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Development of a Plasmid-Based System for Studying DNA Repair Mechanisms in *Escherichia coli*

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Nucleotide excision repair is a DNA repair mechanism involved in the repair of ultraviolet radiation damage. The key proteins in this system are the four Uvr proteins: UvrA, UvrB, UvrC, and UvrD. Previous studies have demonstrated that the nucleotide excision repair system is capable of repairing altered DNA structure, such as thymine dimers, in genomic DNA. Ultraviolet irradiated plasmid also generates thymine dimers, so we hypothesize that the nucleotide excision repair system can repair *in vitro* ultraviolet C damaged plasmids. This study aims to investigate the role of UvrB in the repair of *in vitro* ultraviolet C irradiated plasmid by using wild type and *ΔuvrB Escherichia coli*. Our results demonstrate that as the duration of plasmid irradiation increases, cell viability in both wild-type strain and *ΔuvrB* strain decreases. The *ΔuvrB* strain showed no difference in transformation frequency compared to wild-type strain, which suggests that the absence of UvrB in *Escherichia coli* does not cause additional deficiency in repairing ultraviolet C damaged plasmids. The result here suggests that there may be alternate deoxyribonucleic acid repair mechanisms that can repair ultraviolet damage or there may be other proteins that can compensate for the loss of UvrB.

Exposure of *Escherichia coli* (*E. coli*) to genotoxic stress such as ultraviolet (UV) light induces the SOS response, which is a cellular response to DNA damage (1). UV light is electromagnetic radiation ranging from 10nm to 400nm. This radiation is present in sunlight and can be further classified by wavelengths. UVB (280–315nm) and UVC (100–280nm) are two types of UV, and are reported to induce altered DNA structure such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts (6-4 PPs) in DNA (2, 3). DNA damage arrests DNA replication by blocking DNA polymerase I activity (4). The SOS response repairs damaged DNA by upregulating genes involved in the inhibition of cell division, DNA repair, recombination, and mutagenesis (1). One of these DNA repair systems, nucleotide excision repair (NER), is responsible for repairing a variety of DNA lesions, including CPDs and 6-4 PPs (2, 3).

The NER system consists of UvrA, UvrB, UvrC, UvrD, DNA polymerase I and DNA ligase (3). NER begins with dimeric UvrA ($UvrA_2$) forming a complex with UvrB (5). UvrB is generally recognized as a monomer, although a recent study suggests that UvrB dimerizes when complexing with UvrA (6). $UvrA_2B$ binds and scans along DNA to look for altered structures such as CPDs and 6-4 PPs (3). When a lesion is detected, $UvrA_2$ dissociates from UvrB, while UvrB remains bound to the damaged site, effectively marking the location of the lesion (5). UvrB recruits UvrC to the damaged site to form a UvrBC complex (3). UvrC makes incisions at the 5' and 3' ends of the damaged DNA strand (7, 8). UvrD, a DNA helicase II, removes both UvrC and the oligonucleotide between the two incisions, leaving a gap to be filled by DNA polymerase I (3, 9, 10). Finally, the nick is sealed by DNA ligase (3). Because *E. coli* genomic DNA and plasmids both have a circular topology, we expect that a mutation in the NER system would also cause a deficiency in repairing damaged plasmid DNA and prevent replication of the plasmid.

Previous studies have investigated the role of NER in the repair of UV damaged DNA in both prokaryotic and eukaryotic cells (11, 12, 13). The SOS-related genes are also important for successful transformation of UV irradiated plasmids in *E. coli* (14). Our goal is to further characterize whether NER, specifically UvrB, could help repair *in vitro* UV damaged plasmid DNA in *E. coli*. We hypothesized that UvrB would be necessary for the repair of UV damaged DNA due to the central role it plays in mediating the damage recognition step with the incision step of NER.

In this study, we used wild-type and *ΔuvrB* knockout *E. coli* strains to examine the role of UvrB in the repair of *in vitro* UVC-damaged DNA by measuring transformation frequency. We set up a model system using plasmid that was UV damaged *in vitro*, under the assumption that damaged plasmid would not be replicated in the cell. We observed that as UV exposure increased, transformation frequency decreased. This relationship indicated that our experimental system was functional, and could be used as an assay. *ΔuvrB* and wild-type cells were compared at the 32s UV treatment time point, and showed similar transformation frequency. Due to assay variability, we were not able to draw firm conclusions, however, we have identified limitations with our assay and modifications that may yield more consistent data.

