

Role of RecA in the Protection of DNA Damage by UV-A in *Escherichia coli*.

Amanda Chau, Karen Giang, Marcus Leung and Natalie Tam

Department of Microbiology and Immunology, UBC

Solar Water Disinfection (SODIS) is a simple, yet effective method for water decontamination by utilizing heat and UV-A from sunlight to kill microbes. However, the mechanism for UV-A mediated cell death is unknown. UV-A has been shown to cause DNA damage by the induction of reactive oxygen species (ROS). This damage might cause cell death in SODIS. Therefore, we investigated whether limiting the repair of DNA damaged by UV-A can increase bacterial susceptibility to UV-A. RecA is a DNA repair regulator protein that is activated upon stresses such as heat and reactive oxygen. In this study, we used *Escherichia coli* strains with and without RecA to determine survival frequencies after UV-A irradiation. *recA*⁺ cells with functional RecA showed a higher survival frequency compared to *recA*⁻ mutants after 120 minutes of UV-A exposure, while a smaller difference was observed when cells were cultured without light. These results suggest that the absence of RecA is correlated with an increased vulnerability of cells under UV-A exposure. This difference in sensitivity is most likely due to the inability of mutant bacteria to repair DNA damages. Further studies are required to determine not only the effects of RecA on repairing DNA damage, but also other bactericidal mechanisms exerted by UV-A because the presence of RecA was insufficient to fully protect the treated cells.

Contaminated water is a major threat to the developing world. Thirty percent of the world's population lacks access to clean water and consumption of contaminated water contributes to 1.6 million deaths per year (1). These deaths are largely due to gastrointestinal infections from fecal coliforms, including *Escherichia coli*, *Citrobacter*, *Enterobacter* and *Klebsiella* species (16). The Solar Water Disinfection (SODIS) system is an effective, simple and inexpensive way to decontaminate water by inactivating microbes. In SODIS, contaminated water in polyethylene terephthalate (PET) bottles is subjected to solar irradiation for 6 to 48 hours depending on the intensity of sunlight (2). Although solar UV radiation consists of UV-A (400-320 nm), UV-B (320-290 nm), and UV-C (290-100 nm), only UV-A and UV-B reaches the earth's surface. Of these, 90% consists of UV-A and thus UV-A and heat are thought to be the mechanisms for decontaminating water in SODIS (2).

UV-A light is longer than the wavelength absorbed by DNA and as a result, the predominant form of DNA damage by UV-A is caused indirectly through generation of reactive oxygen species (ROS) (19). ROS, such as hydrogen peroxide (H₂O₂), damage DNA strands by oxidizing the DNA bases to form pyrimidine adducts and dimers (19). RecA, a multifunctional protein, is a key regulator in DNA repair under stresses such as oxidation and heat (18). In the DNA repair pathway, RecA mediates the SOS

response system, which regulates more than 50 genes involved in DNA repair (13). Under normal conditions, the SOS system is negatively regulated by the LexA repressor protein which directly binds to operator sites targeted by RecA. However, the emergence of excision gaps, daughter-strand gaps, and double-strand breaks from UV-A radiation, leads to recruitment of RecA (24, 25). Assembly of RecA into filaments bound with ATP leads to the formation of RecA*, which is capable of cleaving LexA, and results in the upregulation of SOS genes (25). LexA has differential affinities to operators of the SOS regulon; consequently, operators with lower affinity for LexA are transcribed first. Early expressed SOS response genes, including *uvrA* and *uvrB*, are involved in nucleotide excision repair (NER), which is an important DNA repair system (9). However, if NER is not capable of repairing all DNA breaks and chromosomal damages, transcription of other SOS DNA repair genes are also induced (23).

