Interstrand Crosslink Resistance in *Escherichia coli*

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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 crosslink. These lesions completely block replication and transcription, and are widely used in chemotherapies; yet how these lesions are processed in the cell remains poorly understood and insight into these processes could lead to better therapies that evade resistance. Previous studies isolated an *Escherichia coli* mutant demonstrating hyper-resistance to interstrand crosslink-inducing agents suggesting that *E. coli* could serve as a model system for understanding crosslink resistance in cancer cells. Here we sought to provide evidence for the genetic capacity of *E. coli* to acquire resistance to the interstrand crosslinking treatment 8-methoxypsoralen-UVA and to determine possible cellular mechanisms of crosslink resistance. Using iteratively 8-methoxypsoralen-UVA treated cells, we isolated strains that were $>10^4$-fold more resistant to this interstrand crosslink-inducing agent compared to the parent strain. We show that the hyperresistant strain does not form or accumulate interstrand crosslinks following increasing exposure to 8-methoxypsoralen-UVA treatment, unlike its parent. Additionally, isolates of this selected strain exhibit a >10-fold increase in resistance to UV-induced monoadducts, a different form of DNA damage. Taken together, these results suggest that the primary mechanism of crosslink resistance in this strain involves a step prior to intracellular accumulation of 8-methoxypsoralen. Enhanced DNA repair capacity may also contribute secondarily to crosslink resistance. Finally, our results suggest that mechanisms of *E. coli* interstrand crosslink tolerance could serve as a model system for understanding the development of drug resistance in human cells.

Introduction

Crosslinking agents are an important class of clinical compounds that have wide use as potent chemotherapeutics and in the treatment of psoriasis and various anemias (Bredberg, Lambert, Lindblad, Swanbeck, & Wennersten, 1983; Gupta & Anderson, 1987). Subsequent to cellular internalization, crosslinking agents first intercalate between DNA bases, and then form covalent bonds with cellular DNA through interactions mediated by the reactive functional groups found on these chemicals. Crosslinking agents may form one or multiple covalent interactions, including monoadduct covalent bonds on a single base; intrastrand crosslinks, forming covalent bonds on the same strand; or interstrand crosslinks, forming covalent bonds on opposing DNA strands (Schärer, 2005). The most damaging interaction, interstrand crosslinks, leads to significant cellular toxicity and cell death by preventing the separation of DNA strands during genome replication and transcription. Cisplatin and carboplatin, mitomycin C, and the psoralens are among the most commonly used crosslinking agents in clinics (Guainazzi & Schärer, 2010). In spite of the successful application of interstrand crosslinking agents in chemotherapy as evidenced by tumor regression, medical professionals have repeatedly documented the emergence of cancers that are resistant to these types of bifunctional drugs (O'Grady et al., 2014). Thus, the development of crosslink resistance in cancer cells represents a major limitation to this therapy and highlights the importance of understanding the cellular mechanisms underlying chemoresistance.

Psoralen as an Interstrand Crosslinking Agent

Psoralen and its derivatives, have been shown to be effective treatments against the integumentary disorders psoriasis, vitiligo, and cutaneous T-cell lymphoma (Arroyo & Tift, 2003; Wackernagel, Hofer, Legat, Kerl, & Wolf, 2006). The three-ring, planar structure of psoralen allows the molecule to interact with DNA through intercalation (Cimino, Gamper, Isaacs, &

Hearst, 1985). Thereafter, irradiation with long-wavelength ultraviolet light (UVA) can result in the formation of covalent psoralen-DNA adducts (Cole, 1970; Dall'Acqua, 1977). While psoralens can photo-react with all pyrimidines, these compounds show a preference for thymine particularly in the 5'TpA sequence context (Dall'Acqua, 1977; Kanne, Straub, Rapoport, & Hearst, 1982). Psoralen-DNA monoadducts are formed through cycloaddition of either the pyrone or furan ring to an adjacent thymine following absorption of one photon of light (Cimino et al., 1985; Kanne et al., 1982). A subset of these monoadducts, furan-side monoadducts, can then be converted to form an interstrand crosslink with a thymine on the opposite DNA strand after absorption of a second photon of UVA light (Sastry, Ross, & P'arraga, 1997).

