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Resistance to DNA Interstrand Crosslinks in *Escherichia coli* Arises through Prevention Rather than Repair

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by

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Abstract

DNA interstrand crosslinks are particularly lethal lesions that form in DNA when certain molecules intercalate between complementary strands of DNA and form covalent bonds with both strands. Once formed, these lesions present an absolute block to replication and transcription, ultimately resulting in cell death. Because of this lethality, chemicals that form DNA interstrand crosslinks are found in nature as defensive chemicals produced by plants and microbes. Moreover, crosslinking agents have proven effective the treatment of dysplastic conditions and are often first line chemotherapeutics.

However, cancer cells can become resistant to DNA interstrand crosslinks. Unlike other DNA lesions, the double-stranded nature of interstrand crosslinks prevents utilization of one strand as a template for the other strand's repair. Several complex models have been proposed for how a cell may repair interstrand crosslinks, typically involving the sequential contribution of multiple repair pathways. However, these models remain speculative, and the capacity for repair is limited, potentially as low as a single lesion in *Escherichia coli*, making the question of how cells acquire crosslink resistance a clinically and intellectually important one to address.

In this thesis, I investigate how crosslink resistance develops. In Chapter I, I review historical studies on crosslinking agents and possible mechanisms by which resistance to these agents could arise. In Chapter II, I identify causal mutations in *rpoA* and *acrR* leading to psoralen-UVA resistance *E. coli* and begin to characterize their mechanism of action. In Chapter III, I characterize the regulation of AcrAB-TolC, one of the primary drivers of resistance, and describe how global regulatory elements impact its

expression and influence resistance to psoralen-UVA. Finally, in Chapter IV, I conclude with the implications of these findings and suggest future avenues of research.

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Chapter I: Introduction

To maintain genome stability, cells must be able to respond to the continual formation of a wide variety of DNA lesions. Many of the better characterized DNA lesions, such as the pyrimidine dimers produced by ultraviolet light, typically affect only a single strand of DNA (1–3). This allows the cell to remove the offending lesion and use the unaffected complementary strand as a template for synthesis of new DNA (3–6). However, lesions that affect both strands of DNA simultaneously preclude the use of these repair processes and require alternative strategies. Of these, DNA interstrand crosslinks are particularly challenging to deal with, and much remains unknown about cells process these lesions (7).

DNA interstrand crosslinks are formed by the covalent bonding of a single molecule to both complementary strands of DNA (8–13). The exact conformation of these crosslinks varies, but their formation depends on the ability of the crosslinking molecule to first intercalate between the strands of DNA (8–13). As such, molecules capable of forming DNA interstrand crosslinks are typically planar and hydrophobic to facilitate intercalation (14). Once intercalated, covalent bonds are formed with opposing nucleotides through varying mechanisms, creating a crosslink (8–13). Psoralen, the crosslinking agent used in this study, relies on the absorption of photons from the UVA spectrum to form bonds with the DNA (12, 13, 15, 16). The strong linkage formed by these molecules prevents the separation of complementary strands of DNA and thus presents a block to the essential cellular processes of replication and transcription,

eventually leading to cell death (7, 17). This lethality is the hallmark of these compounds and defines their roles in both nature and in human use.

In nature, crosslinking agents are found as secondary metabolites produced by plants and microbes as defensive toxins (18, 19) (20). Psoralens are produced by several families of plants, including figs, citrus, and many members of the carrot family, and are particularly notable for causing phototoxicity (13, 21, 22). Psoralens, and other furanocoumarins, have been suggested to be produced primarily as a defense against fungal pathogens, but they have been demonstrated to exhibit significant antimicrobial activity against bacteria and viruses as well (14, 18, 23–25). Some have even suggested that psoralens serve a role in modulating plant growth at certain life stages (14), though it is unclear how the plants producing these compounds would avoid their toxic effects. Another well-known class of crosslinking agents, the mitomycins, are toxins produced by bacteria in the genus Streptomyces (26, 27). The specificity of these compounds towards the sequence CpG, which is found more commonly in bacteria than in other organisms, along with their significant antimicrobial activity, suggests that the mitomycins serve primarily in microbe-microbe competition (10, 28, 29). For these reasons, mitomycins are considered antibiotics (26, 27, 30).

These substances have had a significant impact on human medicine. The ancient Egyptians and ancient Indians discovered the photosensitizing properties of the psoralens and used them to treat vitiligo and other skin conditions (31). Moreover, the phototoxicity caused by psoralens and other furanocoumarins later became infamous for causing dermatitis in celery workers (32, 33) and furanocoumarins have also been implicated as

the primary cause of the grapefruit effect, in which grapefruit juice interferes with the action of several common medications (34–37). The actual mode of action of crosslinking agents would not be discovered until after the 1940's and 1950's, when researchers in noticed that nitrogen mustards, derivatives of the mustard gas used in the World War 1, were remarkably effective in killing actively growing tumors (38, 39, 39–41). The finding that crosslinking agents were more effective at killing cells when they were rapidly dividing quickly led to their adoption as chemotherapeutics and prompted further investigations into the underlying mode of action.

Today, psoralens, mitomycin C, and derivatives of the nitrogen mustards are clinically important treatments for a wide range of dysplastic conditions in which their cytotoxicity is put to use for the destruction of abnormal and potentially malignant cells (42, 42–45). In particular, their potency as antitumor agents led to them becoming first line chemotherapeutics (46–52). Psoralen, in addition to its uses in treating psoriasis and vitiligo, is utilized in the treatment of cutaneous T-cell lymphoma (44, 49, 53, 54). Mitomycin C is employed against non-small cell lung cancer (54). Cisplatin, a later development and well-known synthetic crosslinker, can be curative for testicular cancers (46, 54). Despite the lethality of interstrand crosslinks, cancer cells have demonstrated the ability to become resistant to crosslinking agents (55–59). However, much remains unknown about how this resistance arises, which is the focus of this thesis.

One of the most common hypotheses for how interstrand crosslink resistance could arise is through upregulation of repair. However, as mentioned earlier, the fact that DNA interstrand crosslinks affect both strands simultaneously means that repair of these

lesions would be more complex and cannot occur through simple excision and resysnthesis. In 1965, Kohn et al used alkaline CsCl gradients to demonstrate that DNA interstrand crosslinks would disappear over time from the DNA of *E. coli* recovering following treatment with nitrogen mustards (60). These authors postulated that although perhaps not sufficient, lesion excision remained the initial step in a repair pathway (60). They also noticed that the disappearance of crosslinked DNA correlated with the ability of strains to remove UV-induced thymine dimers, implying that the process responsible for crosslink may be analogous to that for the removal of UV lesions (60). Nearly simultaneously, Brookes and Lawley obtained similar results using chromatography to monitor the loss of these lesions and suggested, based on cross-sensitivity of UV sensitive mutants to nitrogen mustards, that the endonuclease responsible for removal of UV lesions also acted on nitrogen mustard lesions (61). It was later confirmed that the *uvrABC* excinuclease complex, which is the major component of the nucleotide excision repair pathway responsible for repair of UV lesions, could recognize interstrand crosslinks and make dual incisions around the lesions (62).

Following the discovery that interstrand crosslinks could be excised from DNA (62), R.S. Cole et al used CsCl density profiles and alkaline sucrose sedimentation profiles to monitor the formation of DNA products following treatment with psoralen-UVA and proposed that excised DNA was replaced via strand exchange by the homologous recombination protein *recA* (63). Based on this, they proposed a basic model by which interstrand crosslinks could be repaired (63) in which the *uvrABC* complex initially makes dual incisions on one strand containing the crosslink. Then, an unspecified

nuclease removes additional bases on one side of the lesion to expose a length of single-stranded DNA that would be sufficient to permit *recA*-mediated homologous recombination to fill in the gapped DNA created opposite to the lesion. Once recombination is complete, another round of excision by *uvrABC* from the second DNA strand could release the still dangling lesion and allow repair synthesis by Pol I to fill the gap and restore the integrity of the DNA. Since this model's proposal in 1973, other related models pathway have been described, all of which use multiple existing repair pathways which intermediates would expected operate sequentially, involving excision followed by recombinational gap repair, translesion synthesis, or double strand break repair pathways to further the reaction to a repairable intermediate (64–69).

