Resistance to Interstrand Cross-links in *Escherichia coli*

by

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Abstract

8-methoxypsoralen is a DNA-intercalating agent which can photoreact with pyrimidine bases on opposing DNA strands to form an interstrand cross-link. This type of lesion completely blocks replication and transcription and is lethal to dividing cells, making it highly effective in chemotherapy. Yet how these lesions are processed in the cell remains poorly understood. Further insight into how these lesions are processed by the cell could lead to better therapies and prevent the development of resistance to these agents.

Here I developed a screen to determine if *E. coli* can develop resistance to cross-link agents, similar to cancer cells that develop resistance to these agents. Following repeated exposure of a culture to lethal doses of cross-linking, I identified and isolated three mutant lines with extreme resistance to the cross-linking agent, 8-methoxypsoralen. In at least one mutant, the mutation appears to confer resistance by preventing cross-link formation. I discuss possible mechanisms and future directions to identify the pathways by which this resistance arises.

Introduction

Cross-linking agents are commonly used in the treatment of psoriasis and certain types of anemia, as well as in certain chemotherapeutics. These agents interact with DNA bases on opposing strands and form covalent bonds to form interstrand cross-links. They can also interact with one base to produce a monoadduct, or two bases on the same strand to form an intrastrand cross-link. Interstrand cross-links are the most lethal of all of these interactions because they prevent the DNA from unwinding during DNA replication or transcription and therefore represent an absolute block to both of these metabolic processes (Derheimer et al., 2009). The lethality of interstrand cross-links also arises because the covalent bonding to both DNA strand prevents the normal process of nucleotide excision repair which requires an undamaged complementary strand following incision (Guainazzi & Schärer, 2010). These features make cross-linking agents especially effective where excessive cell proliferation is present. However, despite their effectiveness, cancers can develop resistance to these agents (Deans & C West, 2011). An understanding of how cellular resistance is conferred would be important and potentially suggest methods in which the development of resistance could be diminished or prevented.

Psoralen as a Cross-linking Agent

Psoralens have been effectively used in treatment of diseases characterized by unwanted cell proliferation, such as psoriasis and cutaneous T-cell lymphoma (Arroyo & Tift, 2003; Wackernagel et al., 2006). Psoralen can intercalate into DNA to form adducts with adjacent pyrimidine bases when excited with UVA light. The photoreaction of psoralen with DNA happens in three steps; first, the psoralen intercalates between two adjacent base pairs, second, photoreaction yields a monoadduct, and third, another photon is absorbed, forming an interstrand

cross-link (Kanne et al., 1982). Therefore, irradiating with UVA light can form covalent adducts on both strands of DNA, resulting in an interstrand cross-link.

Interstrand Cross-link Resistance

There are several ways in which cancer cells can acquire resistance to DNA damaging agents. In some cases, cells acquire resistance to DNA-damaging drugs by lowering drug uptake, or increasing efflux of the drug through regulation of the specific drug pump. (Cheung-Ong et al., 2013). Another mechanism of resistance is increased expression of P-gp (P-glycoprotein), an ATP-binding pump that controls efflux of DNA-damaging drugs like psoralen. (Gottesman, 2002). In this study, I explored whether cellular resistance to the DNA interstrand cross-linking agent, 8-methoxypsoralen, can arise in *E.coli* cells repeatedly exposed to this agent and began to characterize the mechanism by which resistance occurs.

Materials and Methods

Bacterial Strains and Plasmids

The parent strain used is SR108, a *thyA36 deoC2* derivative of W3110 (Mellon & Hanawalt, 1989). pBR322 contains a ColE1 origin of replication and is a medium copy number plasmid (Bolivar et al., 1977).

