Characterization of Pathways Dependent on the *uvrE*, *uvrA1*, or *uvrA2* Gene Product for UV Resistance in *Deinococcus radiodurans*

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The genome of a radiation-resistant bacterium, *Deinococcus radiodurans*, contains one *uvrE* gene and two *uvrA* genes, *uvrA1* and *uvrA2*. Using a series of mutants lacking these genes, we determined the biological significance of these components to UV resistance. The UV damage endonuclease (UvrE)-dependent excision repair (UVER) pathway and UvrA1-dependent pathway show some redundancy in their function to counteract the lethal effects of UV. Loss of these pathways does not cause increased sensitivity to UV mutagenesis, suggesting either that these pathways play no function in inducing mutations or that there are mechanisms to prevent mutation other than these excision repair pathways. UVER efficiently removes both cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) from genomic DNA. In contrast, the UvrA1 pathway does not significantly contribute to the repair of CPDs but eliminates 6-4PPs. Inactivation of *uvrA2* does not result in a deleterious effect on survival, mutagenesis, or the repair kinetics of CPDs and 6-4PPs, indicating a minor role in resistance to UV. Loss of *uvrE*, *uvrA1*, and *uvrA2* reduces but does not completely abolish the ability to eliminate CPDs and 6-4PPs from genomic DNA. The result indicates the existence of a system that removes UV damage yet to be identified.

UV light damages DNA, generating cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) (3). The biological consequences of this are severe because these two major UV photoproducts interfere with the functions of the DNA replication and transcription machinery. Therefore, organisms have had to develop mechanisms to deal with these lesions during evolution. A radiation-resistant bacterium, *Deinococcus radiodurans*, exhibits extreme resistance to UV, being about 20 times more resistant than *Escherichia coli* (15). This has led to the notion that *D. radiodurans* possesses highly efficient mechanisms to recover from UV-induced damage. The *Deinococcus* genome contains *uvrE*, *uvrA1*, and *uvrA2* genes, which may encode proteins that contribute to the resistance to UV, whereas homologues of *phr*, flap endonuclease (FEN), and translesion synthesis (TLS) polymerase have not been identified (15). This suggests several possibilities in terms of resistance to UV. The first is that *D. radiodurans* has evolved UV damage endonuclease (UvrE)-dependent excision repair (UVER) as an alternative pathway to photoreactivation, although the subsequent process likely depends on a different mechanism from that associated with flap endonuclease (17). The second possibility is that two different UvrA proteins are involved in nucleotide excision repair (NER) in *D. radiodurans*. Also, the possible lack of TLS sug-

gests the presence of unique mechanisms to overcome a situation in which the replication fork is stalled at the site damaged by UV.

Recently, it was reported that loss of *uvrE* in the *uvrA1* background sensitizes the *Deinococcus* strain to UV (2). The result indicates that as in *Schizosaccharomyces pombe*, the NER and UVER pathways possess a redundant function in the recovery from UV damage in *D. radiodurans* (2, 17). However, it remains unclear which mechanisms play a major role in resistance to UV in *D. radiodurans*, because the deinococcal ability to remove lesions from the genomic DNA and its biological significance in this species have not been elucidated. To determine the capacity of UVER and NER to eliminate CPDs and 6-4PPs and a possible role of UvrA2 in UV resistance, we examined the sensitivities and repair kinetics of a series of mutants lacking *uvrE*, *uvrA1*, and/or *uvrA2*. We also analyzed the survival of strains deficient in excision repair pathways or homologous recombination (HR) to determine the relative contributions of these pathways to UV resistance. Here we provide evidence that (i) UvrA2 plays a minor role in UV resistance, (ii) UVER eliminates both CPDs and 6-4PPs, (iii) NER removes 6-4PPs but does not significantly contribute to the repair of CPDs, and (iv) HR plays a more significant role than UVER or NER in the resistance to UV.

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MATERIALS AND METHODS

Strains and growth conditions. Strains used in this study are listed in Table 1. All strains derived from *D. radiodurans* MR₁ were grown at 30°C in TGY broth (0.5% tryptone, 0.3% yeast extract, and 0.1% glucose) or on TGY plate (1.5% agar) (3). *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar.