Many of these models were derived from experiments *in vitro*, based on the known properties of purified enzymes (70–73). However, *in vivo* evidence for these pathways and intermediates was generally lacking and remained speculative. To begin to examine these possible models, J. Cole et al recently examined the contribution of key genes in the possible crosslink repair mechanisms *in vivo* by testing the sensitivity of *E. coli* deletion mutants to interstrand crosslinks (74). Importantly, the researchers pointed out that all known crosslinking agents also produce a variety of other forms of DNA damage, including monoadducts, which refers to lesions that are attached to a single strand in contrast to interstrand crosslinks (8, 75, 76) The formation of psoralen interstrand crosslinks, for example, proceeds through a monoadduct intermediate on one strand of DNA before the second covalent bond is formed with the complementary strand (16, 75) (77). Though interstrand crosslinks have been shown to be the primary drivers of

cytotoxicity (7, 78, 79), monoadducts and other forms of DNA damage induced by crosslinking agents also contribute to toxicity if they are unable to be removed by the cell (7, 16, 75, 78, 80, 81).This means that hypersensitivity of a specific repair mutant does not necessarily indicate that the gene product contributes to interstrand crosslink repair, complicating the interpretation of these types of observations.

As such, the researchers decided to compare the survival of various mutants following treatment with psoralen, to those treated with angelicin, a structurally related furanocoumarin that can only form monoadducts (77). After testing the survival of deletion mutants for key genes in nucleotide excision repair, homologous recombination, translesion synthesis, and double strand break repair, J. Cole et al found that none of the tested mutants showed specific sensitivity to interstrand crosslinks and concluded that the mechanisms believed to operate in the repair of interstrand crosslinks had a limited contribution to this process (74). In support of this finding, they also used an alkali gel and Southern blot analysis to quantify the number of crosslinks that correlated with lethality in wild-type *E. coli* and found that a single interstrand crosslink in the genome was sufficient for lethality, indicating that the overall repair capacity of the cell for interstrand crosslinks was extremely limited (74). These findings echoed those of Szybalski and Iyer, who similarly found that a single mitomycin C-induced crosslink was lethal to *E. coli*. (28). Additionally, though there are few studies that address the number of crosslinks required for lethality in human cells, existing estimates are between 200 – 900 crosslinks (17, 82–84). Given that the human genome is 1000-fold larger than that of

E. coli and consists of less than 3% essential sequence (85, 86), it seems likely that the capacity for repair of interstrand crosslinks is similarly limited in humans.

The lack of an efficient repair mechanism raises the question of how resistance to clinically important crosslinking drugs arises. One possibility is that resistant cells drastically upregulate existing general repair mechanisms such that the repair capacity of the cells becomes sufficient to keep up with the formation of these lesions. For example, significant upregulation of psoralen monoadduct excision by *uvrABC* or other enzymes could theoretically prevent the formation of lethal DNA interstrand crosslinks, leading to increased resistance. As most of the proposed pathways for interstrand crosslink repair rely on nucleotide excision as the initial step for processing of these lesions in addition to its role in repairing monoadducts, upregulation of this process seems the most likely means by which repair could result in crosslink resistance. However, it remains possible that a novel repair mechanism could also emerge given the strong selective pressure applied by crosslinking agents.

It is also important to consider various non-repair mechanisms that could contribute to resistance. One of the simplest mechanisms would be to prevent the crosslinking drug from entering the cell by altering the permeability of the cell membrane. In *E. coli*, which is the model organism used in this study, control of membrane permeability is often achieved through regulation of two major porin proteins, OmpF and OmpC, that create open pores in the outer membrane (87, 88). These porins proteins are believed to be relatively non-selective (89) and allow the passage of a wide variety of small molecules, including many drugs (90–92). As such, downregulation of

these porins reduces the number of openings in the outer membrane through which drugs may pass. This general mechanism is often seen in resistance to antibiotics and has been implicated as a contributor to multi-drug resistance (91, 93–95).

Another broad mechanism that could be acting in resistant cells is the chemical inactivation or metabolic degradation of the drug either within the cell or in the extracellular environment. Though a wide range of examples of such mechanisms exists, one of the best-known is the production of beta-lactamase enzymes by bacteria (96). These enzymes break down the beta-lactam ring that is the hallmark of many important antibiotics, such as pencillin, thus inactivating the drug (96, 97). Though no known enzymes act on crosslinking agents this way, *Streptomyces lavendulae*, which produces mitomycin C, employs another means of inactivation to protect itself from the lethal effects of its own toxin. It produces a protein, MRD, which selectively binds to mitomycin C and prevents the reduction reaction necessary for its activation (98). Currently, this particular mechanism has only been observed in *Streptomyces* that produce mitomycin, but it is easy to imagine how a similar mechanism could emerge in other species.

Finally, it is important to note that the resistance of *Streptomyces* to mitomycin is also dependent on rapid active transport of the drug out of the cell by the protein MCT (99). In addition to changes in membrane permeability, upregulation of drug transport out of the cell, known as efflux, is one of the most common means by which multi-drug resistance arises (91, 100–102). In *E. coli*, there are several efflux pumps that can contribute to drug resistance, though the AcrAB efflux pump is believed to have the

greatest influence on multi-drug resistance (103, 104). Though part of a different family than AcrAB, the P-glycoprotein efflux pump present in humans and a wide range of other organisms has similarly been implicated as a major contributor to multidrug resistance, including in cancers (105–108)

In *E. coli*, many of the mechanisms of drug resistance described above are coregulated by three tightly interconnected regulons that make up a global stress response pathway that governs resistance to a range of stressors, including antibiootics and oxidative damage (109–111). This pathway depends on the action of three global effector proteins MarA, SoxS, and Rob. Each protein is upregulated in response to different signals: MarA is upregulated when its local regulator MarR recognizes salicylate and related compounds (112, 113); SoxS depends on activation by SoxR upon recognition of superoxide, a reactive oxygen species (114, 115); Rob contains its own sensing domain and primarily responds to bile salts (116, 116). Despite the differences in the induction of these proteins, MarA, SoxS, and Rob share around 50% sequence identity and are capable of regulating the same set of approximately 50 genes (117), including AcrAB-TolC (118, 119) and OmpF (120), that contribute to resistance to various toxic compounds (109, 114, 121). Given the wide reach of the Mar/Sox/Rob pathway (117), one or more of the stress resistance operons it regulates could be responsible for resistance to DNA interstrand crosslinks.

Presently, it is unclear which if any of the mechanisms described here are responsible for resistance to DNA interstrand crosslinks, but the importance of crosslinking drugs as chemotherapeutics makes the issue of resistance especially

pressing. For this reason, elucidating how resistance to DNA interstrand crosslinks develops is the focus of this study. Here, I describe how highly psoralen-UVA resistant strains of *E. coli* were generated and used to demonstrate how this resistance arises.

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Chapter II

Mutations in AcrR and RNA Polymerase Confer High-Level Resistance to Psoralen-UVA Irradiation

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Chapter II: Mutations in AcrR and RNA polymerase confer high-level resistance to psoralen-UVA irradiation.

Abstract

DNA interstrand cross-links, such as those formed by psoralen-UVA irradiation, are highly toxic lesions in both humans and bacteria, with a single lesion being lethal in *Escherichia coli*. Despite the lack of effective repair, human cancers and bacteria can develop resistance to cross-linking treatments, although the mechanisms of resistance remain poorly defined. Here, I subjected *E. coli* to repeated psoralen-UVA exposure to isolate three independently derived strains that were >10,000-fold more resistant to this treatment than the parental strain. Analysis of these strains identified gain-of-function mutations in the transcriptional regulator AcrR and the alpha subunit of RNA polymerase that together could account for the resistance of these strains. Resistance conferred by the AcrR mutation is mediated at least in part through the regulation of the AcrAB-TolC efflux pump. Resistance via mutations in the alpha subunit of RNA polymerase occurs through a still-uncharacterized mechanism that has an additive effect with mutations in AcrR. Both *acrR* and *rpoA* mutations reduced cross-link formation *in vivo*. I discuss potential mechanisms in relation to the ability to repair and survive interstrand DNA cross-links.