DNA Interstrand Crosslink Resistance

Previous research has implicated multiple mechanisms to explain the observed interstrand crosslink resistance characteristically seen in recurrent post-chemotherapy cancer cells. Many cancers owe their increased survival to the presence of an upregulated ATP-dependent drug pump that may employ reduced cytoplasmic uptake of the crosslinking drugs, increased efflux from the cell, or both (Gottesman, 2002). Downregulation of drug-specific receptors has been observed as well (Cheung-Ong, Giaever, & Nislow, 2013). Other mechanisms proposed to reduce crosslink formation or increase resistance to crosslinks include increased expression of detoxification genes, and prevention of apoptosis (Huang, Mohanty, & Basu, 2004; D. Wang & Lippard, 2005). In addition, enhanced activity of nucleotide excision repair, base excision repair and translesion DNA synthesis enzymes have also been proposed to be involved in resistance to interstrand crosslinks in human cells through either the removal of these replication-blocking lesions or their bypass (Ho & Schärer, 2010; Kaina & Christmann, 2002; Wilson & Seidman, 2010).

Given the observed similarities in DNA damage processing between *E. coli* and eukaryotic cells, we proposed to develop *E. coli* as a model system for understanding crosslink resistance in cancer cells (Deans & C West, 2011; Kim & Wilson, 2012). As an approach to identify novel genes contributing to interstrand crosslink resistance, we developed a selection system to generate *E. coli* that were resistant to 8-methoxypsoralen and UVA exposure and characterized the resulting strains for their mode of crosslink resistance.

Materials and Methods

Bacterial Strains.

SR108, a *thyA36 deoC2* derivative of W3110 (Mellon & Hanawalt, 1989), was used as the parent for all strains in this study. SR108 *recA*::Tn*10* (HL921) has been previously described (J. Courcelle, Carswell-Crumpton, & Hanawalt, 1997). pBR322 is a medium copy number, Co1E1-based, 4.4-kb plasmid (Promega).

Interstrand crosslink resistance selection.

A fresh overnight culture of SR108 was grown in Luria-Bertani medium supplemented with 10 µg/ml thymine (LBthy) at 37°C. The following day, cells were treated with 20 µg/ml 8-methoxypsoralen for 10 minutes, then 0.1-ml aliquots were plated on LBthy agar plates supplemented with 20 µg/ml 8-methoxypsoralen and subsequently irradiated using two 32-W UVA bulbs (Sylvania), with a peak emittance of 320 nm at an incident dose of 6.9 J/m^2/s for the indicated doses. All surviving colonies at a UVA dose producing incremental resistance to 8-methoxypsoralen-UVA treatment were collected the following day and grown in LBthy medium at 37°C overnight. Resistant populations were then re-exposed to 8-methoxypsoralen and

increasing UVA doses. A portion of culture from each successive selection passage was stored for genome sequencing and further cellular characterization.

Psoralen-UVA survival assays.

Fresh overnight cultures were grown and diluted 1:100 in Davis media supplemented with 0.4% glucose, 0.2% casamino acids, and 10 µg/ml thymine (DGCthy) and grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.3. At this time, 20 µg/ml 8-methoxypsoralen was added to the culture and incubated at 37°C for 10 minutes. Following incubation, 0.1-ml aliquots of each cultures were removed and serially diluted in 10-fold increments in DGCthy medium. Triplicate 10-µl aliquots of each dilution were then spotted on LBthy agar plates supplemented with 20 µg/ml 8-methoxypsoralen. The cells were then UVA-irradiated for the indicated doses. Viable colonies were counted following 37°C incubation overnight to determine the surviving fraction.

To ensure that any phenotype observed was due to psoralen-UVA treatment and not UVA irradiation, overnight cultures were subcultured in DGCthy, serially diluted in 10-fold increments in DGCthy medium as described above, then spotted on LBthy plates before irradiation with the indicated UVA doses. Viable colonies were counted following 37°C incubation overnight to determine the surviving fraction.

UVC survival assays.

Fresh overnight cultures were grown and diluted 1:100 in DGCthy and grown at 37°C to an OD_{600} of 0.3. Following incubation, 0.1-ml aliquots of each cultures were removed and serially diluted in 10-fold increments in DGCthy medium. Triplicate 10-µl aliquots of each dilution was then spotted on LBthy agar plates. The cells were then irradiated with UVC light using a 15-W germicidal lamp (Sylvania, 254 nm) at an incident dose of 0.9 $J/m^2/s$ for the

indicated doses. Viable colonies were counted following 37°C incubation overnight to determine the surviving fraction.

In vivo detection of DNA interstrand crosslinks.