The mechanisms of bacterial cell death in SODIS remain elusive. However, in non-SODIS experiments UV-A irradiation causes cell death in both eukaryotic and bacterial cells through ROS mediated DNA damage (15, 21). Thus, we hypothesize that DNA damage contributes to the cytotoxic effects of UV-A in SODIS. To test whether DNA damage is involved in UV-A mediated killing, we examined the ability of *E. coli* K-12 isogenic

strains differing only in the presence or absence of *recA* to survive UV-A exposure.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* K-12 substrains BW25113 (*recA*⁺ positive control) and JW2669-1 (*recA* isogenic mutant) were kindly provided by The Coli Genetic Stock Center, Yale University. JW2669-1 contains a deletion mutation in the *recA* gene and was used for the analysis of RecA effects on bacterial survival, while BW25113 was used as a positive control as it contains functional *recA*. Both strains also have $\Delta(\textit{araD-araB})567$, $\Delta\textit{lacZ4787} (::\textit{rrnB-3})$, λ -, *rph-1*, $\Delta(\textit{rhaD-rhaB})568$, *hsdR514*.

Media. Luria-Bertani (LB) broth (1% w/v tryptone, 0.5% w/v yeast extract, and 0.5% w/v NaCl pH 7.0) was used for all experiments requiring suspended bacterial growth. LB agar plates with 1.5% agar were used for plating.

Characterization of growth. Overnight cultures were prepared by inoculating a single colony of each strain into 125 mL Erlenmeyer flask containing 30 mL LB media. The flasks were incubated overnight at 37°C in a shaking incubator at 200 rpm. The following day, 5 mL of each overnight culture was diluted 1-in-10 in LB broth in a 250 mL Erlenmeyer flask and incubated in a 37°C shaking incubator at 200 rpm. Using the Spectronic-20D spectrophotometer (Milton Roy), turbidity measurements were taken from culture samples at 600 nm (OD₆₀₀) every 15 minutes for the initial 90 minutes, and subsequently every 30 minutes thereafter. For OD₆₀₀ readings above 0.60, appropriate dilutions using fresh LB broth were made to adjust the turbidity to fall between 0.10 and 0.60. LB broth was used as blank. Turbidity measurements were stopped when cultures entered stationary phase and the OD readings remained constant for at least three successive data points.

UV-C sensitivity assay to test the presumptive RecA phenotype in each strain. The UV-C sensitivity assay was modified from Algu *et al.* (3). Overnight cultures of both *E. coli* BW25113 and JW2669-1 were prepared in LB broth and incubated at 37°C in a shaking incubator at 200 rpm. 5 mL of each overnight culture was diluted 1-in-10 in LB broth and incubated at 37°C in a shaking incubator until culture reached the turbidity for stationary phase that was determined by previous characterization of growth. Approximately 2×10^9 bacterial cells from

each strain were serially diluted (10^{-3} , 10^{-4} , and 10^{-5}) and 40 μ L was plated on LB agar plates. Each strain of bacteria had two sets of serially diluted plates where one set was subjected to 8 mJ/m², while the other set was subjected to 16 mJ/m² UV-C light (254nm) produced by the UV Stratagene Model 2400 (Stratagene). Control plates, not exposed to UV-C, were prepared with strains diluted 10^{-6} , and 10^{-7} . Plates were incubated in the dark at 37°C overnight and survival frequency of the two strains were determined by counting the concentration of viable cells in the UV exposed samples divided by the concentration of viable cells in the control samples that were not exposed to UV-C light.

Western blot analysis for RecA protein.