TABLE 1. Strains used in this study

Strain	Description	Reference(s) or source
<i>Deinococcus radiodurans</i>		
MR ₁	Wild type	9
TNK001	As MR ₁ but $\Delta uvsE$	This study
302	As MR ₁ but $\Delta uvrA1$	9, 11
TNK002	As MR ₁ but $\Delta uvrA2$	This study
TNK003	As 302 but $\Delta uvsE$	This study
TNK004	As TNK001 but $\Delta uvrA2$	This study
TNK005	As 302 but $\Delta uvrA2$	This study
TNK006	As TNK003 but $\Delta uvrA2$	This study
TNK007	As MR ₁ but <i>recA</i>	This study
<i>Escherichia coli</i> DH5 α -MCR	F' <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ ϕ 80 <i>lacZ</i> Δ 15 Δ <i>lacX</i> 74 <i>endA1 recA1 deoR</i> Δ (<i>ara-leu</i>)7697 <i>araD</i> 139 <i>galU galK nupG rpsL</i>	Invitrogen

Measurement of survival rate and mutation frequency. *D. radiodurans* cultures in the early stationary phase were washed twice with phosphate buffered saline (PBS) containing 0.02% Triton X-100 to ensure the separation of individual CFU, resuspended in PBS, and used for UV exposure. UV irradiation was conducted using a germicidal lamp (GL-15; Matsushita, Osaka, Japan) at a rate of 2.3 J/m²/s. Survival was determined by plating serial dilutions of irradiated cells on TGY plates and incubating at 30°C for 3 days. Strain TNK007 was scored 4 days after plating. For mutation experiments, UV-irradiated strain MR₁ (1,500 J/m²) and UV-irradiated strains TNK003 and TNK006 (120 J/m²) were inoculated on TGY plates and incubated for 3 days to fix the mutation. Cultures were harvested and suspended in PBS containing 0.02% Triton X-100. Serial dilutions with PBS were plated onto TGY plates containing 50 μ g/ml rifampin (Rif) and incubated for 4 days before scoring. The total number of CFU was determined by plating serial dilutions on TGY plates. The frequency of mutation conferring resistance to Rif was determined as the ratio of mutant bacteria on Rif plates to total viable bacteria on TGY plates.

Construction of strains. Gene disruption was performed by targeted mutagenesis as described previously (4). To create strain TNK001, a 3.6-kbp genomic DNA fragment containing the *uvsE* gene, encoding UV damage endonuclease, was cloned into pBS (SK+) at *AclI* and *EcoRI* sites, generating pTNK001. This plasmid was digested with *HindIII* and *SphI* (these restriction sites are located in the *uvsE* gene) and ligated with a 0.8-kbp fragment from pKatAAD, a streptomycin-resistant version of pKatCAT (4). The resulting plasmid was designated pTNK002. The *uvsE* disruption plasmid was introduced into *D. radiodurans* MR₁, and the recombinants were selected on TGY plates containing 8 μ g/ml streptomycin. The disruption of *uvsE* was confirmed by PCR using 5'-TGGCG TAAGAAATGGGAGCGCGA-3' and 5'-CAGTCGTCAGAAACCCCGTCAT C-3'. These primers generate a 1.1-kbp fragment from MR₁ genomic DNA and a 1.6-kbp fragment if *uvsE* is replaced by the deletion cassette. The recombinant from which only the 1.6-kbp version was produced was designated TNK001. TNK003 was constructed in the same manner as TNK001 was constructed from strain 302: *uvrA1* was mutagenized by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine treatment (9).

To generate strain TNK002, a 2.8-kbp *SalI*-*SmaI* fragment of genomic DNA corresponding to the downstream region of *uvrA2* was inserted upstream of a hygromycin resistance cassette in pKatHPH3, a hygromycin-resistant version of pKatCAT (4). The resulting plasmid was ligated with a 3.2-kbp *EagI*-*HindIII* fragment, upstream of *uvrA2*, generating pTNK007 in which a hygromycin resistance cassette is fused downstream and upstream of *uvrA2*. *D. radiodurans* MR₁ cells were transformed with the hybrid fragment and spread on TGY plates containing 37.5 μ g/ml hygromycin. Recombinants were screened for a disrupted *uvrA2* using primers 5'-TTCGGTTGTGGTCACATCGTC-3' and 5'-CGTGTT CCACACGAACAGG-3'. These primers amplify a 1.6-kbp fragment from the MR₁ genome, and a 1.8-kbp fragment is generated when *uvrA2* is replaced with the disruption cassette. Amplification of genomic DNA from strain TNK002 produced the 1.8-kbp version. Strains TNK004, TNK005, and TNK006 were constructed by inactivating *uvrA2* in strains TNK001, 302, and TNK003, respectively.

Strain TNK007 was generated as described previously (12). Briefly, a *recA229::cat* fragment of pKSCR3 was used to transform strain MR₁, and genomic DNA of a recombinant selected on TGY plates containing 3 μ g/ml chloramphenicol was screened by PCR using 5'-AACGCAGCAAGGCCATCG AAAC-3' and 5'-ACCAGCAGTTCGTCGGTGTTC-3'. These primers am-

plify a 0.3-kbp fragment from MR₁ genomic DNA or a 1.2-kbp fragment corresponding to the disruption cassette when *recA* is disrupted.