MATERIALS AND METHODS

Preparation of bacterial strains and plasmids. *E. coli* strains BW25113 and JW0762-2 (Δ*uvrB*) were obtained from the Keio Knockout Collection. BW25113 is the parent strain of JW0762-2, and both strains are derived from *E. coli* K-12 (15). Detailed strain information is summarized in **Table S2**. pUC19 plasmid was commercially purchased from New England Biolabs.

DNA agarose gel electrophoresis. Undiluted UVC irradiated pUC19 plasmids were run on a 0.7% agarose gel at 120 volts (V) for 1 hour (hr) (Fig. 1)**.**

UVC irradiation of pUC19 plasmid. pUC19 plasmid concentrations were serially diluted to obtain dilutions of 10^0 to 10^{-3}. The plasmids were then aliquoted into 32 wells on Corning® UV

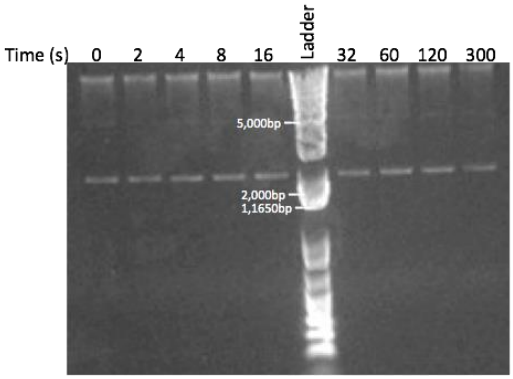

FIG. 1 Electrophoresis of UVC irradiated pUC19 plasmid. Undiluted (10^0) pUC19 irradiated with various time lengths (0-300s) were run as samples. 1kb Plus DNA Ladder was used.

transparent 96-well plate. Each well contains 75 µL. The Bio-Rad GS Gene Linker® UV Chamber, which produces radiation at 254nm, was set at 30mJ and used to irradiate the plasmids for 2 seconds (s), 4s, 8s, 16s, 32s, 60s, 120s and 300s. The plasmids were irradiated at the same time until a time point is reached. Once a time point is reached, the irradiated plasmids for that time point at dilutions 10^0 to 10^{-3} are transferred out of the wells. The 96-well plate was placed back into the UV chamber to continue the process of irradiation until the next time point was reached. This was performed until all the plasmids in the wells have been irradiated for the targeted experimental time length.

Preparation of competent cells. 100mL flasks of LB broth were inoculated with 1mL overnight cultures of BW25113 and JW0762-2, respectively. The inoculated broths were incubated on a 37°C shaking incubator at 200rpm until an optical density (OD_{600}) of 0.3 was reached. The cultures were chilled on ice for 15mins, and then transferred to centrifuge tubes for centrifugation at 4000rpm for 10mins at 4ºC. The supernatant was discarded and the pelleted cells were resuspended in 25mL ice-cold 0.1M $CaCl_2$. The cells were incubated on ice for 30mins before centrifugation at 4000rpm for 10mins at 4°C. The supernatant was discarded and the cell pellets from each strain were pooled back together during resuspension. Each strain was resuspended in a total volume of 5mL using an ice-cold 15% glycerol 0.1M $CaCl_2$ solution. The cells were stored in 50µL aliquots at -80°C.

Heat-Shock Transformation. All heat-shock transformations were performed with undiluted (10^0) pUC19 plasmid (22.2 ng/µL) irradiated with various irradiated time length. 50µL aliquots of BW25113 and JW0762-2 competent cells were thawed on ice. 3µL of pUC19 were added to each 50µL aliquot and incubated on ice for 30mins. The cells were heat-shocked in a 42°C water bath for 30s and then placed on ice for 2mins. 1mL of pre-warmed LB was added to each aliquot and incubated in a shaking incubator at 200rpm for 1hr at 37°C. After incubation, the cells were serially diluted before being plated on LB plates supplemented with different antibiotics. BW25113 were plated on LB and 100µg/mL LB-Ampicillin (LB-Amp) plates. JW0762-2 were plated on 50µg/mL LB-Kanamycin and LB-Kanamycin/Ampicillin (LB-Kan/Amp) plates supplemented with 50µg/mL kanamycin and 100µg/mL ampicillin.