Cultures containing the plasmid pBR322 were grown overnight in DGCthy supplemented with 50 μg/ml ampicillin at 37°C. A 0.1-ml aliquot of this culture was pelleted and resuspended in 10-ml DGCthy medium without ampicillin and grown in a 37°C shaking water bath to OD_{600} of 0.4. Cultures were treated with 20 μg/ml 8-methoxypsoralen for 10 min at 37°C and subsequently irradiated with increasing doses of UVA light. At the indicated doses, 0.75-ml aliquots were collected and transferred to an equal volume of ice-cold 4x NET buffer (10 mM Tris, pH 8.0; 40 mM EDTA, pH 8.0; 400 mM NaCl).

Cells were pelleted, resuspended in 130-μl lysis buffer (1 mg/ml lysozyme, 0.5 mg/ml RNaseA in 10 mM Tris, 1 mM EDTA, pH 8.0) and incubated at 37°C for 30 min. Then, 10 μl of 10 μg/ml proteinase K and 10 μl of 20% Sarkosyl were added to the samples, and incubation continued for 1 hr at 37°C. Samples were extracted with four volumes of phenol:chloroform, followed by two volumes of chloroform, and then dialyzed against 200 ml of 1 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0) for 45 min using 47-mm Millipore 0.025-μm pore disks. The DNA was digested with PvuII (Thermo Fisher) overnight at 37°C to linearize the plasmid. Samples were electrophoresed on a 0.75% alkaline agarose gel in 30 mM NaOH and 1 mM EDTA at 30 V for 16 h. DNA in the gels was then transferred to Hybond N+ nylon membranes (GE Healthcare) using standard Southern blotting techniques. The plasmid DNA was detected by probing with ^{32}P-labeled pBR322 that was prepared by nick translation (Roche) using >6000 Ci/mmol [α^{32}-32P]dCTP (PerkinElmer). Southern blots were visualized using a Storm 840 phosphoimager (GE Biosciences) and its associated ImageQuant analysis software.

The fraction of 8-methoxypsoralen crosslinks 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 crosslinks in untreated samples.

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

where XD represents crosslinked DNA and TD represents total DNA.

Results

E. coli have the genetic capacity for interstrand crosslink resistance.

To determine whether *E. coli* encode genes that confer resistance to interstrand crosslinks, we developed a random mutagenesis and functional selection scheme to generate *E. coli* strains that were resistant to 8-methoxypsoralen and UVA exposure. Beginning with wild-type cells, we iteratively exposed cultures to UVA irradiation in the presence of 8-methoxypsoralen, each time selecting for increasingly resistant cells to treat in subsequent generations. For each round of selection, cultures were exposed to incremental doses of UVA irradiation in the presence of 8-methoxypsoralen and surviving colonies were then collected in bulk from plates exposed to a UVA dose that produced lethality in most, but not all of the growing cells (Figure 1A). Using nine rounds of this selection protocol, we were able to generate a population of cells that were resistant to and produced a lawn at a UVA dose of 28.32 kJ/m^2 compared to a lawn growth at 3.54 kJ/m^2 UVA light for the parent strain.