Selection for 8-methoxypsoralen UVA resistance

SR108 was freshly streaked on Luria-Bertani agar plates supplemented with 10 μg/ml thymine (LBthy). Two colony isolates from the same plate (hereafter referred to as strain A and strain B) were inoculated in 2ml LBthy medium and incubated overnight at 37°C and were used throughout the entire experiment. 20 μg/ml 8-methoxypsoralen were added to each overnight culture and incubation continued for 20 minutes at 37°C before the experiment began. Then, aliquots of 0.1 ml were plated on several LBthy agar plates supplemented with 20 μg/ml 8-methoxypsoralen. Each plate was then exposed to UVA irradiation using two 32-W UVA bulbs (peak emittance 320 nm) at an incident dose of 5.05 J/m²/s for increasing periods of time. The plates containing the exposed cultures were then incubated overnight at 37°C. The next day, the plate that was exposed to the highest dose and still displayed 'significant' survival (ie. almost, but not quite, a lawn of bacteria) was chosen as the population that could be enriched for mutants that conferred resistance to psoralen plus UVA. Cells from this plate were resuspended and grown in LBthy medium overnight at 37°C, and re-exposed to 8-methoxypsoralen and increasing UVA doses. At this point, the selection process was repeated. A portion of culture from each successive selection passage was frozen in LBthy media supplemented with 20% glycerol and stored for future characterization.

Psoralen-UVA (PUVA) survival assays

Fresh overnight cultures were diluted 1:100 in Davis medium supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μg/ml thymine (DGCthy), and grown at 37°C to mid-log phase (OD600 ~0.4). At this time, 20 μg/ml 8-methoxypsoralen were added to the cultures and incubation continued for 20 min at 37°C. Following this incubation period, 0.1 ml of each culture was serially diluted in 10-fold increments in DGCthy medium. Three 10 μl aliquots were spotted on LBthy plates supplemented with 20 μg/ml 8-methoxypsoralen, and exposed to UVA irradiation for the indicated doses. Plates were incubated at 37°C overnight, and visible colonies were counted to determine the percent survival, relative to unirradiated cultures.

UVC survival assays

Fresh overnight cultures were diluted 1:100 in DGCthy, and grown at 37°C to mid-log phase (OD600 ~0.4). 0.1 ml of each culture was serially diluted in 10-fold increments in DGCthy medium, and three 10μl aliquots were spotted on LBthy plates, and exposed to UVC irradiation for the indicated doses found in figure 3. Plates were incubated 37°C overnight, and visible colonies were counted to determine the percent survival, relative to unexposed cultures.

Southern blot analysis for detection of DNA interstrand cross-links

Strains transformed with pBR322 were grown overnight in DGCthy medium supplemented with 50 mg/ml ampicillin at 37°C. Cells in a 100-μl aliquot of each strain were collected and resuspended in 10ml DGCthy medium without ampicillin and grown in a 37°C shaking water bath to mid-log phase. 20 μg/ml 8-methoxypsoralen was added to cultures and incubation continued for 20 min at 37°C, and then exposed to increasing doses of UVA light. At the indicated doses, 0.75-ml aliquots of each culture were added to an equal volume of ice-cold 2x NET buffer (400 mM NaCl; 40 mM EDTA, pH 8; 10 mM Tris, pH 8)before the cells were pelleted and stored at -80°C until all samples were collected. Each frozen pellet was resuspended

in 140-ml lysis buffer (1 mg/ml lysozyme, 0.5 mg/ml RNaseA in 10 mM Tris, 1 mM EDTA) and incubated at 37°C for 30 minutes. Then, 10 µl of 20% Sarkosyl and 10 µl of 10 mg/ml proteinase K were added to each sample and incubated at 37°C for another 30 minutes. The samples were extracted with 4 volumes of phenol:chloroform, followed by 2 volumes of chloroform, and then dialyzed against 200 ml of 1mM Tris (pH 8), 1mM EDTA (pH 8) for 30 minutes by dropping 100 µl of each DNA sample onto 47-mm Millipore 0.025 mm pore disks that were floating on the dialysis buffer. The purified genomic DNA was then digested with PvuII overnight at 37°C to linearize the plasmid. Samples were electrophoresed on a 0.75% alkaline agarose gel in 30 mM NaOH, 1 mM EDTA at 30 V (1 V/cm) for 16 hr. DNA was transferred to Hybond N+ nylon membranes using standard Southern blotting techniques. The plasmid DNA was detected using a ^{32}P-labelled pBR322 probe that was prepared with a random primer labeling kit (Agilent) using >6000 Ci/mmol [α-^{32}P]dCTP (PerkinElmer). Southern blots were visualized using a Storm 840 phosphoimager (GE Biosciences) and its associated ImageQuant analysis software.