Transformation frequency calculation. The transformation frequencies were obtained by dividing the number of transformants from plates containing ampicillin (i.e. LB-Amp or LB-Kan/Amp) by the number of colony-forming units (cfu) from LB or LB-Kan plates.

RESULTS

Irreversible DNA damage is induced by overexposure to UV light without the loss of plasmid quantities. UV-damaged DNA was generated by the Bio-Rad GS Gene Linker® UV Chamber as described in the methods for the purpose of studying the role of the NER system in *E. coli* DNA repair. Our result showed that when pUC19 plasmids were exposed to UV light at 254nm and 30mJ for longer than 60s, little to no transformed *E. coli* survived on ampicillin-selective plates (Figs. 2-3). Transformed colonies would have acquired antibiotic resistance from the pUC19 plasmid after uptake. The lack of colonies surviving from heavily irradiated plasmids suggest that either plasmids with irreparable antibiotic resistance genes were taken up, or a different factor prevented the heavily-irradiated plasmids from being successfully transformed into our competent cells. The Bio-Rad GS Gene Linker® UV Chamber is normally used to cross-link DNA, so it is possible that the effective concentration of the heavily-irradiated plasmids was changed due to DNA aggregation. Spectrophotometry and gel electrophoresis were used to examine quantities of irradiated pUC19 plasmids. All time points from 0s to 300s had similar plasmid concentrations of approximately 22.2ng/µL obtained by measuring

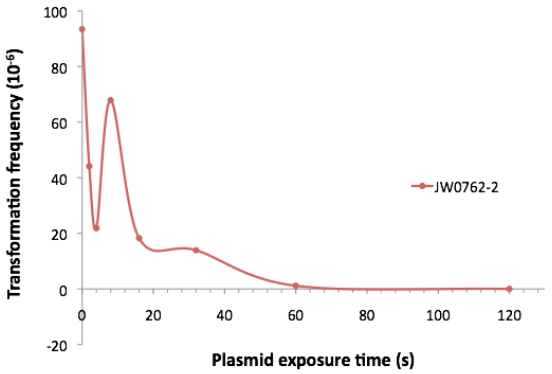

FIG. 2 JW0762-2 transformation frequency for run 1. Transformation frequency (10^{-6}) trend of JW0762-2 is shown in the y-axis. Plasmid exposure time in seconds is shown in the x-axis.

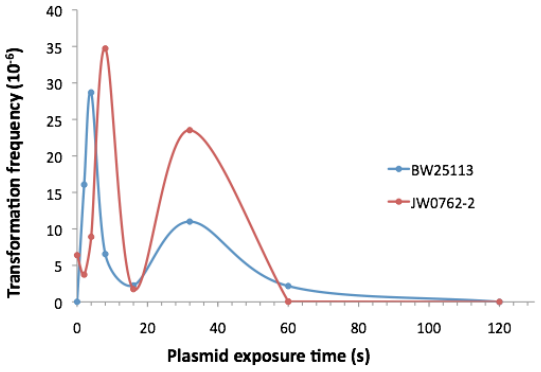

FIG. 3 Comparison of BW25113 and JW0762-2 transformation frequency for run 2. Transformation frequency (10^{-6}) trend of JW0762-2 and BW25113 are shown in the y-axis. Plasmid exposure time in seconds is shown in the x-axis.

absorbance at 260nm using Nanodrop2000. All irradiated plasmids showed the same number of bands migrating the same distance as the wild-type (time 0s) plasmid on a 0.7% agarose gel (Fig. 1)**.** These results indicate that the irradiated DNA samples were of similar concentration and structural integrity as measured by spectroscopy and gel electrophoresis, respectively.