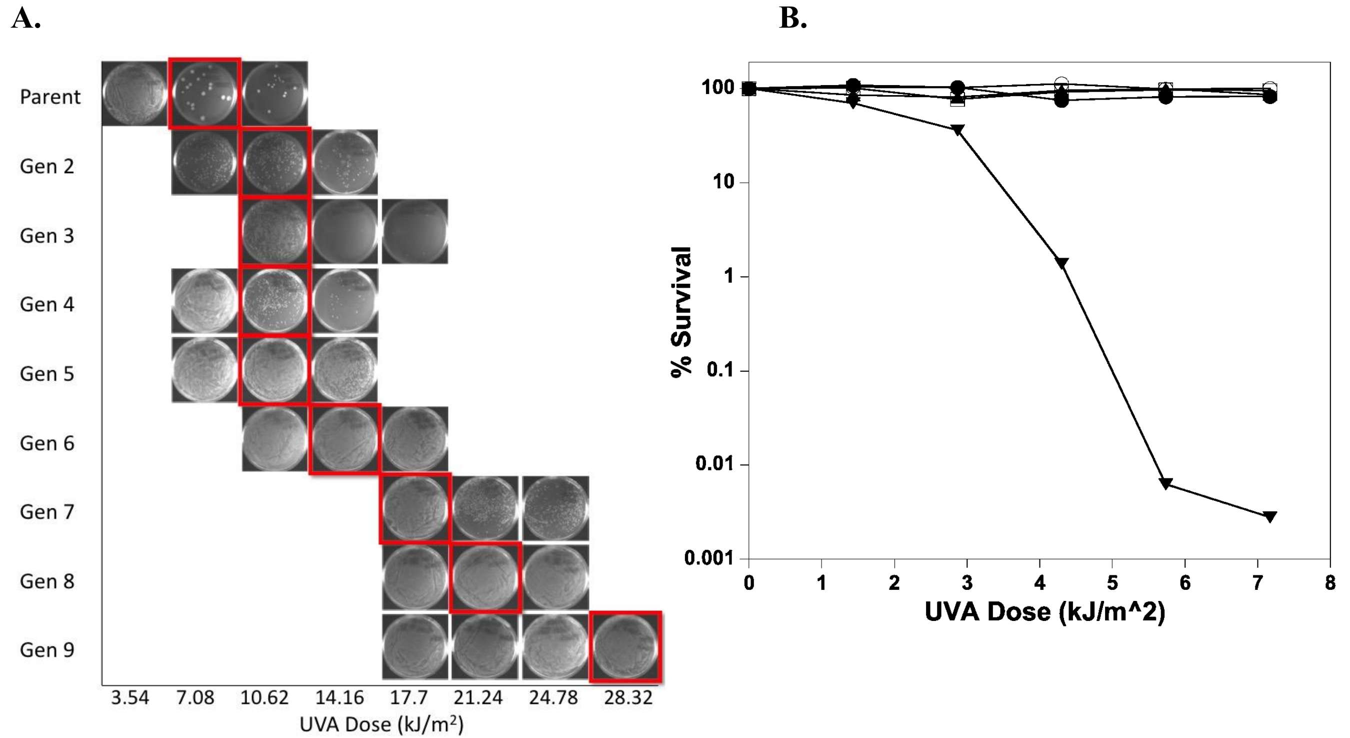

Figure 1. Wild-type cells exposed to successive rounds of 8-methoxypsoralen-UVA treatment develop resistance to this agent. A) Survival of cells exposed to 8-methoxypsoralen-UVA over several rounds of selection. Top row shows viability of parental strain; subsequent rows below show viability over successive generations. Red box denotes the population that was collected, propagated and used for selection in the next round. B) The survival of parent (filled upside-down triangles), Gen 6 isolate 1 (filled triangles), Gen 6 isolate 2 (filled circles), Gen 6 isolate 3 (open circles) and Gen 6 isolate 4 (open squares) cells after exposure to 20 µg/ml 8-methoxypsoralen and the indicated doses of UVA is plotted. The graph represents the results from one independent experiment.

To directly quantify the resistance of these selected mutants to 8-methoxypsoralen and UVA treatment, we isolated four individuals at random from selection round 6 (Gen 6) and examined their ability to survive exposure to this agent. All four individuals from this sixth selection round were hyperresistant to UVA irradiation in the presence of 8-methoxypsoralen and their viability was unaffected by any of the UVA doses we used in this experiment (Figure 1B). At the highest UVA doses applied (6 kJ/m^2 and higher), all four isolates displayed $>10^4$-fold increased viability compared to the parent strain. We interpret these results to indicate that *E. coli* cells have the genetic capacity to develop resistance to crosslink-inducing agents and by extension to interstrand crosslinks.