Western blots were performed to verify the expression of RecA in *E. coli* BW25113 and the absence of *E. coli* RecA in JW2669-1. Samples of each bacterial strain were obtained from cultures used in preparation for the UV-A experiment. Bacterial lysates were generated from an adapted lysis protocol from Chen *et al.* (6). Volumes of bacterial cultures containing 10^9 bacterial cells from each strain were collected. Cells were pelleted for 45 seconds at 7,500 x g. Supernatants were discarded and cell pellets were resuspended with 100 μ L of lysis buffer (100mM Tris pH 7.8 and 100mM NaCl), vortexed for 30-60 seconds, then left on ice for 15 minutes. 150 μ L of 2X SDS-PAGE sample buffer (0.5M Tris-HCl pH 6.8, 5% v/v glycerol, 2% w/v SDS and 2% v/v β -mercaptoethanol, 0.02% w/v bromophenol blue) was added to the cell suspensions and then heated at 95°C for 10 minutes. The heated suspensions were centrifuged for 5 minutes at 7,500 x g. 15 μ L of the supernatant was loaded into a 5% stacking, 12% separating acrylamide gel for separating proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). BenchMark™ Pre-Stained Protein Ladder (Invitrogen) was included along with samples. The gel was run at 150 V for 60 minutes under running buffer (0.19 M glycine, 25 mM Tris base, 1.73 mM SDS) and was transferred to nitrocellulose membranes (BioRad) at 90 V for 90 minutes in transfer buffer (25 mM Tris base, 0.19 M glycine, 20% v/v methanol). The membrane was blocked overnight at 4°C in 5% skim milk in TBS-T (0.1% v/v Tween-20, 50 mM Tris base pH 7.5, 150 mM NaCl). It was then washed and probed with rabbit polyclonal anti-*E. coli* RecA primary antibody (MBL RK-61-003) diluted 1-in-1000 in 5% skim milk in TBS-T. The secondary antibody used was a goat anti-rabbit IgG affinity purified peroxidase-labelled antibody (KPL 474-1506) diluted 1-in-5000 in 5% skim milk in TBS-T. Enhanced

chemiluminescence Western Blot Detection Reagents (Amersham Biosciences RPN2108) were used according to manufacturer's protocol. The membrane was exposed for 10 minutes on X-ray film (Kodak).

UV-A treatment. Stationary phase bacterial cultures of *E. coli* BW25113 and JW2669-1 were obtained following the characterization of growth procedure. The appropriate volume of bacterial culture to yield a final bottle concentration of 5×10^5 bacterial cells/mL was centrifuged at $7,500 \times g$ for 10 minutes at room temperature. Pellets were resuspended in 1 mL of sterile distilled water and transferred to 500 mL PET bottles (Costco Kirkland Signature) with 399 mL of sterile distilled water. The samples were subjected to UV-A irradiation provided by two 20 watt Sylvania black light with a

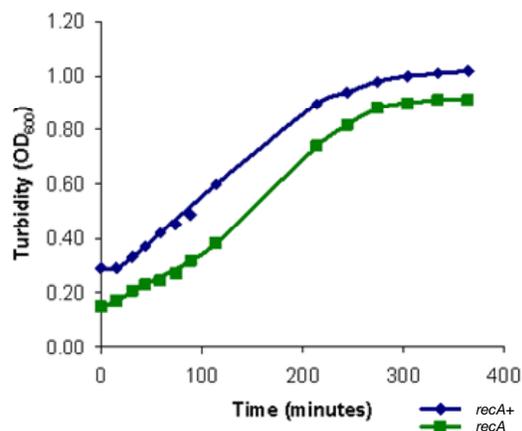


FIG 1. Growth characteristics of *recA*⁺ (BW25113) and *recA* (JW2669-1) *E. coli* cultures in LB grown at 37°C to determine time required to reach stationary phase.

TABLE I. Effect of UV-C radiation on survival frequency of *recA*⁺ (BW25113) and *recA* (JW2669-1) cultures plated on LB plates exposed to either 8 mJ/cm² or 16 mJ/cm² UV-C.

UV-C Intensity (mJ/cm ²)	Survival Frequency (%)	
	<i>recA</i> ⁺	<i>recA</i>
8	0.09	0.00
16	0.00	0.00

wavelength of 366 nm at 213 W/m². All bottles were laid horizontally to provide even light distribution and the Sylvania black light was placed 5 cm above the top of the side edge of the bottles. Controls were set up identical to UV-A irradiated bottles except they were covered in aluminium foil and kept in a dark cupboard. Samples were taken from control and UV-A-irradiated bottles at 0 and 120 minutes. 50 µL of each sample was plated on LB agar plates at final dilutions of 10⁻², 10⁻³, and 10⁻⁴. The dilutions are chosen to ensure that the number of colonies was within the statistically acceptable colony range of 30-300. Plates were incubated at 37° C in the dark overnight. Colony counts were performed the following day and survival frequencies were determined by comparing viable concentrations at 120 minutes compared to time 0 minutes for the same treatment. Subtraction of survival frequencies of UV-A treated cells from survival frequencies of dark treatment was used to determine the effect of UV-A on cell viabilities within each strain.