Wild type and *ΔuvrB* strains exhibited no significant difference in their ability to repair irradiated plasmids. The ability to uptake and maintain UV-damaged DNA in cells is examined by ampicillin resistance of wild type (BW25113) and *ΔuvrB* mutant (JW0762-2) strains transformed with UVC-irradiated pUC19 plasmids. We exposed pUC19 plasmids to different amounts of UV light (2s, 4s, 8s, 16s, 32s, 60s, 120s, and 300s). The length of UV exposure corresponds to the severity of DNA damage from low to high. We expected higher DNA repair ability in transformants that contain highly damaged DNA, but can still survive on ampicillin plates. As such, we expected to see more colonies on ampicillin-selective plates with the wild type in comparison to the *ΔuvrB* mutant. In order to normalize our data we measured transformation frequency (percent of colony-forming units transformed), which was calculated by dividing the number of transformants from plates containing ampicillin by the number of colony-forming units (cfu) displayed on plates without ampicillin.

Transformation experiments for the entire set of time points from 0-300s were performed twice independently; the results are represented by Figures 2 and 3. For plasmids with shorter UV exposure, we observed irregular trends of frequency for both strains. The transformation frequency of BW25113 at 0s was 328X lower than JW0762-2 (Fig. 3). BW25113 has a peak at 4s, which was not present for the same time point in either replicates of JW0762-2 (Fig. 3). JW0762-2 frequency decreased for time points 0-4s in run 1, but the same time points in run 2 showed first a decreasing and then an increasing trend (Figs. 2-3). JW0762-2 had a peak appear at 8s before decreasing sharply at 16s in the replicated results (Figs. 2-3).

The 32s time point initially showed the most clear difference between our wild-type and mutant strains in terms of transformation frequencies, with JW0762-2 having a 25% higher frequency than BW25113 (data not shown)**.** To further explore this point, we did three replicates of BW25113 and JW0762-2 at 32s and averaged the transformation frequencies in order to determine statistical relevance (Fig. 4)**.** Upon averaging the accumulated data, we found that transformation frequencies at 32s were statistically similar between BW25113 and JW0762-2.

Transformants with 300s UV irradiated plasmids showed no viable colonies on ampicillin-selective plates (data not shown). Cells transformed with 60s or 120s irradiated plasmids displayed zero to low transformation frequencies, suggesting that plasmids over-exposed to UV light lead to damaged plasmid that could not be repaired in *E. coli* cells (Fig. 2-3). Together, our results suggest that BW25113 and JW0762-2 have similar transformation frequency for plasmids irradiated longer than 32s, but our assay results in variability for absolute transformation frequency with plasmids irradiated for less than 60s.

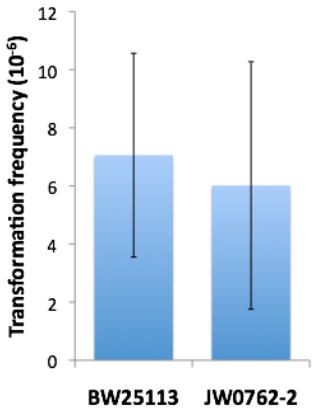

FIG. 4 Average transformation frequency for UVC irradiated pUC19 plasmid at t = 32s (n = 3). The transformation frequency at t = 32s was averaged from replicate 1 of run 2 and both replicates of run 3 for a total sample size of 3.

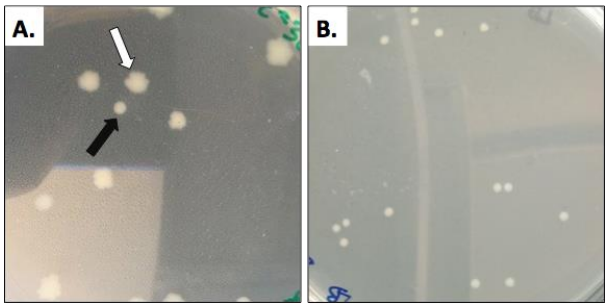

FIG. 5 Colony morphologies of JW0762-2 and BW25113. (A) JW0762-2 transformed with irradiated pUC19 at t = 32s on LB-Kan plate at 10^{-6} plating dilution. Large colony with irregular form is indicated with the white arrow. Small colony with circular form is indicated with the black arrow. **(B)** BW25113 transformed with pUC19 at t = 16s on LB plate at 10^{-8} plating dilution.