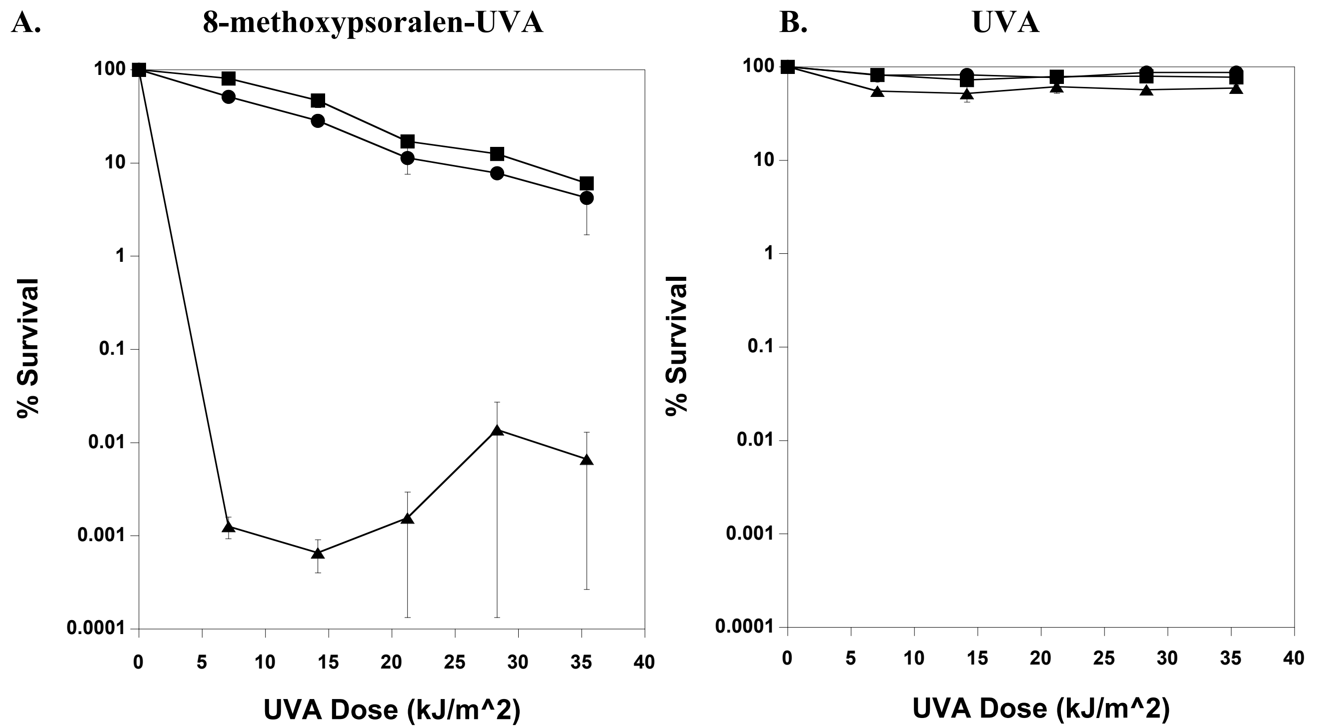

Figure 2. Gen 6 isolates have an extended survival capacity in the presence of DNA interstrand crosslinks. A) The survival of parent (filled triangles), Gen 6 isolate 1 (filled squares) and Gen 6 isolate 2 (filled circles) after exposure to 20 µg/ml 8-methoxypsoralen and the indicated doses of UVA is plotted. B) The survival of parent (filled triangles), Gen 6 isolate 1 (filled squares) and Gen 6 isolate 2 (filled circles) after exposure to the indicated doses of UVA in the absence of 8-methoxypsoralen is plotted. The graphs represent the results from two independent experiments. Error bars represent SEM.

Our data suggested that Gen 6 individuals are able to tolerate doses of UVA irradiation up to 7.08 kJ/m^2 in the presence of 8-methoxypsoralen without detriment to cell viability. We next examined whether there was an upper limit to the ability of these strains to survive in the presence of DNA interstrand crosslinks by extending the range of UVA exposure. To determine the extended capacity for 8-methoxypsoralen-UVA resistance in Gen 6 isolates 1 and 2 directly, we compared the survival of these cells following irradiation with UVA doses up to five times greater than we previously used in the presence of 8-methoxypsoralen. As shown in Figure 2A, Gen 6 isolates 1 and 2 were only modestly sensitive to DNA interstrand crosslinks at the highest dose of UVA irradiation (35.4 kJ/m^2) used and displayed ~10-fold decrease in cell viability compared to untreated cells. In contrast, the viability of parental cells was reduced by 10-fold at a UVA dose of 4 kJ/m^2 (see Figure 1B). Compared to parental cells, Gen 6 isolates 1 and 2 showed $>10^3$ increased viability at the highest UVA doses used (21.24 kJ/m^2 and higher). This result suggests that Gen 6 isolates have an extended survival capacity for DNA interstrand crosslinks.

UVA irradiation has previously been shown to generate oxidative damage to cellular DNA (Santos et al., 2013). To confirm that the hyperresistance we observed in Gen 6 isolates was due to DNA interstrand crosslinks and not to oxidative DNA damage, we determined the survival of parental and Gen 6 cells following UVA irradiation. As show in Figure 2B, neither parental nor Gen 6 cells were hypersensitive to UVA irradiation in the absence of 8-methoxypsoralen. This result indicates that the hyperresistance we observe for Gen 6 isolates is specific for DNA interstrand crosslinks induced by 8-methoxypsoralen-UVA treatment.