The fraction of 8-methoxypsoralen cross-links formed at each dose was calculated as the ratio of the DNA band running at about twice the molecular weight of the linear band to the total DNA per lane and normalized to the fraction of cross-links in untreated samples.

Fraction of cross-linked DNA = $(XD_{dose(x)}/TD_{dose(x)})-(XD_{untreated}/TD_{untreated})$

where XD represents cross-linked DNA and TD represents total DNA.

Results

E. coli have the capacity to develop resistance to the interstrand cross-linking agent, 8methoxypsoralen plus UVA.

I developed a genetic screen to select for cells that became resistant to psoralen-UVA treatment. To this end, two separate cultures of a parent strain of *E. coli* were plated on media containing 8-methoxypsoralen and exposed to increasing doses of UVA light (PUVA) before the cells were allowed to recover and grow overnight. Cells from the plate that received the highest dose and still produced a lawn were considered to potentially contain mutations that benefited their ability to survive this treatment, along with the remaining unmutated cells that, simply by random chance, received less lesions or were 'lucky enough' to survive. In this way, the cells on the plate were considered to be a culture 'enriched' for beneficial mutations that allow for survival from psoralen-UVA treatments. The process was then repeated, with the idea that cells containing the beneficial mutation would continue to survive and grow, whereas non mutant cells would be more likely to be killed. Thus, over each repetition, the proportion of cells in the culture having the beneficial mutation would increase, until eventually only the resistant cells remain. As shown in figure 1, cultures on plates containing the original parent strain were unable to survive doses exceeding 3 kJ/m^2. The ability of these cultures to survive increased each successive generation. After seven rounds of mutagenesis and enrichment, resistant populations were able to produce a lawn at a UVA dose of 24.24 kJ/m2.