RESULTS

Growth curve. In the absence of UV-A radiation, the growth profile for the *recA*⁺ and *recA* strains were similar. Both strains reached stationary phase at approximately 300 minutes where turbidity measurements persisted at approximately 1.0 OD₆₀₀

for at least 90 minutes (Fig. 1). The isogenic strains had comparable doubling times of 87 and 83 minutes for the *recA*⁺ and *recA* strains, respectively. However, the 1-in-10 dilution of overnight cultures at 0 minutes produced an initial OD₆₀₀ reading of the *recA*⁺ culture, which was two times higher than *recA*. The turbidity in the *recA*⁺ strain remained about 0.15 OD₆₀₀ higher than the *recA* strain throughout the growth curve. The *recA* strain had a slower phase of growth for the first 90 minutes. The *recA*⁺ strain had a lag for about 15 minutes.

UV-C sensitivity assay. UV-C light was used to validate RecA phenotypes of BW25113 and JW2669-1. Both strains were tested for susceptibility to either 8 mJ/cm² or 16 mJ/cm² UV-C because *recA*⁺ was previously shown to survive in 8 mJ/cm² but not 16 mJ/cm² (3). As seen in table I, *recA*⁺ cells had a higher survival frequency (0.09%) at 8 mJ/cm² than mutant cells (0.00%), while no cells of either strain survived at 16 mJ/cm².

Western blot. To confirm the absence of RecA expression in the mutant strain during UV-A treatment, samples were taken from both RecA⁺ and RecA⁻ cultures prior to dilution within PET bottles for UV-A exposure. A band between 37.1 kDa and 48.8 kDa was seen in lane 1 with RecA⁺ lysates corresponding to the size of the 38 kDa RecA protein (Fig. 2). In contrast, a band of this size was absent in lane 2 with the RecA⁻ bacterial lysates. Equal loading

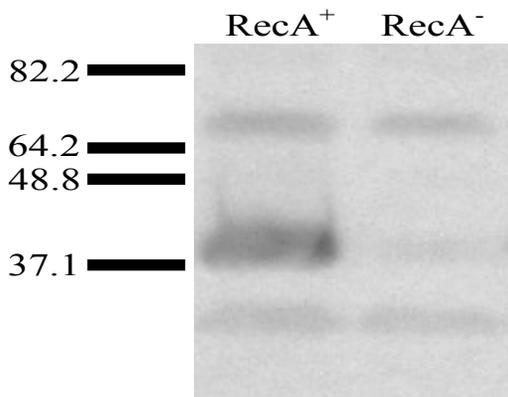


FIG 2. Expression of RecA in RecA⁺ and RecA⁻ *E. coli* strains prior to UV-A exposure to confirm the genotype of the *E. coli* strains. Numbers are the sizes in kilodaltons

of protein in the two lanes was assessed by detection of equal intensity bands at ~65 kDa and ~30 kDa.

UV-A treatment. Average survival frequency was higher for RecA⁺ cells than RecA⁻ cells after 120 minutes of UV-A exposure (Fig. 3). Survival frequencies were 105 ± 29% and 59 ± 23% for RecA⁺ and RecA⁻, respectively (Fig. 3). In comparison, average survival rates for RecA⁺ and RecA⁻ cells kept in the dark were relatively similar with frequencies of 139 ± 8% and 116 ± 40%, respectively (Fig. 3). Although results were not statistically significant, due to the broad error range, general trends were recognized. In the dark, the strains had similar survival frequencies while in UV-A, there was a greater difference in survival between the two strains. In addition, both RecA⁺ and RecA⁻ had better survival in the dark than under UV-A. This trend not only indicated improved survival in the dark for both strains, but also a growth of cells as survival