Enzyme-linked immunosorbent assays (ELISAs) for CPDs and 6-4PPs. ELISAs were performed to determine the repair kinetics of CPDs and 6-4PPs as described previously (7, 8). Briefly, cells irradiated with 600 J/m² of UV were resuspended in TGY and incubated at 30°C with shaking. Aliquots were harvested at various time points for the preparation of DNA. The amount of CPDs or 6-4PPs in genomic DNA from each aliquot was measured by an ELISA. To detect CPDs, 6 ng of DNA was reacted with TDM-2 antibodies. For the detection of 6-4PP with 64M-2 antibodies, 30 ng of DNA was used.

RESULTS

To determine the relative contributions of UVER and NER to UV resistance, we examined the sensitivity to UV of mutants lacking *uvsE*, *uvrA1*, and/or *uvrA2*. In our conditions, cells with a single mutation of *uvsE* or *uvrA1* showed changes in sensitivity. The *uvrA1* strain displayed slightly higher sensitivity than the wild type, while an extremely high dose of radiation was lethal in the *uvsE* mutant (Fig. 1). These observations are consistent with the notion that in *S. pombe*, NER is more relevant to UV resistance than UVER (16, 17), although a previous study reported that the loss of

FIG. 1. UV survival curves for *D. radiodurans* strain MR₁ and mutants lacking *uvsE*, *uvrA1*, and/or *uvrA2*. Each point represents the average $\pm$ standard deviation (error bar) of at least three independent experiments. WT, wild type.

FIG. 2. UV survival curves for three mutant *D. radiodurans* strains (*uvsE uvrA1*, *uvsE uvrA1 uvrA2*, and *recA* mutants). Values are means $\pm$ standard deviations (error bars).

uvrA1 or *uvsE* alone had no effect on survival in *D. radiodurans* (2). This may reflect the difference in the capacity to recover from UV damage between rapidly growing cells and early stationary-phase cells in *Deinococcus*. It has been reported that the contributions of NER and UVER pathways to UV resistance in *S. pombe* depend on the growth phase (16). Our data suggest that the roles of deinococcal UVER and NER in the recovery from UV damage are increased in stationary-phase cells than in exponential-phase cells. Figure 1 also indicates that the loss of *uvrA2* had no deleterious effect on survival of wild-type, *uvrA1* mutant, and *uvsE* mutant cells.

When *uvsE* was disrupted in the *uvrA1* background, the resulting strain displayed synergistically decreased UV resistance (Fig. 2), showing the overlap in the functions of the UVER and NER pathways to recover from UV damage as previously reported (2). In addition, we have found that inactivation of the *uvrA2* gene in the *uvsE uvrA1* background had no effect on survival (Fig. 2), suggesting a minor role for UvrA2 in the resistance to UV. Furthermore, loss of *uvsE*, *uvrA1*, and *uvrA2* was found to cause no increase in UV-

FIG. 3. Mutation frequencies for *D. radiodurans* strain MR₁ and *uvsE uvrA1* and *uvsE uvrA1 uvrA2* mutant strains with (+) or without (-) exposure to UV. Values are means $\pm$ standard deviations (error bars). WT, wild type.

FIG. 4. Removal of CPDs and 6-4PPs from genomic DNA in UV-irradiated *D. radiodurans* strain MR₁ and *uvsE*, *uvrA1*, *uvsE uvrA1*, and *uvsE uvrA1 uvrA2* mutant strains. Each point represents the average $\pm$ standard deviation (error bar) of at least three independent experiments. WT, wild type.

induced mutations (Fig. 3), implying that UVER, NER, and UvrA2 do not significantly contribute to the resistance to the mutagenic effect of UV in this species. In terms of UV resistance pathways other than excision repair mechanisms, HR could be critical in this species, because a deinococcal *recA* strain was extremely sensitive to UV (5). We have found that the *recA* single mutant showed greater sensitivity to UV than the *uvsE uvrA1* and *uvsE uvrA1 uvrA2* mutants (Fig. 2). Therefore, it is suggested that the function of RecA is more significant than that of two different excision repair pathways for UV resistance in this species and that the recovery from UV damage of the *uvsE uvrA1* and *uvsE uvrA1 uvrA2* strains depends on the remaining mechanisms of resistance, including HR.