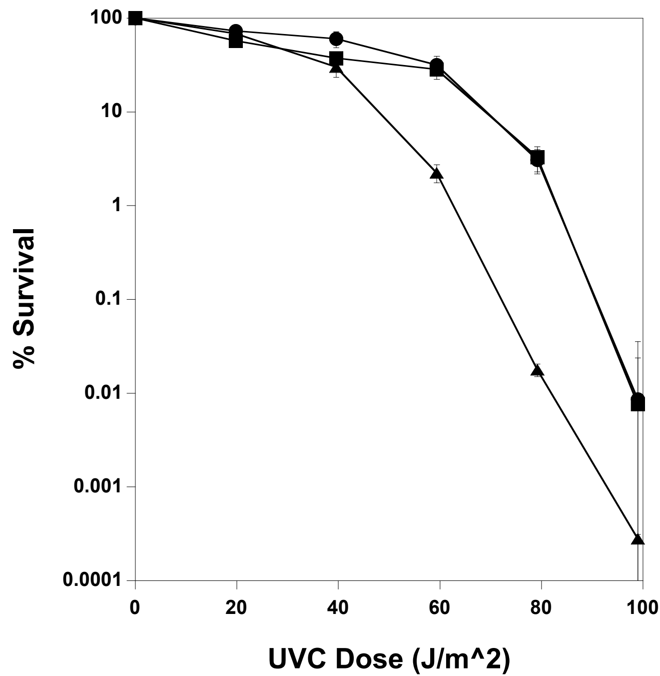

Figure 3. Gen 6 isolates are modestly resistant to UV-induced DNA lesions. The survival of parent (filled triangles), Gen 6 isolate 1 (filled squares) and Gen 6 isolate 2 (filled circles) after exposure to the indicated doses of UVC irradiation is plotted. The graph represents the results from two independent experiments. Error bars represent SEM.

Taken together, our results suggest that *E. coli* is able to acquire resistance to DNA interstrand crosslinks. In human cells, enhanced DNA repair of replication-blocking lesions has been proposed to mediate DNA interstrand crosslink resistance (Ho & Schärer, 2010). If this same pathway operates in *E. coli* to confer interstrand crosslink resistance, then we predicted that Gen 6 isolates would exhibit greater resistance to an unrelated replication-blocking lesion. To address this possibility directly, we compared the survival of parental and Gen 6 isolates following UVC (254 nm) irradiation. UVC irradiation induces cyclobutane pyrimidine dimers and (6-4) photoproducts, which block the progress of DNA polymerases.

Following exposure to UVC light, wild-type cells and Gen 6 isolates revealed similar levels of survival at moderate doses (40 J/m^2 and lower, Figure 3). At higher doses of UVC (60 J/m^2), Gen 6 isolates exhibited increased resistance to UVC irradiation compared to wild-type. However, this resistance was >10-fold higher than the parental strain and was modest in effect compared to what was observed following DNA interstrand crosslink induction (>10^3 to 10^4). This result suggests that while increased DNA repair capacity might contribute to DNA interstrand crosslink resistance, it is not the primary mechanism by which Gen 6 isolates survive these lesions.