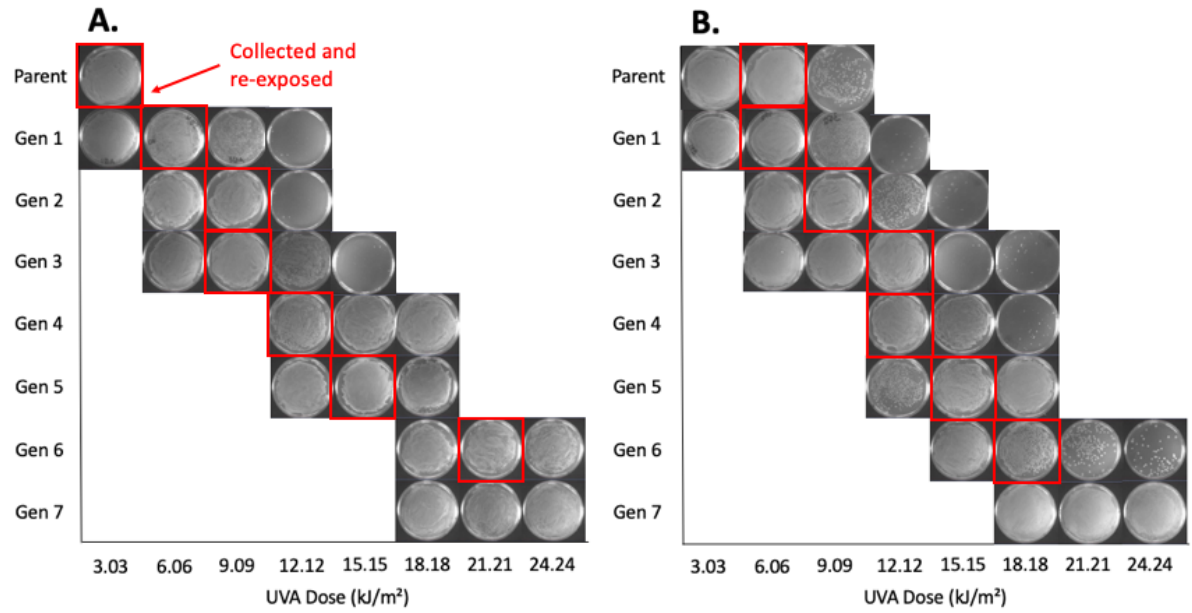

Figure 1. *E. coli* can develop resistance to prosalen- UVA treatment. Two separate cultures (A and B) of wild-type cells were plated on media containing 20 µg/ml 8-methoxypsoralen and exposed to increasing doses of UVA. The cells from the plate that contained the highest dose and still produced numerous viable cells were collected, grown, and the enrichment/selection process was repeated. Over successive rounds, the resistance of the culture increased significantly. Images of the plates with surviving cells at each dose are shown above.

To quantify the level of resistance in the final enriched culture, four cells from each of the two enriched populations were isolated as colonies that grew from the enriched culture. To ensure that the isolated cells were actual mutants of the original *E. coli* strain, and not contaminant of other species or strains, I grew the cells in media lacking thymine. My original parental strain was a thymine auxotroph. One of the of the eight cells that were isolated grew in media lacking thymine (not shown). Since the thymine mutation is a point mutation, it is possible that either this isolate reverted to thy+, or is a containimant that occurred during the enrichment process. Irrespective, this isolate was not further characterized. As shown in Figure 2, each of the isolates following selection exhibited a high degree of resistance to psoralen plus UVA treatment. Whereas the survival parental cell was reduced by more than five orders of magnitude when exposed to 30 kJ/m^2 of UVA radiation in the presence of 10 µg/ml 8-methoxypsoralen, the

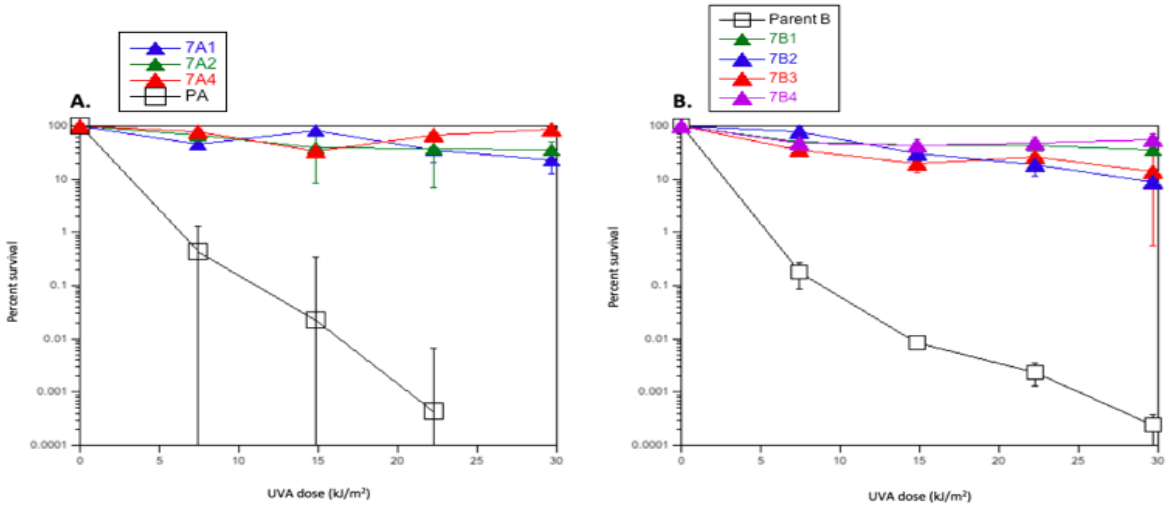

Figure 2. **Several isolates from the selection process displayed extreme resistance to psoralen plus UVA treatment.** The survival of the parents versus each mutant isolate when exposed to PUVA treatment is plotted. 8methoyxpsoralen was used at 20µg/ml. Parental strains A and B are denoted by squares. Isolates after repeated selection are denoted by colored triangles. Graphs represent the average of three experiments. Error bars represent the standard error of the mean.

survival of each isolate was more than 10,000 fold more resistant at this dose. The observations demonstrate that mutations in *E. coli* can confer extreme resistance to at least some forms of cross-linking agents, similar to that seen to develop in specific human cancers.