Introduction

The clinically important drugs psoralen, cisplatin, nitrogen mustard, and mitomycin vary widely in their origins. Some are naturally occurring: psoralens are defensive toxins produced by a variety of plants such as figs and celery (1), and

mitomycins are antibiotics produced by bacteria of the genus *Streptomyces* (2). In contrast, nitrogen mustards were originally synthesized in the 1920s and 1930s for use in chemical warfare (3). Despite their differences, these drugs have a common mechanism of toxicity due to their ability to create particularly destructive lesions in DNA called interstrand cross-links (4, 5). Interstrand cross-links are generated when molecules such as psoralen intercalate between complementary strands of DNA and form covalent bonds with both strands (4, 6–8). In the case of psoralen and its derivatives, bond formation additionally requires the absorption of photons from the UVA wavelength (7, 9). Interstrand cross-links prevent the separation of the DNA strands, making essential cellular processes like transcription and replication impossible. Eventually, such interference is lethal to the cell (10). Furthermore, DNA interstrand cross-links are recalcitrant to known mechanisms of repair since no complementary strand is available for repair synthesis to occur (10).

The formidable lethality of interstrand cross-links makes cross-linking drugs potent treatments for conditions in which the destruction of rapidly dividing or metabolically active cells is required, such as in the treatment of cancers (11, 12). Cross-linking drugs are often first- and second-line chemotherapeutics, including cisplatin, which is curative for many testicular cancers (13–16). Despite the potency of these drugs, cells, including those in cancerous tumors, can develop resistance to these therapies (17–19). Much remains unknown about how resistance to cross-linking agents arises (19–21). Resistance may result from changes in the membrane permeation of the cross-linking drug or from the active efflux of the drug out of the cell (22), as seen in multidrug-

resistant cancers (23, 24). Others have identified various gene products that seem to interact with interstrand cross-links and hypothesized that cells can remove interstrand cross-links from DNA and repair the genomic damage caused by these lesions (25–27).

Several DNA repair mutants that render cells hypersensitive to cross-linking agents have been isolated (25, 27–30). Notably, in humans, mutations in 22 genes result in the hereditary genetic disorder Fanconi anemia. Cells from patients exhibit hypersensitivity to cross-linking agents and accumulate chromosome breaks following treatment with cross-linking agents (31, 32), suggesting defects in the repair of these lesions. As in *Escherichia coli*, many of the cross-link-hypersensitive Fanconi anemia genes render cells hypersensitive to other types of DNA damage, including monoadducts formed by the same cross-linking agents (33, 34). Based on these hypersensitivities, a number of complex models have been proposed in which either base or nucleotide excision repair acts sequentially before and after translesion synthesis or recombination to effect repair (29, 35–37). However, *in vivo* evidence of intermediates predicted by these models is generally lacking. Complicating the interpretation of DNA repair mutant hypersensitivity is that all cross-linking drugs also induce other forms of DNA damage, including monoadduct intermediates (6, 38). Mutants proposed to be involved in cross-link repair are also hypersensitive to monoadducts, which may account for the sensitivity of these mutants to cross-linking drugs. By comparing psoralen derivatives that form only monoadducts to those that form both monoadducts and cross-links, Cole et al. found that the hypersensitivity of most mutants could be attributed to defects in monoadduct repair alone (39). Additionally, when the cross-links in DNA were quantified, the authors found

that between one and two cross-links were sufficient to render *E. coli* inviable (9, 39). Using other cross-linking agents, others have arrived at similar conclusions (40). In human cells, studies measuring cross-link repair are limited but similarly suggest that the repair capacity is minimal, with lethality occurring at between 200 and 900 lesions per cell (41–44). In all of these studies, the estimates depend on extrapolation since lethality occurs at doses below the direct detection limit for cross-links.

These studies make it clear that the capacity to repair interstrand cross-links is limited and raise the possibility that effective repair mechanisms do not exist in the cell. This implies that alternative mechanisms are responsible for the development of resistance to cross-linking drugs like psoralen, which is the focus of this study. To investigate the mechanisms by which cross-link resistance develops, an iterative selection scheme was utilized to generate three independently derived strains of *E. coli* that are highly resistant to psoralen and UVA (PUVA) treatment. The genomes of these strains were then sequenced, and the mutations involved in psoralen resistance were characterized.

Results

Generation of strains resistant to psoralen plus UVA irradiation.

To select for mutations that confer resistance to psoralen-UVA treatment, three independent cultures derived from a single colony were grown, spread onto plates, and exposed to various doses of UVA irradiation in the presence of 20 μg/mL 8-methoxypsoralen. The cells were then collected from the plate at the dose where survival was first noticeably reduced (Fig 2.1) and were used to inoculate cultures for the next

successive round. Following six (for isolate 1) or seven (for isolates 2 and 3) rounds of selection, each culture exhibited a high level of resistance to psoralen-UVA treatment. Independent colonies were isolated from each culture and designated resistant isolates 1, 2, and 3. To quantify the level of the psoralen-UVA resistance of each isolate, 10-fold serial dilutions of a culture grown overnight were spotted onto plates containing 20 μg/mL 8-methoxypsoralen and exposed to increasing doses of UVA. Following incubation overnight at 37°C, the surviving colonies were counted and compared to those on the unexposed plate to determine survival. As shown in Fig. 2.2, the survival of the three isolates increased more than 10^4-fold relative to the parent strain at high psoralen-UVA doses. The survival of cells exposed to UVA alone or psoralen alone remained unaffected at these doses (Fig. 2.3). These results indicate that *E. coli* cells contain within their genomes the ability to become resistant to treatment with psoralen-UVA, a cross-linking agent.

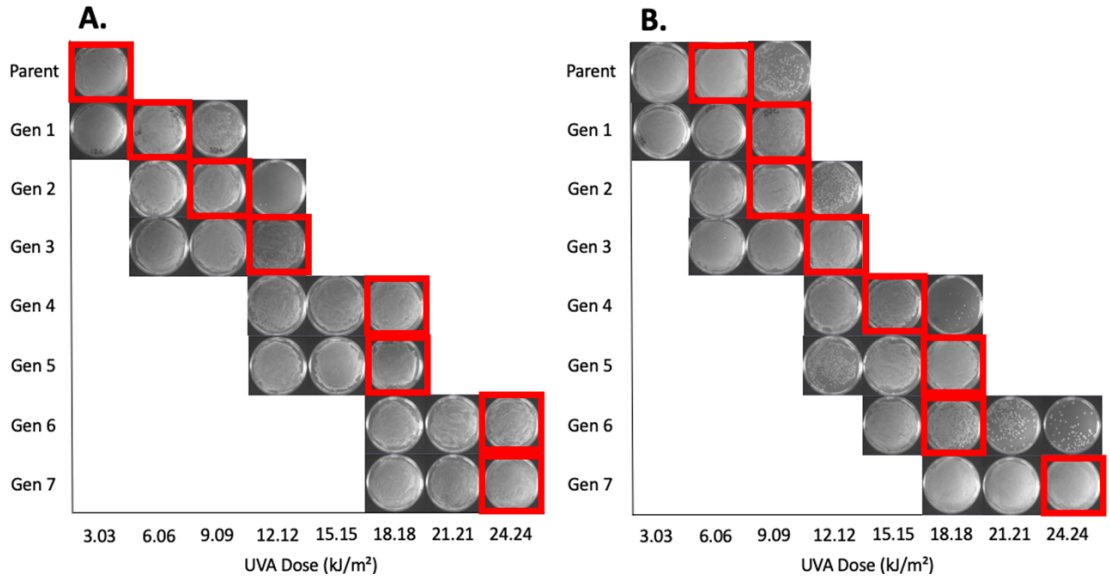

Fig. 2.1. Selection for the development of resistance to psoralen-UVA treatment. Two separate cultures (A and B) of strain SR108 were plated on media containing 20 µg/ml 8-methoxypsoralen and exposed to increasing doses of UVA. The cells from the plate where viability began to become compromised were collected, grown, and the psoralen-UVA treatment/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. A third resistant strain was subsequently isolated using this same approach.

