), with LD_{90} values of 195 ± 15 J/m^2 (*ywjD uvrA*) and 210 ± 18 J/m^2 (wild type), respectively. Together, these results suggest that the type of DNA damage generated by UV-B but not by UV-C is eliminated during spore germination by the concomitant action of YwjD and the NER pathway.

Role of YwjD in resistance of developing *B. subtilis* sporangia to UV radiation. The physiological significance of the expression of *ywjD* during forespore development was further analyzed by examination of the effects of loss of *ywjD* on the resistance of T_5 sporulating cells of various strains to UV-B and UV-C. T_5 sporulating cells were chosen for this analysis because the levels of β-galactosidase from *ywjD-lacZ* were maximal at this time (Fig. 3A). This analysis revealed that the absence of YwjD slightly but significantly increased the susceptibility of T_5 sporangia to 254-nm UV radiation (Fig. 5A), since the calculated LD_{90}s for sporangia of the *ywjD* and wild-type strains were 62 ± 8 and 95.3 ± 7 J/m^2, respectively.

Although the contribution of the NER system in conferring protection to vegetative cells and spores from the noxious effects of UV radiation has been well established (6, 41, 51, 52), the role played by this pathway in protecting sporangia from UV radiation is unknown. Our results showed that in comparison to the parental wild-type strain, the absence of UvrA, an essential component of the NER pathway (53), greatly increased the susceptibility of the *B. subtilis* T_5 sporangia to UV-C radiation (Fig. 5A), and this susceptibility was further increased by the loss of both YwjD and UvrA (Fig. 5A). Sporangia from the wild-type, *ywjD*, *uvrA*, and *ywjD uvrA* strains exhibited significantly different LD_{90}s to UV-C of 95.3 ± 7, 64.6 ± 3.1, 13.5 ± 2.5, and 2.9 ± 0.5 J/m^2, respectively.

We next investigated the relative contributions of YwjD and/or NER in protecting the T_5 sporangia from artificial UV-B light. The results revealed that sporulating cells from the *uvrA* strain were more sensitive to UV-B than those from the wild-type strain, whereas the loss of *ywjD* alone did not increase the susceptibility of sporangia to UV-B (Fig. 5B). However, sporulating cells lacking both YwjD and UvrA were more sensitive to UV-B than *uvrA* sporangia (Fig. 5B). The LD_{90}s for UV-B with strains lacking YwjD and/or UvrA were 189.6 ± 7.8 J/m^2 (wild type), 191.7 ± 5 J/m^2 (*ywjD*), 35.7 ± 4.3 J/m^2 (*uvrA*), and 21.3 ± 2.9 J/m^2 (*ywjD uvrA*).

DISCUSSION

UV-damage endonucleases are distributed among organisms of different species, and they are involved in processing PD-containing DNA using alternative excision repair mechanisms (13). Although it has been demonstrated that these enzymes incise DNA immediately 5′ to PDs, the postincision event(s) that completes this repair mechanism is not fully understood (13). The *in vivo* function of the *N. crassa* and *S. pombe* UVDE homologs was inferred from their ability to decrease the UV-C radiation susceptibility of *E. coli* strains deficient on NER, recombination and/or photolyase activities (47, 52). In *D. radiodurans*, such susceptibility was only evident when both the UvsE protein and the NER pathways were inactivated (48).

It was previously shown that expression of a *B. subtilis* genomic sequence, termed Z49782, encoding a predicted protein with regional homology to SP-Uve1, decreased the UV susceptibility of an *E. coli* strain deficient in light-dependent photoreversion, recombination, and NER (47). An inspection of the *B. subtilis* chromosome (16; GenoList/SubtiList from Institut Pasteur Genopole) indicated that Z49782 encoded a product that was similar to an ORF termed *ywjD*, although the former contained an extra C-terminal 24-residues long peptide. The results of an *in silico* analysis revealed that YwjD shares 52 to 54% amino acid sequence homology with UV-damage endonucleases of fungal and bacterial origin that belong to the Uve/Uvs/UvdE protein family (1). We demonstrated here that purified His_6-YwjD protein generated from the correct *ywjD* ORF, namely, 960 bp (16), specifically nicked a UV-irradiated plasmid substrate, strongly suggesting that YwjD possesses properties required for involvement in repair of UV-C-induced PD. The substrate specificity of YwjD remains to be determined, since the UV irradiated plasmid used in this assay may contain UV-induced lesions in addition to CPDs such as pyrimidine(6-4)pyrimidone photoproducts (13).

Analysis of the *B. subtilis* chromosome (16; GenoList/SubtiList

from Institut Pasteur Genopole) reveals that *ywjD* is flanked upstream by *ywjE* encoding a cardiolipin synthetase and downstream by *ywjC* a gene whose function has not been determined. The intergenic region between *ywjE* and *ywjD* is only 12 bp, suggesting that both genes are part of a bicistronic operon (and see below). In contrast, previous results have shown that *ywjC* belongs to the σ^B regulon (26); therefore, a promoter recognized by σ^B presumably directs the independent transcription of this gene.