Irregular colony morphology of JW0762-2 strain. Colonies of JW0762-2 showed unusual colony morphology as compared to BW25113 strains on agar plates (Fig. 5A-B). BW25113 displayed consistent size and shape among colonies (data not shown), while JW0762-2 exhibited variable size and shape. JW0762-2 were found to either be small and round, or large and irregularly shaped **(**Fig. 5A-B). This was observed from colonies that were spread plated following transformation with pUC19, but was also observed in untransformed JW0762-2 that were streak plated to a fresh LB-Kan plate for the purpose of strain passaging and propagation.

DISCUSSION

NER is important in the repair of UV damaged DNA and has been well characterized in dealing with CPD and 6-4 PP DNA lesions (2, 3). We attempted to study the role of a central NER protein, UvrB, in repairing UVC damaged plasmid *in vivo*. We were specifically interested in the role of UvrB in the repair of damaged

plasmids, and this was reflected by our experimental approach involving the irradiation of purified pUC19 plasmid *in vitro* and subsequent transformation into UvrB-proficient and UvrB-deficient strains. Transforming our cells first before irradiating the transformed colonies with UVC would cause genomic DNA damage in addition to damaging our targeted plasmid DNA, which would have introduced other variables into our study (23). Hence, we irradiated our plasmids *in vitro* instead of *in vivo*. Following the transformations, we collected and tabulated plate counts of ampicillin-sensitive transformants as well as colony forming units on non-selective media.

Both BW25113 and JW0762-2 had low transformation frequencies when transformed with plasmids irradiated for 60-300s. We suspect this may be because the plasmids contained too many CPDs and 6-4 PPs to be repaired. Even an NER-proficient strain such as BW25113 may not have been able to repair the UV damage efficiently, resulting in low transformation frequencies. It is also possible that the SOS response and thus NER was not induced properly following the plating of our transformed cultures, resulting in cell death irrespective of *uvrB*. The presence of ssDNA induces the SOS response, which is produced and accumulates during the replication of UV-damaged DNA containing CPDs and 6-4 PPs. The DNA lesions block the DNA polymerase from proceeding in the replication fork while the upstream helicase continues unwinding the dsDNA, generating long segments of ssDNA (24, 25). Because replication must first occur in order to generate ssDNA to trigger the SOS response, it is possible that the heavily irradiated plasmids had accumulated excessive UV-damage to the point where it is no longer able to recruit DNA polymerases in the first place, failing to induce the SOS response and therefore not undergo NER. An alternative possibility may be that the cells have higher selectivity in DNA uptake and become less competent when heavily damaged plasmid DNA is present in the environment but this is purely speculation.

In contrast, we consistently obtained transformant colonies with both BW25113 and JW0762-2 for plasmids irradiated for a shorter amount of time (0-32s). Despite this, it was difficult to determine clear trends for 0-16s due to the presence of peaks and troughs that occurred at different time points between strains. This was seen in (Fig. 3) where a peak is present at 4s for BW25113 and at 8s for JW0762-2. Even between replicates of the same strain, there are still differences in where the peaks and troughs were found. For JW0762-2, there is a trough at 4s in Figure 2, but not at 4s in Figure 3. Currently, we do not have a clear explanation for these results, but we suspect they might be artifacts caused by pipetting errors. Our workflow involved many pipette manipulations to produce even one plate, so it is possible that a very minor pipetting error in the beginning of the workflow exerted a significant effect on our plate counts. Increasing the number of replicates may provide an explanation for these results, however our current experimental setup is very labour intensive for the amount of data it produces.

In the second run of the experiment, JW0762-2 transformed with 32s-irradiated plasmid showed a higher number of colony forming transformants than BW25113, but a lower number of colony forming units than BW25113. Due to this observation, we produced two more replicates of the experiment for 32s only. The average of the three replicates as shown in Figure 4 suggested that there is no statistically significant difference in transformation frequency. Based on this data. the results that were observed in the second run may have been similar to the results for 0-16s, or perhaps there is an alternative repair mechanism active in JW0762-2 but not in BW25113, as we had expected BW25113 to have the higher transformation frequency (26).

As well, we considered plasmid aggregation due to cross-linking from UV exposure as a possible factor that may have influenced our results (27). Aggregation of the DNA may affect DNA uptake of the competent cells, preventing transformation and yielding colony counts that would have been a result not attributed to NER. We tested for aggregation using spectrophotometry and gel electrophoresis but neither tests suggested there was aggregation present.