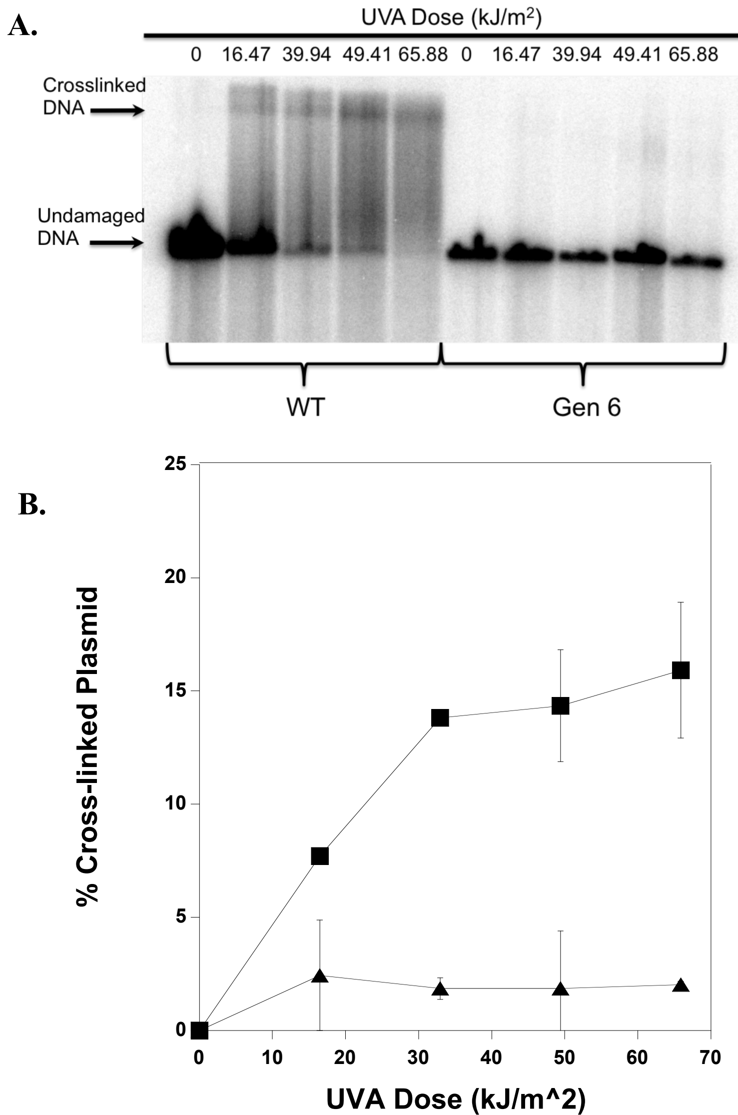

Figure 4. Gen 6 cells do not accumulate DNA interstrand crosslinks. A) DNA interstrand crosslink formation as a function of dose. Wild-type (WT) and Gen 6 isolate 1 cells containing plasmid pBR322 were UVA-irradiated in the presence of 10 µg/ml 8-methoxypsoralen. To observe crosslink formation, total genomic and plasmid DNA was purified from cells at each dose, restricted with PvuII to linearize the plasmid, and analyzed by Southern blot following alkali gel electrophoresis using ^{32}P-labeled pBR322 as a probe. A representative blot is shown. B) The percent of crosslinked plasmid DNA at each dose is plotted for WT (filled squares) and Gen 6 isolate 1 (filled triangles). Graphs represent the average of two experiments. Error bars represent SEM.

Alternative models propose increased efflux or reduced uptake of crosslinking agents by cells as a route to DNA interstrand crosslink resistance (Damia & D'Incalci, 1998; Gottesman, 2002). In this scenario, cells exhibit resistance to DNA interstrand crosslinks as a result of decreased intracellular accumulation of crosslinking agents and consequently reduced or absent crosslink formation. To determine whether the hyperresistance in Gen 6 isolates arises by this mechanism, we quantified crosslink formation on an endogenous plasmid (pBR322) in Gen 6 and wild-type cells in vivo as a function of dose. pBR322 is a medium copy number plasmid, which increases the sensitivity of our crosslink detection assay over a similarly sized genomic region. Gen 6 and wild-type cultures containing the plasmid pBR322 were grown in media containing 10 µg/ml 8-methoxypsoralen and UVA irradiated with increasing doses. Total (genomic and plasmid) DNA was purified, linearized and separated on an alkali agarose gel. Southern blot analysis was employed to directly identify and quantify the crosslinks formed at each dose (Perera, Mendenhall, Courcelle, & Courcelle, 2016). In wild-type cells, increasing UVA doses resulted in increasing amounts of crosslink formation (Figure 4). At the highest UVA dose (65.88 kJ/m^2), all the plasmid DNA in wild-type cells was observed to migrate as damaged DNA and approximately 20% of the plasmid DNA was found to be crosslinked. In contrast, the plasmid DNA contained in the Gen 6 isolate remained relatively crosslink-free even at the highest dose of UVA irradiation (65.88 kJ/m^2, Figure 4). This result supports the idea of an active transport mechanism removing 8-methoxypsoralen from cells prior to DNA interaction in Gen 6 cells. However, it does not exclude the possibility that DNA interstrand crosslink resistance in Gen 6 cells is due to the activities of multiple cellular pathways.

Discussion

The purpose of this study was to determine whether *E. coli* are capable of acquiring resistance to DNA interstrand crosslinks. Using iterative rounds of 8-methoxypsoralen-UVA treatment and selection, we generated cell populations capable of surviving a UVA dose that was ~10-fold higher than that tolerated by the parent strain. These cells exhibited >10^4-fold increased resistance by UVA dose compared to their progenitor, suggesting that *E. coli* do indeed contain the genetic capacity to acquire interstrand crosslink resistance. Interstrand crosslink resistance in this strain correlated with a significantly reduced accumulation of crosslinks in cells challenged with 8-methoxypsoralen-UVA treatment and a modest increase in resistance to other forms of DNA damage.

Due to the significant cellular toxicity resulting from the inhibition of replication and transcription by interstrand crosslinks in all cells, agents that induce these lesions have been widely adopted as chemotherapeutics and resistance to these covalent linkages have become an important area of study (Deans & C West, 2011). Our observation that *E. coli* can acquire interstrand crosslink resistance following repeated exposure demonstrates that this bacteria may be a good model system for understanding the development of interstrand crosslink resistance or tolerance over time. If this is the case, the functional homology between metabolic processes in *E. coli* and mammalian cells would suggest that similar pathways may be exploited by cancer cells to accumulate resistance to crosslinking chemotherapeutic agents over the course of cancer treatment, ultimately leading to cancer recurrence (Guainazzi & Schärer, 2010).