To begin to characterize the mechanism by which cells gained resistance to psoralen interstrand cross-links, we first examined whether the cells upregulated their ability to repair other forms of DNA damage. Irradiation with UVC produces cyclobutane pyrimidine dimers and 6-4 photoproducts that similarly represent blocks to both transcription and replication, but form intrastrand, rather than interstrand, cross-links (Chan GL et al.,1985; Mitchell & Nairn, 1989; Setlow et al., 1963). Therefore, I sought to determine if the resistance to interstrand cross-linking agents in the resistant isolates also increased their resistance to UVC-induced damage.

Each isolate was also exposed to UVC light to find if any of the isolates had an increased resistance. While most isolates had a similar downward trend as the wild type, isolate 7A1 showed slightly increased resistance compared to the wild type, with survival 100 fold higher

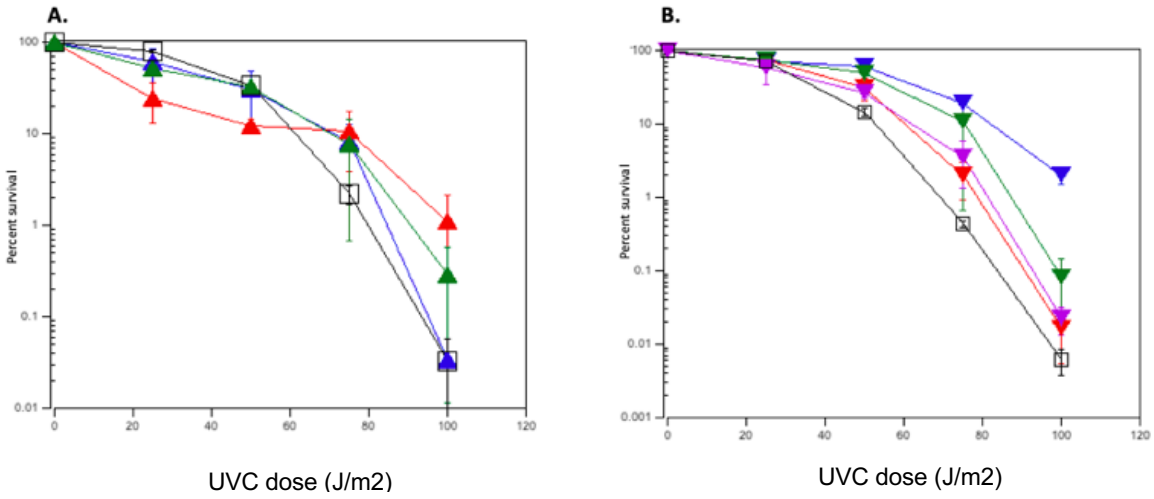

Figure 3. One cross-link resistant isolate exhibited a modest increase in resistance to UVC irradiation. The survival of each isolate and parent when irradiated using 254 nm UVC light is plotted. Parents are denoted by squares, and each isolate is represented by colored triangles. Isolate 7A1, pictured in blue triangles, has slightly increased resistance. The figures represent the average from two independent of experiments. Error bars represent the standard error of the mean.

than the parental cell line at 100 J/m2, the highest dose examined. The increased resistance to UVC could suggest that the isolate contains a mutation that upregulates repair of both forms of lesions. Alternatively, it could suggest that the isolate contains a mutation that upregulates pigmentation which may shield, or partially protect the DNA from wavelengths in the UVC and UVA range. In all other isolates, the resistance appeared to be specific to damage induced by the psoralen plus UVA treatment.