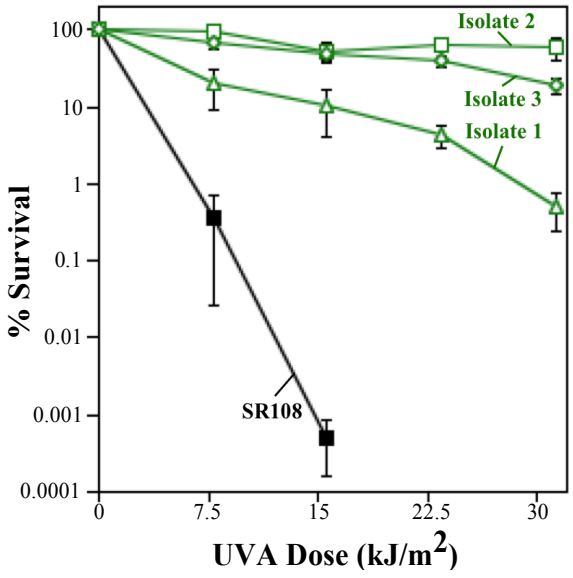

Fig. 2.2. Isolates acquired high-level resistance to psoralen-UVA treatment following repeated exposure and selection. The survival of resistant isolate 1 (green open triangles), resistant isolate 2 (green open squares), resistant isolate 3 (green open diamonds), and the SR108 parent (black filled squares) following irradiation with the indicated UVA doses in the presence of 20 µg/mL 8-methoxypsoralen is plotted. The survival of the parental strain was below the detectable limit at UVA doses of >15.6 kJ/m^2. Plots represent the averages from at least three independent experiments. Error bars represent the standard errors of the means.

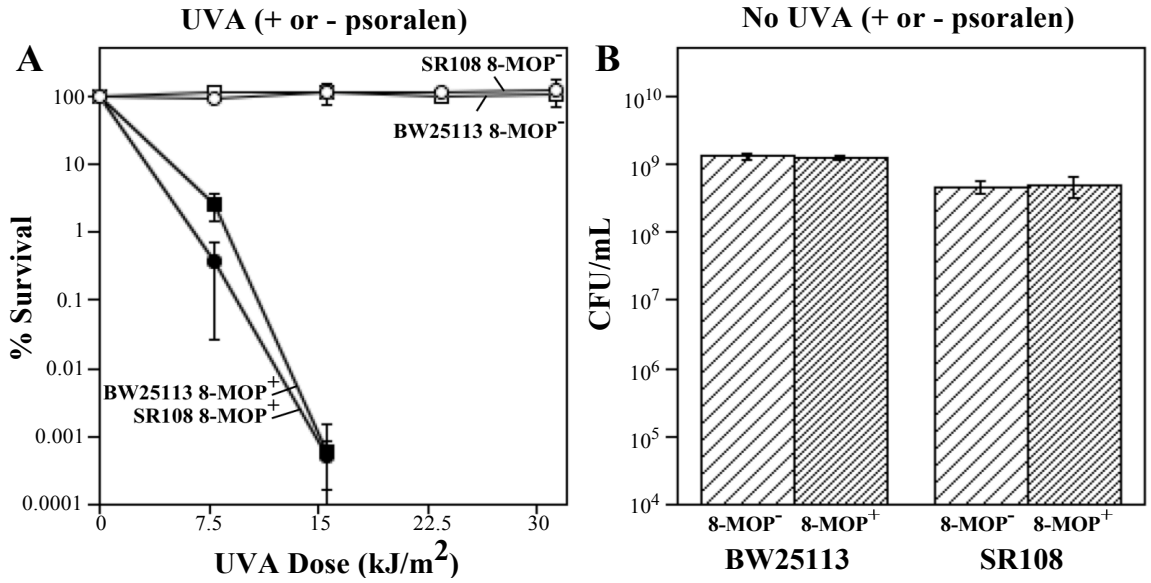

Fig. 2.3. SR108 and BW25113 do not significantly differ in sensitivity to psoralen-UVA. UVA or psoralen alone do not contribute to lethality. (A) Survival of BW25113 and SR108 in the presence of psoralen (filled squares and filled circles respectively) and BW25113 and SR108 without psoralen (empty squares and empty circles respectively) at the indicated UVA doses is plotted. (B) Number of colony-forming units for BW25113 (left) and SR108 (right) with and without psoralen and no exposure to UVA irradiation are plotted. Plots represent the average of at least two independent experiments. Error bars represent the standard error of the mean.

Identification of mutations in resistant strains.

To identify the mutations responsible for conferring psoralen-UVA resistance, the genome of each isolate was sequenced using high-throughput sequencing and compared to the SR108 parent genome. Although several mutations were identified in each strain (Table 2.1), three genes were found to be mutated across multiple isolates. All three resistant isolates contained mutations in *acrR*, a transcriptional regulator (45). Resistant isolates 2 and 3 had point mutations in *rpoA*, which encodes the alpha subunit of the bacterial RNA polymerase (46). Finally, resistant isolate 2 contained a mutation in *rclA*, encoding an oxidoreductase involved in resistance to reactive chlorine species (47, 48). Similarly, resistant isolate 3 contained an intergenic mutation between *rclR*, the local activator of *rclA* expression, and a putative oxidoreductase, *ykgE* (47). It should be noted that while both isolate 1 and isolate 3 had the same large deletions of a region near the terminus, this deletion has been observed previously in our SR108 parent strain, appears to be driven by recombination between IS*5* insertion elements, and does not exhibit any noticeable phenotype in our SR108 background. I therefore elected to further characterize the gene products of the *acrR*, *rpoA*, and *rclA* mutants for their potential role in psoralen-UVA resistance.

Table 2.1. Full list of mutations in psoralen-resistant strains.

Resistant Isolate	**Mutations[a]**
1	*ycdS*(W555C), *ycfQ* (frameshift), *flhC* (frameshift), *yghA/exbD*(frameshift), *yrbG* (frameshift), Δ*abgT- ydeN*, *ykgD/ykgE* (INS), *acrR*(INS)
2	*rclA*(A368G), *acrR*(L34Q), *fadR* (frameshift), *purL*(D162D), *ygjR*(M184T), *yiiF/yiiE* (frameshift), *rpoA*(E273G), *Lamb/malM* (A→G)
3	*dgt*(L260*), *gmhB*(D127E), *acrR* (frameshift), *ybeL/ybeQ* (frameshift), *ybfA* (frameshift), *rhsC*(Y1276C), *ssuC* (frameshift), *ychN*(L28S), *oppB*(frameshift), *cspI/ydfP* (frameshift), *torY* (frameshift), *wcaL(frameshift), yfeU(Y146Y), yfhM(P1609P), ygcE/ygcF (frameshift), ygiV/ygiW (T→A), qseB(Y41C), thrU/coaA (G→A), katG(D140E), wzxE* (frameshift), *aldB/yiaW* (frameshift), *yiaM* (frameshift), *rhsB* (frameshift), *rpoA* (P323R), *trkA* (H51R), *melR* (L219P), *yjiD* (frameshift), Δ*abgT-ydeN*
[a] INS, insertion; /, intergenic	

Gain-of-function mutations in *acrR* and *rpoA*, but not *rclA*, confer resistance and can account for increased survival against psoralen and UVA irradiation.

To characterize these three candidate gene products, I focused on resistant isolate 2, which contained mutations in all three candidate genes, had the fewest total mutations, and exhibited levels of resistance as high as or higher than those of the other isolates (Fig. 2.2). Furthermore, despite numerous attempts, I have been unable to productively infect isolates 1 and 3 with phage P1 for transduction. To determine if any of these mutations contributed to psoralen-UVA resistance, each mutation was linked to a kanamycin resistance (Kan^r) cassette and transduced from isolate 2 into BW25113, the parental strain used for the Keio collection of deletion mutants (49, 50). The resulting mutations were then examined using the same survival assay as the one used to test the resistant isolates, with BW25113 and resistant isolate 2 serving as negative and positive controls, respectively. As shown in Fig. 2.4A, both mutations *acrR*(L34Q) and *rpoA*(E273G) conferred moderate levels of resistance to psoralen-UVA in an otherwise wild-type background compared to their BW25113 parent. Neither the *acrR*(L34Q) nor the *rpoA*(E273G) mutant was as resistant as resistant isolate 2, arguing that each mutation could only partially account for the resistance phenotype of this isolate. In contrast, the sensitivity of strains containing the *rclA*(A368G) mutation did not change relative to the parental strain (Fig. 2.4A), arguing that this mutation was not associated with the psoralen-UVA resistance phenotype.