Lastly, unusual colony morphology of JW0762-2 was found sporadically on spread plates after transformation with pUC19 and from untransformed JW0762-2 plated on LB-Kanamycin plates. This suggests that there was either contamination or JW0762-2 had accumulated mutations. With irregular colonies arising from media supplemented with kanamycin, we deduced that it is unlikely to have been from contamination due to JW0762-2 being kanamycin resistant. Instead, we suspect that our JW0762-2 strains may have accumulated mutations from repeated cell divisions that were not fixed due to the strain's incapability to undergo NER, giving rise to irregular colony morphology. JW0762-2 may not have been genetically stable enough to undergo rounds of strain passaging and propagation that were required for our experimental procedures.

In conclusion, heavy UVC irradiation of pUC19 resulted in similarly low transformation frequencies between NER-deficient and NER-proficient strains. Also, irradiation for briefer periods of time did not result in statistically different transformation frequencies between NER-deficient and NER-proficient strains. Taken together, our results suggest that *uvrB* deficient and proficient strains show similar transformation frequencies when transformed with *in vitro* UVC-irradiated plasmid, and the role of *uvrB* in repairing UV-damaged plasmid DNA remains unclear.

Our result provides evidence that UV light can induce permanent damage to DNA that cannot be repaired by bacteria *in vivo*. The *uvrB*-deficient strain had a similar ability as *uvrB*-proficient strains to repair UV-damaged DNA *in vivo*, implying that UvrB may not play an important role in repairing plasmid DNA. Irregular morphology found with JW0762-2 provides evidence that the NER system is important in maintaining genomic integrity and preventing accumulation of mutations in *E. coli*.

FUTURE DIRECTIONS

It is still not clear the role of *uvrB* in correcting UV-damaged plasmid DNA *in vivo*. Sequencing UVC-irradiated plasmids from transformed colonies of BW25112 and JW0762-2 (Δ*uvrB*) strains will allow us to investigate if JW0762-2 strains have more mutations on the plasmids. The sequence of non-essential genes on the UVC-irradiated plasmids will be checked for mutations by comparison with the original pUC19 plasmid sequence.

With our experimental model, one would not be able to differentiate between cells that were transformed with an undamaged wild-type plasmid from a damaged plasmid that was then subsequently repaired by NER. Methods that experimentally quantify the amount of DNA lesions, such as CPDs and 6-4 PPs, present after UVC irradiation would allow for molecular explanations for the resulting transformation frequencies. One method to determine the quantity of DNA lesion in the plasmids is to perform an enzyme-linked immunosorbent assay (ELISA). The ELISA method can be used to quantify the damage in nucleic acid samples by the use of antibodies binding to DNA adducts and lesions. Another method is to use atomic force microscopy (AFM). AFM imaging detection paired with agarose gel electrophoresis will allow DNA lesions such as CPD sites to be visualized ranging from individual intact to damaged plasmids.

A more stable and high throughput platform is needed to study DNA repair mechanisms in *Escherichia coli*. Our current approach towards the generation of UVC-irradiated plasmids may have had an effect on our unusual transformation frequency data. This method does not involve randomizing the relative locations of pUC19 in the UV transparent 96-well plate for the UVC irradiation process. By randomizing the location of plasmid in the wells, potential edge effects or biases in how a specific time point was irradiated would be eliminated. Furthermore, our approach towards looking at transformation frequency by colony counts was challenged by serial dilutions using inconsistent pipetting technique. Other groups may want to explore an alternative method with higher throughput compared to plate counts because this would increase ease of reproducibility.

Finally, we observed irregular colony morphology in the JW0762-2 strain, which suggests our JW0762-2 strain may have accumulated mutations. It would be interesting to check the genome sequence composition by doing whole genome sequencing for JW0762-2 strain. Based on the sequencing result, we would be able to compare the degree of mutagenesis in JW0762-2 and the parent strain. We would also know the efficiency of UvrB in the prevention of gene mutation. Lastly, characterizing mutated genes from JW0762-2 may provide insight to its role in colony morphology determination.

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