In the present study, we found that *ywjD* is expressed throughout the life cycle of *B. subtilis*, since *ywjD* mRNA was detected during growth and sporulation. Expression studies with a *ywjD-lacZ* fusion showed that β-galactosidase from this fusion exhibited a temporal pattern of expression similar to that of $E\sigma^G$-dependent genes whose expression occurs in the forespore compartment. Consistent with these results, the sporulation-associated expression of the *ywjD-lacZ* fusion was dependent on the σ^G protein, confirming results of a microarray study that showed that *ywjD* belongs to the σ^G regulon (45). Indeed, both *ywjE* and *ywjD* are members of the σ^G regulon, which is consistent with these two genes being in an operon. In conjunction, these results strongly suggest that expression of *ywjD* during sporulation is carried out by $E\sigma^G$, the holoenzyme that transcribes most sporulation genes in the forespore compartment (9, 27, 32).

Based on the results of expression analysis of *ywjD* and the significant expression of this gene during sporulation, the UV resistance properties of developing and dormant spores lacking YwjD and/or UvrA were determined. In our experimental conditions the single disruption of either *ywjD* or *uvrA* did not sensitize spores to UV-B, suggesting that neither of these systems alone is essential to protect to spores from this type of radiation. This result was unexpected since Slieman and Nicholson (44) have demonstrated that UV-B irradiation of dormant *B. subtilis* spores results in a major production of CPDs and both YwjD and NER are supposed to repair this type of lesion (44). However, when both repair systems were inactivated, a dramatic decline in spore resistance to this type of radiation was observed. Taken collectively, our results strongly suggest that UV-B-promoted PDs are eliminated from the spore chromosome during spore germination by NER and by an alternative excision repair process that employs YwjD.

The A-type conformation adopted by the spore chromosome promotes the production of the unique PD SP following UV-C radiation (21, 40). The results described in the present study suggested that YwjD is most probably not involved in processing SP, since spores deficient in this protein were not sensitized to UV-C. A similar result was obtained when both *uvrA* and *ywjD* were disrupted. Therefore, we conclude that damage inflicted by UV-C on the chromosome of spores lacking YwjD and UvrA is most likely repaired by SplB and RecA during spore germination/outgrowth (21, 38, 40).

YwjD and NER also clearly had a role in conferring protection on T_5 sporangia against UV radiation. Analysis of this protection was complicated because there are two cell types in T_5 sporangia, the mother cell and the developing forespore, either of which could in theory give rise to a colony. However, by T_5 in sporulation under these conditions, the majority of sporangia are committed to proceed through sporulation with eventual mother cell lysis before giving rise to a growing cell, and therefore the colonies generated from T_5 sporangia are largely derived from the developing forespores. The UV resistance of T_5 sporangia is thus determined by developing forespores' UV photochemistry, their complement of DNA repair proteins, and their maintenance of sufficient levels of ATP and general enzyme activity to allow DNA repair in the developing forespore itself, as well as possible repair of DNA lesions acquired in sporulation only following spore germination. Keeping these facts in mind, it was clear that NER played the major role in protecting T_5 sporangia from both UV-C and UV-B radiation, since the inactivation of *uvrA* was enough to sensitize these cells to both types of radiation, whereas the disruption of *ywjD* alone only slightly increased the susceptibility of T_5 sporangia to UV-C. However, YwjD did play a role in protecting T_5 sporangia from UV-B, since the disruption of *ywjD* increased the susceptibility of the resulting *ywjD uvrA* strain to this type of radiation. We propose that the different contributions of YwjD in protecting T_5 sporangia from UV-C and UV-B radiation probably depends on the UV photochemistry and the large amount of CPDS induced by this physical factor in developing forespores at this developmental stage. In support of this contention, it has been shown that the spore-specific DNA photochemistry is dependent not only on the SASP that are expressed 3 to 4 h after the onset of sporulation but also on the dehydration state of the spore core, as well as its accumulation of dipicolinic acid, and dipicolinic acid uptake and full spore core dehydration take place 1 to 2 h after SASP accumulation (8, 19). It has also been established that UV-C generally induces a much higher proportion of CPDs than UV-B in SASP-free DNA (36). Taken collectively, our results support the idea that under conditions of extreme UV radiation, the developing spore eliminates PDs by incision/excision (NER) and alternative excision repair (YwjD), assuring completion of the sporulation process. Importantly, the UV sensitivity exhibited by mature and developing spores lacking *ywjD* cannot be attributed to a polar effect, since supplementation of *ywjD* cultures with IPTG to induce the expression of the downstream *ywjC* gene did not affect sporulating cells' UV sensitivity (data not shown). Repair of UV-induced DNA damage is indeed important during spore morphogenesis and may proceed in an error-prone manner since a recent report showed that the absence of translesion DNA polymerases YqjH and YqjW not only resulted in inefficient sporulation but also sensitized sporulating *B. subtilis* cells to UV-C (31). Indeed, repair or bypassing of UV-C-induced PDs in this developmental stage may be directed to actively transcribed genes since these repair processes seem to require the transcriptional repair coupling factor Mfd (31; F. H. Ramírez-Guadiana and M. Pedraza-Reyes, unpublished results).

Finally, the evidence presented here indicates that YwjD catalyzed the incision of CPD-containing DNA. However, the mechanism that completes the repair process in *B. subtilis* is currently unknown. In *S. pombe* two such mechanisms have been proposed: (i) incision on the 3′ side of the UV lesion by the Rad2 flap endonuclease, generating a 3′-OH end that is extended by a DNA polymerase, followed by synthesis of the phosphodiester bond, and (ii) the recombination subpathway, which requires the Exo1 Rhp51 and Rad18 proteins (13). The *B. subtilis* genome lacks sequences encoding proteins with evident homology to flap endonucleases. Therefore, additional work will be necessary to identify the protein(s) involved in the postincision events that complete the repair process initiated by YwjD in *B. subtilis*.

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