To determine whether the resistance-conferring mutations represent a loss or a gain of protein function, I examined the resistance of strains deleted for these genes. If deleting the gene confers resistance similar to that of the point mutation, the mutation

likely results in a loss of the protein's function. rpoA is an essential gene (49), implying that the resistance mutations arising in this gene product are gain-of-function mutations. However, the strains with deletions in *acrR* and *rclA* are viable. As shown in Fig. 2.2B, the deletion of *acrR* renders cells hypersensitive to psoralen-UVA irradiation, in contrast to the point mutation, arguing that *acrR*(L34Q) represents a gain-of-function mutation. The sensitivity of a mutant deleted for *rclA* was similar to those of both the *rclA*(A368G) point mutant and the parental strain. Furthermore, mutants deleted for the genes *rclR* and *ykgE*, which border an intergenic insertion in resistant isolate 3, also showed wild-type sensitivity to psoralen-UVA treatment (Fig. 2.5). These results are consistent with the interpretation that the *rclA* locus is not involved in the resistance phenotype.

The point mutations in *rpoA* and *acrR* could be acting in the same pathway to confer resistance, or they may function through separate mechanisms. If the mutations operate in different pathways to confer resistance, one might expect that cells containing both mutations would be more resistant than either single mutant alone. If the mutations operate in a single pathway, one might expect that the resistance of the double mutant cells would be similar to that of the single mutants. To examine this possibility, I constructed an *acrR*(L34Q) *rpoA*(E273G) double mutant. As shown in Fig. 2.4C, the double mutant was more resistant than either single mutant and exhibited resistance comparable to that of isolate 2. I interpret these results to imply that the mechanisms by which mutations in *acrR* and *rpoA* confer resistance to psoralen-UVA are distinct and likely account for the resistance observed in isolate 2.

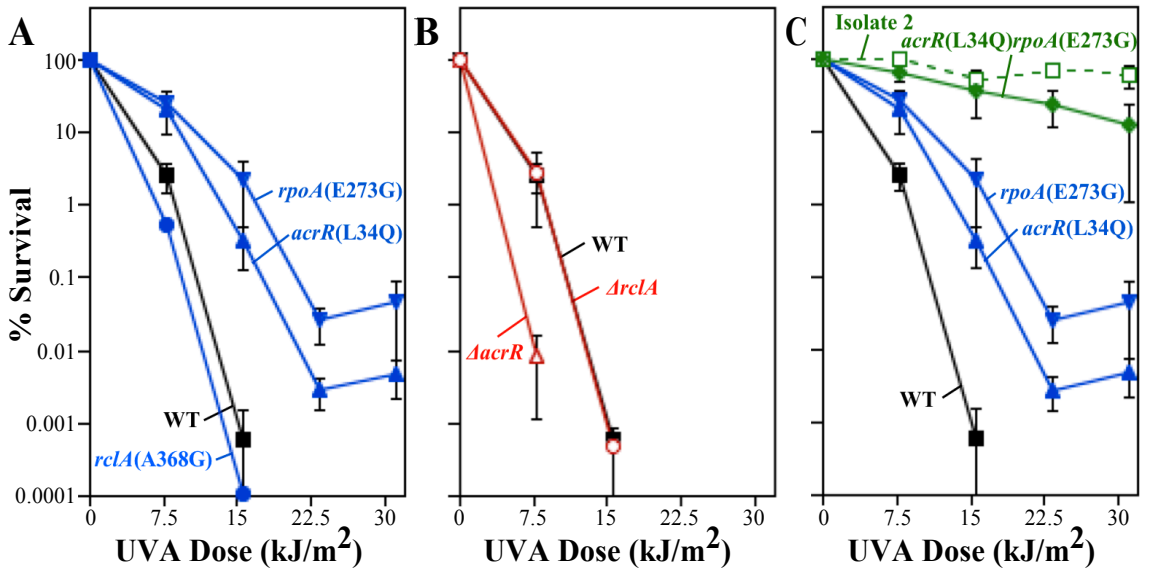

Fig. 2.4. Gain-of-function mutations in *acrR* and *rpoA*, but not *rclA*, confer resistance to psoralen-UVA treatment. The survival of the *acrR*(L34Q) (blue triangles), *rpoA*(E273G) (blue inverted triangles), and *rclA*(A368G) (blue circles) mutants and the BW25113 parent (black squares) (A); the Δ*acrR* (red open triangles) and Δ*rclA* (red open circles) mutants (B); and the *acrR*(L34Q) mutant (blue triangles), the *rpoA*(E273G) mutant (blue inverted triangles), the *acrR*(L34Q) *rpoA*(E273G) double mutant (green diamonds), and resistant isolate 2 (green open squares) (C) in the presence of 20 μg/mL 8-methoxypsoralen at the indicated UVA doses is plotted as described in the legend of Fig. 2.1. The survival of the Δ*acrR* and Δ*rclA* mutants was below the detectable limit at UVA doses of >7.5 and >15.6 kJ/m^2, respectively. Plots represent the averages from at least three independent experiments. Resistant isolate 2 was derived from the SR108 background (indicated by the dotted line), while all other strains are isogenic mutants of BW25113. Error bars represent the standard errors of the means. WT, wild type.

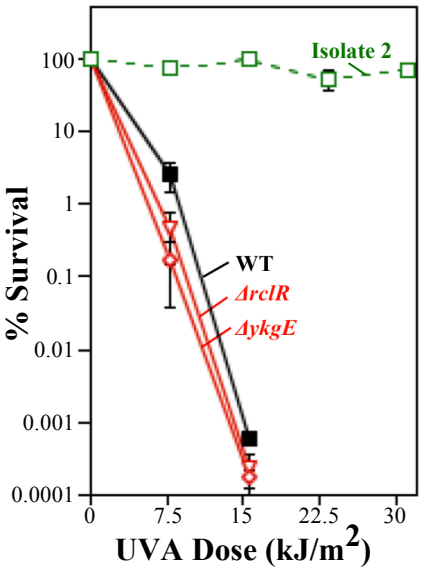

Fig. 2.5. Δ*rclR* and Δ*ykgE* show wild type sensitivity to psoralen-UVA. Survival of BW25113 (black, filled square), Δ*rclR* (red, open inverted triangle), Δ*ykgE* (red, open diamond) in the presence of 20 μg/ml 8-methoxypsoralen at the indicated UVA doses is plotted. Plots represent the average of at least three independent experiments. Error bars represent the standard error of the mean.

Neither *acrR*(L34Q) nor *rpoA*(E273G) alters the growth rate of cells.

One general mechanism that increases resistance to DNA damage is a reduction in the growth rate of cells. In general, this allows more time for repair to occur and reduces the frequency with which replication encounters DNA damage (51–53). To examine whether slower growth may be contributing to the resistance of these strains, I compared the growth rates of these mutants to those of their parents. The results showed that the growth rates of resistant isolates 2 and 3 were modestly slower than that of the SR108 parental strain from which they were derived (Fig. 2.6A). However, when the *acrR*(L34Q) or *rpoA*(E273G) mutation was moved into an otherwise wild-type BW25113 background, no difference in the growth rate was observed for either the single or double mutant (Fig. 2.6B). These results suggest that the reduced growth rates of isolates 2 and 3 are likely caused by secondary mutations that accumulated in these strains. Notably, all growth rates remained relatively similar between strains, and no severe growth impairments were observed that could account for the extreme resistance observed in these strains.