To determine the capacities of deinococcal UVER and NER pathways to remove CPDs and 6-4PPs, we measured the repair rate for each photoproduct by an ELISA (Fig. 4). We have found that both CPDs and 6-4PPs were removed in *uvsE*, *uvrA1*, and *uvsE uvrA1* mutants, although the kinetics of the respective repair process was influenced by the pathway inactivated. When *uvrA1* was lost in addition to *uvsE*, the repair of 6-4PPs was less efficient than that in the *uvsE* mutant, whereas the rates of removal of CPDs in the *uvsE* strain and *uvsE uvrA1* strain were comparable. This result indicates that the NER pathway of *D. radiodurans* removes 6-4PPs from UV-irradiated DNA but plays a minor role in the removal of CPDs. In contrast, loss of *uvsE* in the *uvrA1* background reduced the repair rates of CPDs and 6-4PPs relative to those of the *uvrA1* mutant, revealing that UVER removes both lesions. On the other hand, loss of *uvrA2* did not remarkably affect the repair kinetics in the *uvsE uvrA1* strain (Fig. 4) or those in the wild-type, *uvsE*, and *uvrA1* strains (data not shown), indicating a minor role for UvrA2 in the repair of CPDs and 6-4PPs.

DISCUSSION

Our epistatic analysis supports the hypothesis that the UVER and NER pathways function independently to counteract the deadly effects of UV in *D. radiodurans* (2). We also determined the relative contributions of UVER, NER, and the RecA-dependent process to the resistance to UV in *D. radiodurans*. In addition, we biochemically demonstrated the properties of UVER and NER of CPDs and 6-4PPs. However, the biological significance of UvrA2 to UV resistance remains unclear. Our data indicate a minor role for UvrA2 in the resistance to UV-induced killing and mutagenesis and in the removal of CPDs and 6-4PPs. This may suggest yet another function of UvrA2 protein in cellular metabolism.

Since the deinococcal UVER pathway efficiently eliminates both CPDs and 6-4PPs, this pathway is an important component of the UV damage repair system, as in *S. pombe* (17). In contrast, the deinococcal NER pathway does not significantly contribute to the removal of CPDs, though it efficiently repairs 6-4PPs, suggesting a critical role for the repair pathways in dealing with 6-4PPs. It is also suggested that loss of NER results in biological consequences of UV mainly caused by 6-4PPs. Here we argue that 6-4PPs are mainly responsible for the killing of cells in *Deinococcus*. When both UVER and NER were inactivated, the resulting strain showed a synergistic increase in sensitivity to UV relative to each single mutant. On the other hand, the numbers of CPDs remaining in the UVER-defective strain and UVER plus NER-defective strain were comparable, although the rate of repair of 6-4PPs was reduced in the strain lacking both UVER and NER. Therefore, it is possible that the relative roles of CPDs and 6-4PPs are different in this species; 6-4PPs have a more lethal effect on *D. radiodurans* than do CPDs. In this respect, the response of *D. radiodurans* to UV is similar to that of NER-deficient human cells in which 6-4PPs are more toxic than CPDs (10).

On the basis of photoreactivation studies, it has been reported that cytosine-containing pyrimidine dimers are most responsible for UV-induced mutagenesis (13, 14). Since 67% of the genome is composed of cytosine and guanine base pairs (15), the deinococcal genomic DNA should be susceptible to UV mutagenesis. However, the frequency of mutation was not increased by UV in spite of the loss of UVER, NER, and UvrA2 in our Rif assay. This suggests that *D. radiodurans* does not possess DNA polymerases for TLS or that the TLS is remarkably error free. Alternatively, it is possible that this bacterium possesses mechanisms to remove UV photoproducts in addition to UVER and NER. Actually, our data showed that both CPDs and 6-4PPs were eliminated even in the absence of UVER and NER (Fig. 4). We have also found that the *recA* mutant was more sensitive to UV than the strain lacking both UVER and NER (Fig. 2). Since the deinococcal RecA forms a filament on the double-stranded DNA (6), the damaged strand would be processed by HR in this species. Therefore, HR would help to remove UV photoproducts from genomic DNA in this species. In this context, the major role of RecA in resistance to UV in *D. radiodurans* seems to be different from that in *E. coli*. HR in *E. coli* does not significantly contribute to the elimination of CPDs in the *uvrA* background, whereas RecA plays an indirect role in the repair of damage by amplifying UvrA in the SOS response to fully accomplish NER (7). This also

suggests a difference in the gene regulatory mechanism for UV resistance in the two organisms. In *Deinococcus*, *recA* expression is stimulated by IrrE, a regulator unique to this bacterium, after exposure to ionizing radiation, and the loss of *irrE* reduces the resistance to UV as well as ionizing radiation (1). This implies a significant role for the IrrE-associated regulatory system in resistance to UV as well. Investigations of the transcriptional response to UV may identify novel components responsible for resistance and provide insights into the mechanisms that regulate the expression of excision repair genes and HR genes, leading to a better understanding of exactly how this species copes with exposure to UV.

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