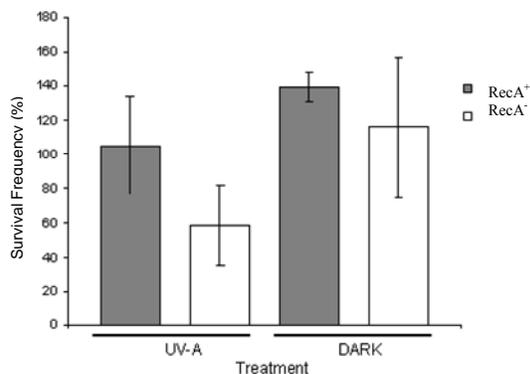


FIG 3. Effects of the presence or absence of 120 minutes of UV-A irradiation on the survival of RecA⁺ (BW25113) and RecA⁻ (JW2669-1) *E. coli*.

frequencies were over 100%. In comparison to the dark control, a 35% decrease in survival was observed in RecA⁺ cells exposed to UV-A. In RecA⁻ cells, approximately 60% difference in percent survival was noted between the UV-A treatment and the dark control. Although the percent of cell death was greater in the mutant strain under both conditions, RecA⁻ cells were twice as likely to die when exposed to UV-A when this background difference was taken into consideration.

DISCUSSION

The similar growth curves observed for RecA⁺ and RecA⁻ cultures are in agreement with previous studies (8, 11). This suggests that under normal conditions, such as in the absence of UV-A radiation, RecA⁻ is not commonly required for replication or there are other proteins that can compensate for the lack of RecA⁻ (7, 8). The consistently higher OD₆₀₀ readings in the stationary phase of RecA⁺ cultures may be the consequence of a lower maximal culture density for RecA⁻ cultures. The difference in the measured OD₆₀₀ readings for RecA⁻ cultures can possibly be attributed to factors including smaller cell size and lower cell numbers. The lower turbidity readings in RecA⁻ have been reported in other studies (11). Growth rates were expected to be higher for RecA⁺ cultures than RecA⁻ cultures (5), although we observed similar growth rates for both strains. This discrepancy in growth rate may result from differing methods in constructing the growth curve such as different initial optical densities, proportion of viable and dead cells. Most notably, there were genetic differences resulting in mutations between our isogenic strains and strains used by Capadello-Kimball *et al.* (5), which can also explain the discrepancy. The main purpose of the growth curve was to determine entry time of stationary phase so that subsequent experiments were conducted with cells in the stationary phase. Stationary phase bacteria, compared to those growing exponentially, have been shown to have decreased sensitivity to stresses due to the physiological adaptation the cells go through under nutrient-limiting conditions (17). This is imparted by RpoS activation, which causes the induction of SOS response regulon (14). The SOS response regulon contains the *recA* gene (14). Another importance of using stationary phase cells is that it better mimics the physiological state of bacteria in water from the environment. These bacteria are often suspended in water with low nutrient levels that limit its growth (12). Therefore, stationary phase cells are more representative of bacteria found in contaminated water for SODIS and experiments performed on this stage of cell growth is

more applicable for understanding the effectiveness of SODIS to eliminate bacteria.

UV-C sensitivity assay and Western immunoblotting were performed to verify the phenotype of *E. coli* BW25113 (*recA*⁺) and JW2669-1 (*recA* mutant). The *recA*⁺ strain was expected to have a higher survival frequency than the mutant strain at UV-C intensity at 8 mJ/cm², while at 16 mJ/cm² both strains were not expected to survive based on results presented in previous studies (3). The mutant strain showed a higher UV-C sensitivity at an intensity of 8 mJ/cm² than the *recA*⁺ strain (Table 1), and this can be explained by the fact that RecA is indispensable for DNA repair systems. UV-C irradiation causes DNA strand lesions and thymine dimers, which leads to cell death (27). The mutant strain was also expected to be deficient in RecA protein expression, which was confirmed by probing for RecA on the Western immunoblot (Fig. 2). This also verifies the phenotype of the two strains. The other resultant bands at approximately 65 kDa and approximately 30 kDa were non-specific, most likely due to the use of polyclonal antibodies.