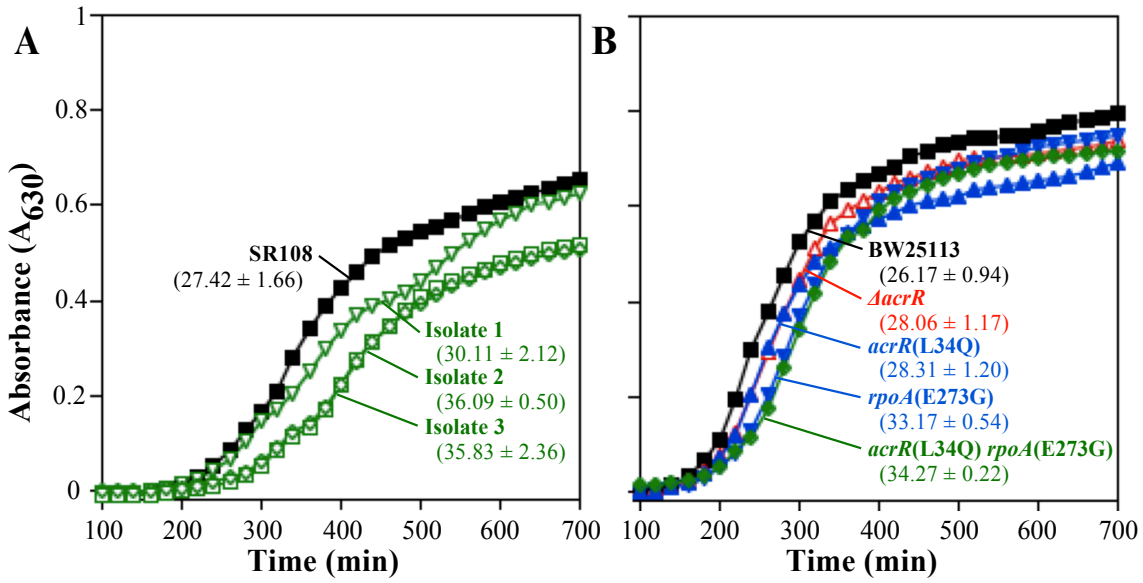

Fig. 2.6. Growth rates remain similar between strains and are unlikely to account for the resistance conferred by *acrR*(L34Q) or *rpoA*(E273G). The absorbance (630 nm) values of SR108 parent (black filled squares), isolate 1 (green open inverted triangles), isolate 2 (green open squares), and isolate 3 (green open diamonds) cultures (A) and BW25113 parent (black filled squares), Δ*acrR* mutant (red open triangles), *acrR*(L34Q) mutant (blue triangles), *rpoA*(E273G) mutant (blue inverted triangles) and *acrR*(L34Q) *rpoA*(E273G) double mutant (green diamonds) cultures (B) grown at 37°C are plotted over time. The doubling times and ranges (in minutes) from duplicate experiments are indicated below each strain.

acrR(L34Q) and _rpoA_(E273G) affect the efflux capacity, but not the repair capacity, of the cell.

Reduced cross-link formation in the resistant isolates could result from an increased repair or efflux capacity of the cell. To address this, I examined survival following UVC irradiation, whose toxicity is associated with the formation of bulky DNA adducts that require removal by nucleotide excision repair. The UVC resistance of the three isolates as well as the *acrR*(L34Q) and *rpoA*(E273G) mutants remained statistically similar to that of the parental strain (Fig. 2.7A, B and D, E). In contrast, the three isolates and the *acrR*(L34Q) and *rpoA*(E273G) mutants each conferred resistance to chloramphenicol, an aromatic molecule that affects translation but does not damage DNA directly (Fig. 2.7C and F). Chloramphenicol has a structure similar to that of psoralen and has been shown to be a substrate for the AcrAB efflux pump (54). Together, these observations are consistent with psoralen-UVA resistance being associated with an upregulation of the efflux capacity but not the DNA repair capacity.

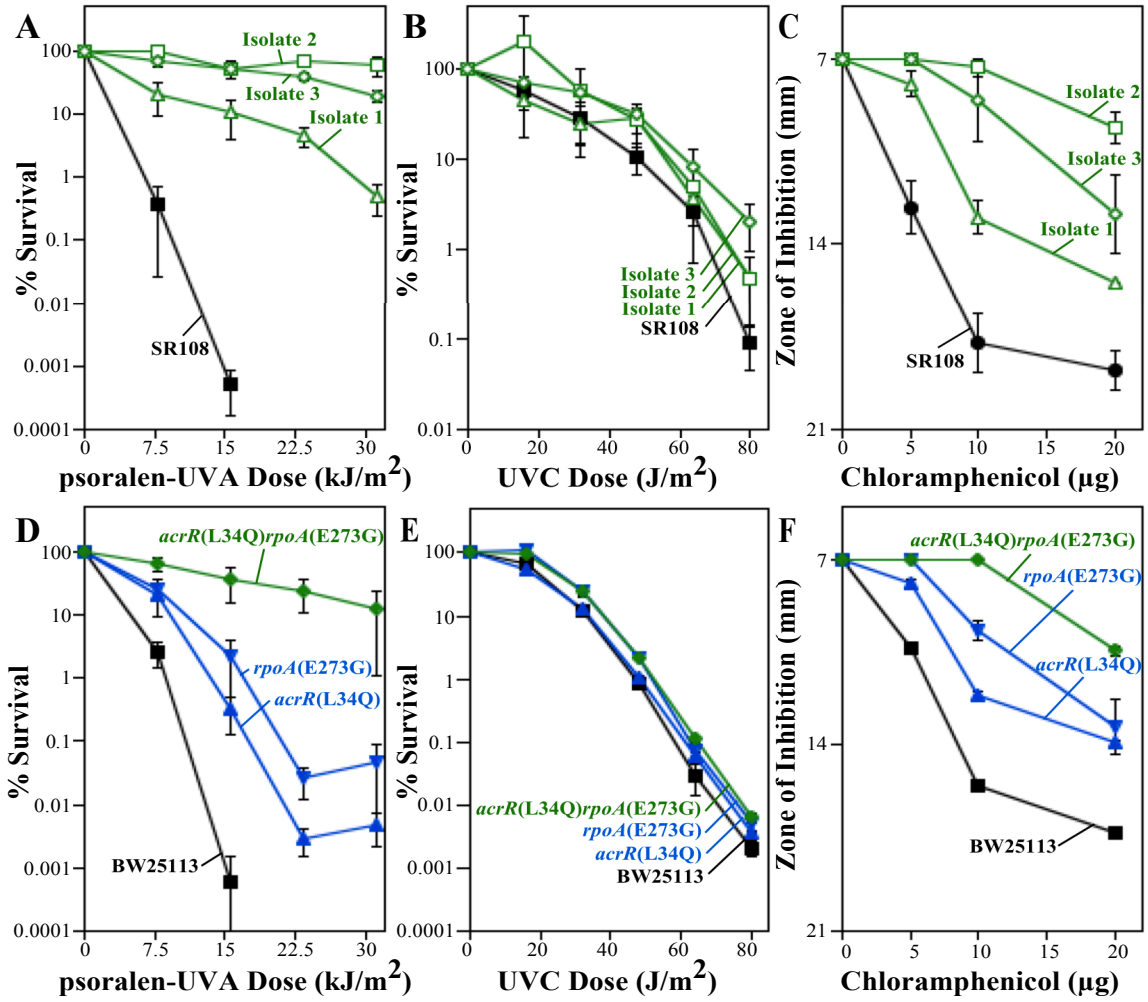

Fig. 2.7. Isolates and mutants that exhibit increased resistance to psoralen-UVA also exhibit increased resistance to chloramphenicol but not UVC irradiation. (A) The survival of resistant isolate 1 (green open triangles), resistant isolate 2 (green open squares), resistant isolate 3 (green open diamonds), and the SR108 parent (black filled squares) in the presence of psoralen is replotted from Fig. 1 for comparison. (B) The survival of resistant isolate 1 (green open triangles), resistant isolate 2 (green open squares), resistant isolate 3 (green open diamonds), and the SR108 parent (black filled squares) following UVC irradiation at the indicated doses is plotted. (C) Diameters of the zones of inhibition around 7-mm paper discs treated with the indicated amounts of chloramphenicol are plotted for resistant isolate 1 (green open triangles), resistant isolate 2 (green open squares), resistant isolate 3 (green open diamonds), and the SR108 parent (black filled squares). (D) The survival of the *acrR*(L34Q) mutant (blue triangles), the *rpoA*(E273G) mutant (blue inverted triangles), the *acrR*(L34Q) *rpoA*(E273G) double mutant (green diamonds), and the BW25113 parent (black squares) is replotted from Fig. 2.2 for comparison. The survival of the *acrR*(L34Q) mutant (blue triangles), the *rpoA*(E273G) mutant (blue inverted triangles), the *acrR*(L34Q) *rpoA*(E273G) double mutant (green diamonds), and the BW25113 parent (black squares) following UVC irradiation at the indicated doses is plotted. (F) The diameters of the zones of inhibition around 7-mm paper discs treated with the indicated amounts of chloramphenicol are plotted for the *acrR*(L34Q) mutant (blue triangles), the *rpoA*(E273G) mutant (blue inverted triangles), the *acrR*(L34Q) *rpoA*(E273G) double mutant (green diamonds), and the BW25113 parent (black squares). Plots represent the averages from at least two experiments. Error bars represent the standard errors of the means.