We tested the idea that enhanced DNA repair might account for increased resistance to crosslinking agents. While the exact cellular mechanisms responsible for increased cell survival to crosslinking agents remains unknown, recent studies have implicated enzymes such as Cho

endonuclease, along with the UvrAB complex, to be involved in interstrand crosslink repair mechanisms, independent from DNA monoadducts (Perera, Mendenhall, Courcelle, & Courcelle, 2016), supporting this hypothesis. Additionally, it seemed plausible that two alternatively proposed repair mechanisms for DNA interstrand crosslinks involving the nucleotide excision repair/lesion bypass (X. Wang et al., 2001); and the nucleotide excision repair/translesion DNA synthesis pathways (Berardini, Foster, & Loechler, 1999; Kumari et al., 2008) could be subject to upregulation in the hyperresistant strain. We found that Gen 6 isolates were >10-fold more resistant to UVC-induced DNA lesions than their parent, however this effect was modest compared to the $>10^4$-fold increase in resistance we observed using our crosslinking agent.

Another plausible mechanism of the DNA interstrand crosslink resistance observed in this study is transmembrane pump activity, which could remove 8-methoxypsoralen from *E. coli* cells prior to DNA intercalation and UVA exposure. The multiple drug resistance protein in cancer stem cells has been frequently documented to remove toxic agents from cells prior to DNA alterations, thus contributing to chemotherapy resistance and reduced crosslink formation (reviewed in DI & ZHAO, 2015). In support of this type of cellular mechanism, human hepatocytes have been shown to potently induce the oxidative and hydrolytic drug-clearance enzymes cytochrome P450 3A4 and carboxylesterase 2 following induction of crosslinks by 8-methoxypsoralen (Yang & Yan, 2007). We examined whether decreased uptake or increased efflux of 8-methoxypsoralen is responsible for all or some of the hyperresistance observed by monitoring the accumulation of DNA interstrand crosslinks over increasing UVA doses. We infer from the absence or reduction in accumulation of interstrand crosslinks in the hyperresistant strain, but not the wild-type cells, that an active transport mechanism is the primary mode of interstrand crosslink resistance. We favor an active transport mechanism over enhanced removal

of interstrand crosslinks as we observed a steady state level of crosslinked DNA across all doses we examined.

Although our results would suggest that an active transport mechanism is the main cellular route to interstrand crosslink resistance in our selected strain, it remains possible that synergy between this pre-target resistance pathway and enhanced DNA repair results in the hyperresistance we observe. Nucleotide excision repair enzymes are responsible for the recognition and removal of a wide variety of bulky lesions derived from different environmental agents (reviewed in Kisker, Kuper, & Van Houten, 2013; Schärer, 2013). Since psoralen-UVA-induced interstrand crosslinks are the product of a continuum of DNA lesions beginning with a monoadduct, increased repair of the first lesion formed could reduce the ultimate formation of interstrand crosslinks. When coupled with a robust transport mechanism that limits intracellular accumulation of bifunctional compounds, enhanced repair of any monoadducts that result from trace entry of psoralen could effectively prevent DNA interstrand crosslink formation and facilitate resistance. Construction of mutants deficient in the recognition step of nucleotide excision repair (function of *uvrA* gene product) in the hyperresistant genetic background may allow for more conclusive identification of such a role for DNA repair in crosslink resistance. If increased repair of 8-methoxypsoralen-bound DNA is responsible for the crosslink-resistant phenotype, then inhibition of the involved complex should cause a reversion in survival towards that seen in repair-deficient mutants.

Finally, our Gen 6 isolates were the product of a single selection experiment and it is likely that we selected and purified only a small subset of crosslink resistance traits early in our trial. It will be important to perform several independent selection experiments and isolate multiple hyperresistant strains for characterization. Additionally, sequencing the genomes of Gen

6 and similar hyperresistant strains may reveal what genes and pathways function in DNA interstrand crosslink processing, tolerance and resistance.

Importantly this study confirmed that *E. coli* do possess the capacity for interstrand crosslink resistance within their genomes. Given the functional conservation between cellular processes in *E. coli* and mammals, further characterization of the genes affected in the hyperresistant strain may provide insights into the cellular pathways involved in DNA interstrand crosslink processing and development of drug resistance.

Acknowledgements

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