In support of our hypothesis, the ratio of cell death from UV-A exposure to dark conditions was twice as high in RecA⁻ cells compared to RecA⁺ (Fig. 3). Dark controls were used in attempt to eliminate cell death not attributed to UV-A. Thus, the decrease in survival frequency in the dark for RecA⁻ suggests that death is partially due to the mutants' intrinsic deficiency of RecA protein. Despite the lower survival frequency in RecA⁻ compared to RecA⁺ under the dark condition, the difference was greater under UV-A treatment indicating that RecA⁻ was more susceptible to UV-A. Thus, RecA contributes to the protection of cells during UV-A since there was lower survival in UV-A compared to the dark for both strains. UV-A irradiation increases DNA lesions, which in RecA⁺ strains, would activate RecA and induce DNA repair (4). In the RecA⁻, the DNA lesion could not be repaired due to deficiency of sensing DNA breaks (20). Therefore, our results imply that DNA damage could be a factor in the mechanism of cell death by UV-A during SODIS, but more importantly imply that similar processes of DNA damage occur under SODIS-like set-ups.

Although, SODIS is clearly successful in decontaminating water (2), we observed an average survival rate of 105% in RecA⁺ cells exposed to UVA. This suggests that either UV-A does not kill bacterial cells. However, the average survival frequency of RecA⁺ cells receiving the dark treatment was 35% higher than the UVA treated cells, which indicates that, as expected, UV-A does decrease the viability of RecA⁺ cells. RecA⁻ cells in the dark also exhibited a survival frequency greater than 100%.

These high survival frequencies indicate that cells continue to divide after being placed in the sterile distilled water at 0 minute and that there may be a faster rate of growth than death under UV-A. This conflicts with our interpretation of the growth curve as cells were collected from early stationary phase and assumed to be non-dividing. Since turbidity increase correlates to culture mass, the slower rate of OD increase occurs sooner than the decrease in cell division; therefore, it is possible that cells were actually extracted from transition phase between exponential growth and early stationary phase. Collecting cells at a later time point in the stationary phase could avoid this problem.

The limited cell death might also reflect the short duration of the UV-A exposure, the difference in UV-A intensity and the difference in temperatures in our experimental conditions when compared to the actual SODIS protocol. The SODIS protocol involves sunlight exposure for a minimum of six hours, which is three times the length of our experiment (2). It has also been documented that microbial inactivation in SODIS has the highest efficiency if cells are subjected to a solar radiation intensity of at least 500 W/m² (26). This solar radiation consists of UV-B (10) which was absent in our experiment. Although only constituting to 10% of sunlight, UV-B is approximately 1000 times more potent in causing DNA damage than UV-A (10). In our experiment, the radiation intensity of 213 W/m² is roughly half the recommended intensity and only consists of UV-A; thus, possibly further decreasing the efficacy of UV-A mediated killing observed. Furthermore, room temperature was used in our UV-A irradiation protocol whereas SODIS protocol recommends 50° C to obtain a three-fold increase in disinfection frequency (2).

In this paper UV-A was isolated from other SODIS decontamination aspects such as UV-B and heat. Our experiments demonstrate the importance of RecA in conferring cell survival during UV-A treatment. RecA is important in DNA repair mechanisms and thus, it can be inferred that DNA damage mediated by UV-A irradiation could contribute to the bactericidal activity of SODIS. Understanding the mechanism by which bacteria are killed will help to develop rational improvements on the efficacy and efficiency of SODIS.

FUTURE EXPERIMENTS

This experiment should be repeated with cells collected from a later time in the stationary phase to ensure cell division is no longer occurring. This will eliminate effects of cell division from the results and thus, trends in killing due to UV-A can be more

clearly distinguished. In addition, decreased survival rates attributed to the absence of RecA can be confirmed in a complementation study. Transformation of *recA* with a plasmid encoding RecA under the control of its own promoter should result in restored survival rates. The link between ROS-mediated DNA damage still needs to be clearly established. The oxidation of C8 in guanine (8-OHdG) is a marker for ROS-damaged DNA and has been recognized by antibodies (22). Therefore, immunofluorescence could be used to detect ROS-mediated DNA damage in UV-A treated RecA⁺ cells relative to the control. A higher level of ROS-damaged DNA would be expected in RecA⁻ mutants compared to RecA⁺ upon exposure to UV-A.

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