***acrR*(L34Q) and *rpoA*(E273G) reduce cross-link formation frequencies *in vivo*.**

Another mechanism by which resistance could be conferred is by decreasing cross-link formation in cellular DNA. To examine this possibility, I utilized an alkali agarose gel and Southern blot analysis to monitor the accumulation of cross-links on plasmids growing in these strains. Cells containing the plasmid pBR322 were treated with psoralen and UVA before the total cellular DNA was purified, digested with a restriction enzyme to linearize the plasmid, and analyzed by denaturing alkali agarose gel electrophoresis. Interstrand cross-links covalently bind both DNA strands, preventing complete strand separation under denaturing conditions, and cross-linked plasmid forms migrate more slowly than the corresponding single-stranded fragments during electrophoresis (55, 56). In theory, the assay should produce gels containing only 2 bands, undamaged DNA and interstrand DNA cross-links. However, 1 to 2 cross-links per chromosome are lethal in *E. coli* (39, 40), and assays detecting these lesions *in vivo* require doses far beyond this, with long irradiation times. The long irradiation periods required allow cross-links formed in cultures to be further processed by enzymes that may unlink, excise, and exonucleolytically degrade the DNA surrounding adducts, resulting in a more diffuse signal that stretches from the cross-link down to the undamaged DNA. To quantify the overall levels of psoralen-UVA-induced adduct formation in cells, the loss of the undamaged single-strand DNA band remaining in the treated samples was compared to that in the unirradiated sample.

When I compared the resistant isolates to their SR108 parent, all three resistant isolates retained significantly more un-cross-linked, linear, single-stranded DNA

following psoralen-UVA treatment (Fig. 2.8A). In the SR108 parent, the fraction of undamaged plasmid remaining was significantly diminished following doses of both 35.1 kJ/m^2 and 70.2 kJ/m^2, whereas the resistant isolates could be seen to retain undamaged plasmid DNA at both the low and high doses used in these experiments. Thus, I interpret the observed persistence of the undamaged linear DNA in the resistant isolates to indicate that the formation of cross-links in DNA is reduced in the three isolates and that this likely accounts for their observed resistance.

To determine if the *acrR*(L34Q) and *rpoA*(E273G) mutations were responsible for the reduced cross-link formation, I next examined the formation of cross-links in strains containing the *acrR*(L34Q), *rpoA*(E273G), and both mutations. The *acrR*(L34Q) mutant partially reduced the formation of cross-links relative to its BW25113 parent, as measured by the loss of full-length undamaged DNA, although this effect appeared modest within the sensitivity of our assay. When the *acrR*(L34Q) *rpoAE*(E273G) double mutant was examined, a clear increase in the amount of undamaged DNA was observed (Fig. 2.8B). The amount of undamaged DNA in the double mutant was comparable to that seen in the original resistant isolate 2. These observations suggest that the two mutations act through separate mechanisms, are additive, and together can account for the reduced cross-link formation seen in the resistant isolate.

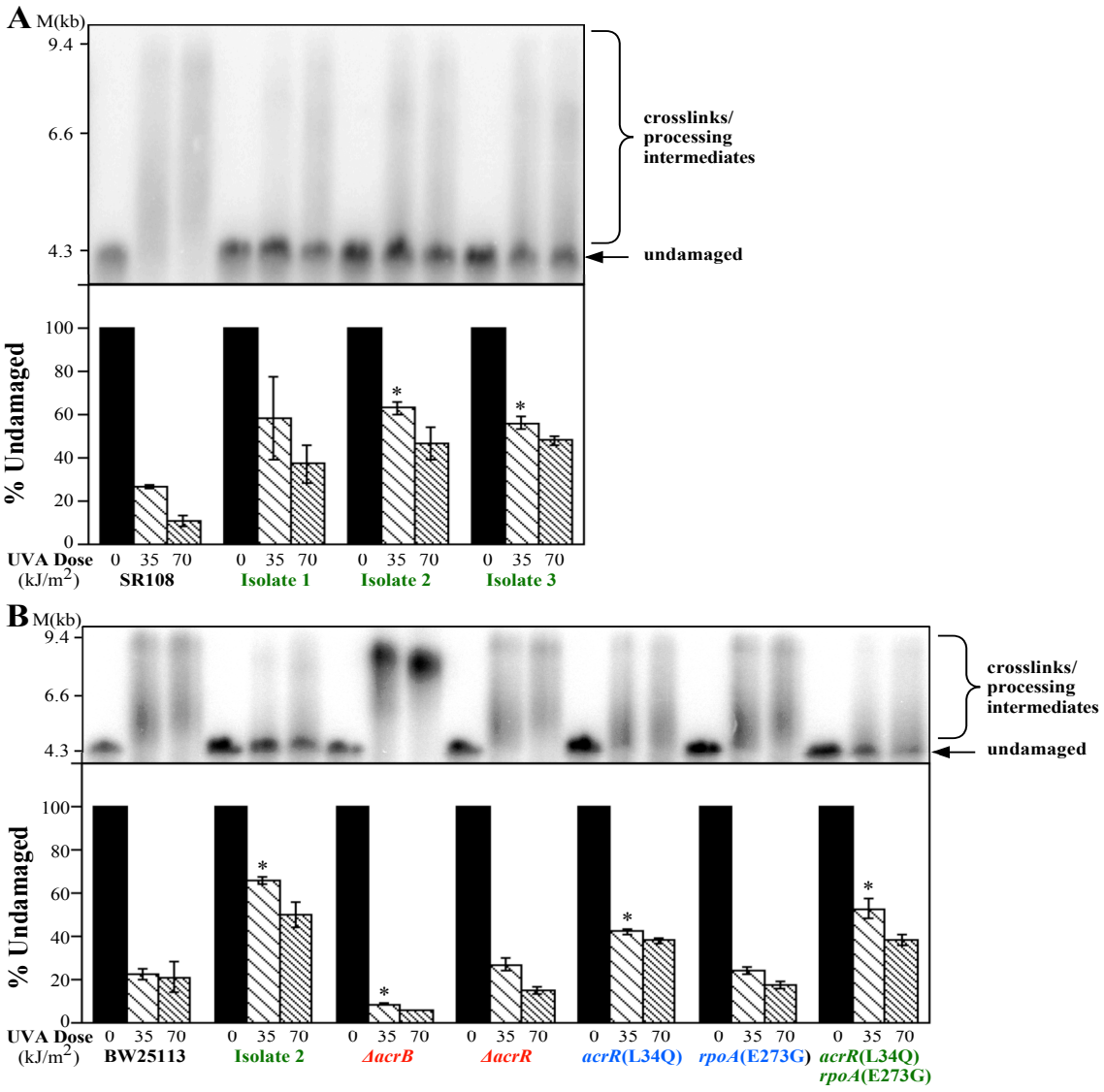

Fig. 2.8. *acrR*(L34Q) *rpoA*(E273G) double mutants exhibit reduced formation of cross-links and other forms of DNA damage, similar to the PUVA-resistant isolates. (A) Resistant isolates do not accumulate PUVA-induced DNA damage. (Top) SR108, isolate 1, isolate 2, and isolate 3 cultures containing the plasmid pBR322 were UVA irradiated at the indicated doses in the presence of 20 μg/mL 8-methoxypsoralen before total genomic DNA was isolated, restricted with PvuII to linearize the plasmid, and examined by Southern analysis following alkali gel electrophoresis using ^{32}P-labeled pBR322 as a probe. Undamaged DNA is indicated by the arrow. A shift from undamaged DNA to a higher-apparent-molecular-weight form indicates the presence of PUVA-induced DNA damage. (Bottom) The percentage of undamaged DNA remaining at each dose is plotted for each strain. (B) The *acrR*(L34Q) *rpoA*(E273G) mutants show reduced formation of PUVA-induced DNA damage. Cultures of the BW251113 parent and the *acrR*(L34Q), *rpoA*(E273G), and *acrR*(L34Q) *rpoA*(E273G) mutants containing the plasmid pBR322 were treated and analyzed as described above for panel A. (Top) Representative Southern blot; (bottom) percentage of undamaged DNA remaining at each dose. Plots represent averages from two experiments. Error bars represent the standard errors of the means. * indicates statistical significance relative to the corresponding wild-type strain ($P < 0.05$ by a *t* test).