Another possible mechanism by which the isolates could have gained resistance to psoralen plus UVA treatment is if the cells became less permeable to the drug or upregulated transporters that pumped the drug out of the cell. To examine this possibility, we examined whether the resistant mutants failed to form cross-links when irradiated with UVA. Cells containing the plasmid pBR322 were used to assess whether the cross-links formed on the plasmid. Cultures containing the plasmid were incubated with 10µg/ml psoralen and irradiated with increasing doses of UVA. After each dose, total genomic and plasmid DNA was purified

from each culture. The DNA was treated with a restriction endonuclease that would linearize the plasmid. The plasmid was electrophoresed through a denaturing, alkali-agarose gel. Under these conditions, plasmids containing interstrand cross-links are prevented from denaturing by the covalent bonds formed by the psoralen, holding both strands together. These molecules are larger than the single strand fragments that don't contain cross-links, and migrate slower, as a band above the linear fragments (figure 4). To detect the plasmid forms, southern blot analysis was used to determine how many cross-links formed at each dosage using a 32P radioactively labeled plasmid as a probe. The amount of undamaged DNA was compared to the total DNA in each lane to determine the percentage of unmodified DNA at each dose (figure 5). In all isolates examined, resistant isolates formed far less interstrand cross-links than the parental strains, with most DNA remaining undamaged. The results are consistent with the idea that the resistant mutants have mutations that prevent psoralen from interacting with the DNA to form cross-links.

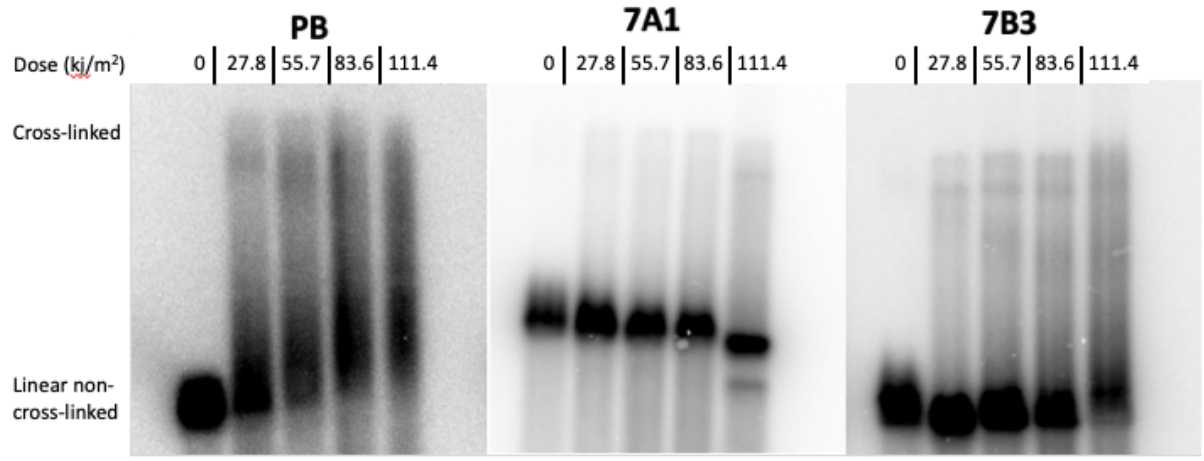

Figure 4. Southern Blot Analysis was used to determine how many cross-links were formed at each dose. Southern blots of samples Parent B, isolate 7A1, and isolate 7B3. Cultures containing the plasmid pBR322 were irradiated in the presence of 10 ug/ml 8MOP. genomic DNA was purfied, digested with a restriction enzyme to linearize the plasmid and electrophoresed in a denaturing agarose gel. Plasmid DNA was then examined using 23P-labeled pBR322 plasmid as a probe. DNA at the bottom of the image shows unmodified DNA, and DNA further up the gel shows damaged DNA. DNA at the top of the image is cross-linked.

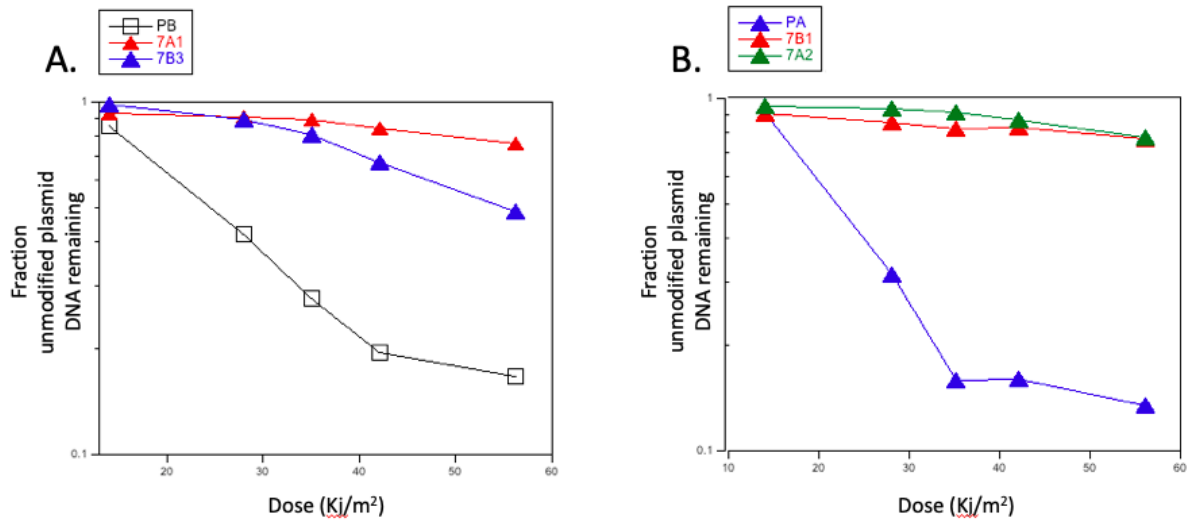

Figure 5. The amount of undamaged DNA was compared to the total DNA in each lane to determine the percentage of unmodified DNA at each dose. The fraction of unmodified plasmid DNA remaining at each UVA dose for several isolates irradiated in the presence of psoralen is plotted. The fraction of unmodified DNA in each case was quantified from blots similar to those shown in figure 4. from data taken from southern blot. Percent of undamaged DNA drops off rapidly in parent strains, while mutant strains remain mostly undamaged. Each plot represents a single experiment in which southern blots were quantified.

Discussion

Through repeated exposure to PUVA treatment, we were able to produce populations of resistant mutants. These populations gained a greater than 10,000-fold increase in resistance when compared to the parent strain. The accumulation of cross-links was also significantly reduced in resistant populations, implying that these strains did gain the ability to repair interstrand cross-links or prevent them from forming in the first place.

The formation of interstrand cross-links is a target for cancer treatment because these lesions are highly lethal to the cell. Resistance to these lesions results in cancer that cannot be treated with these potent chemotherapeutics. Our experiments use *E. coli,* which are functionally similar to mammalian cells in terms of DNA repair and prevention of cross-links.

Though we were able to demonstrate that *E. coli* have the capacity to develop interstrand cross-link resistance, the exact mechanisms remain unknown. One possibility is the activity of

transmembrane pumps removing the 8-methoxypsoralen before UVA exposure causes the interstrand cross-links. Similarly, if the cells became impermeable to the drug, psoralen interactions with DNA would be prevented to confer resistance. Consistent with these possibilities, I observed a significant reduction in cross-link formation in all the resistant isolates I examined.

Another possibility is that the *E. coli* are truly upregulating a mechanism to repair these interstrand cross-links. One isolate appeared to modestly increase its resistance to UVC in addition to PUVA resistance, which would be consistent with an upregulated repair mechanism. However, the increased resistance to UVC was quite modest by comparison to the PUVA resistance observed ,and seemed unlikely to be able to account for the observed phenotype. Further, the repair of interstrand cross-links is not very efficient, and some studies have suggested that an effective mechanism for repairing interstrand cross-links may not be present in *E. coli* (Cole, J.M, J.D Acott, C.T. Courcelle, & J. Courcelle, 2018; Guinazzi & Schärer, 2010). Monoadducts, which affect one base, can be repaired through nucleotide excision. It is possible that repair of these lesions was upregulated in the modestly resistant isolate, as our assay would not differentiate between monoadduct and cross-link repair. Other repair mutants in genes encoding glycosylase and AP endonucleases have shown moderately increased resistance to PUVA treatment, though the mechanisms are still unclear (Couvé-Privat, Macé, Rosselli, & Saparbaev, 2007).

Sequencing the genomes of these mutants and comparing them to the parental strains would be a good next step. Mutations that are identified as different from the parental strains could then be engineered into the parent strain to determine if these mutations are responsible for the resistance I saw in this study.

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