AcrR regulates the expression of the AcrAB-TolC efflux system. This suggests a mechanism in which the upregulation of the efflux system may increase psoralen export, thereby preventing its intercalation with DNA and cross-link formation. To examine this possibility, I examined how the deletion of the AcrB transporter component of the efflux system affected cell sensitivity to psoralen-UVA and cross-link formation. As shown in Fig. 2.8B, the deletion of *acrB* resulted in an increase in cross-link formation in DNA. In addition to the effect on cross-link formation, the inactivation of the efflux system severely hypersensitized the parental strain to psoralen-UVA treatment (Fig. 2.9). Furthermore, the deletion of *acrB* in isolate 2 completely abolished its resistance, rendering the cells as hypersensitive as the Δ*acrB* strain in an otherwise wild-type background (Fig. 2.9). The same was observed for the other two resistant isolates (Fig. 2.10). Taken together, these results indicate that the *acrR*(L34Q) mutation is likely effecting resistance through the regulation of the AcrAB-TolC complex and that AcrAB-TolC is capable of effluxing psoralen to reduce cross-link formation.

To test this possibility directly, the *acrAB* operon was placed into an arabinose-inducible expression plasmid. The overexpression of AcrAB, by itself, in an otherwise wild-type background increased psoralen-UVA resistance, similar to the AcrR mutant allele (Fig. 2.11), demonstrating that resistance can be conferred directly by the upregulation of the efflux pump under AcrR control.

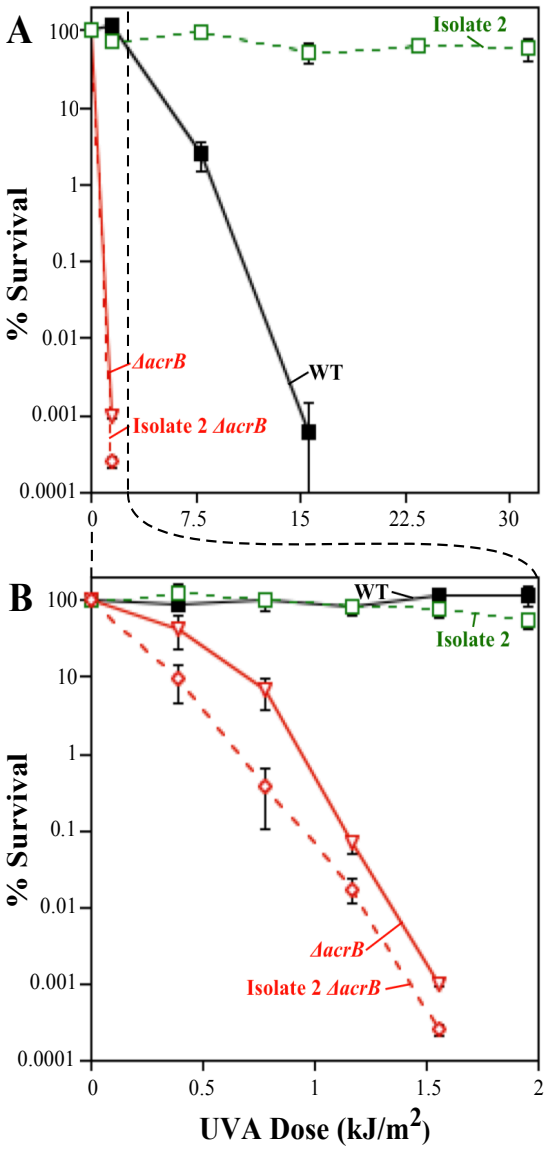

Fig. 2.9. The AcrAB-TolC efflux system is required for resistance to PUVA. (A) The survival of BW25113 (black filled squares), resistant isolate 2 (green open squares), an *acrB* deletion mutant (red open inverted triangles), and an *acrB* deletion mutant in the resistant isolate 2 background (red open diamonds) in the presence of psoralen at the indicated UVA doses is plotted. (B) The survival of BW25113 (black filled squares), resistant isolate 2 (green open squares), an *acrB* deletion mutant (red open inverted triangles), and an *acrB* deletion mutant in the resistant isolate 2 background (red open diamonds) in the presence of psoralen is replotted on a narrower UVA scale. Resistant isolate 2 and resistant isolate 2 Δ*acrB* mutant were derived from the SR108 background (indicated by the dotted line), while Δ*acrB* is an isogenic mutant of BW25113 (WT). Plots represent the averages from at least three independent experiments. Error bars represent the standard errors of the means.

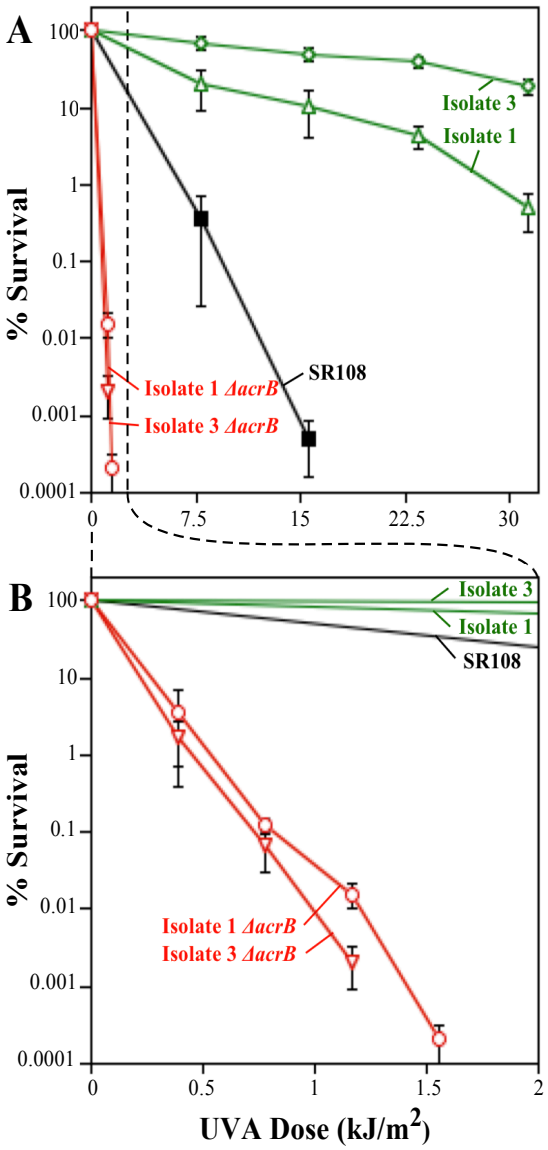

Fig. 2.10. Deletion of *acrB* in resistant isolates 1 and 3 reversed resistance to PUVA. Survival of BW25113 (black, filled square), *acrB* deletion in resistant isolate 1 background (red, open triangle), and *acrB* deletion in resistant isolate 3 background (red, open diamond) in the presence of 20 µg/ml 8-methoxypsoralen at the indicated UVA doses is plotted. Plots represent the average of at least three independent experiments. Resistant isolates 1 and 3 were derived in SR108 background (indicated by dotted line). Error bars represent the standard error of the mean.

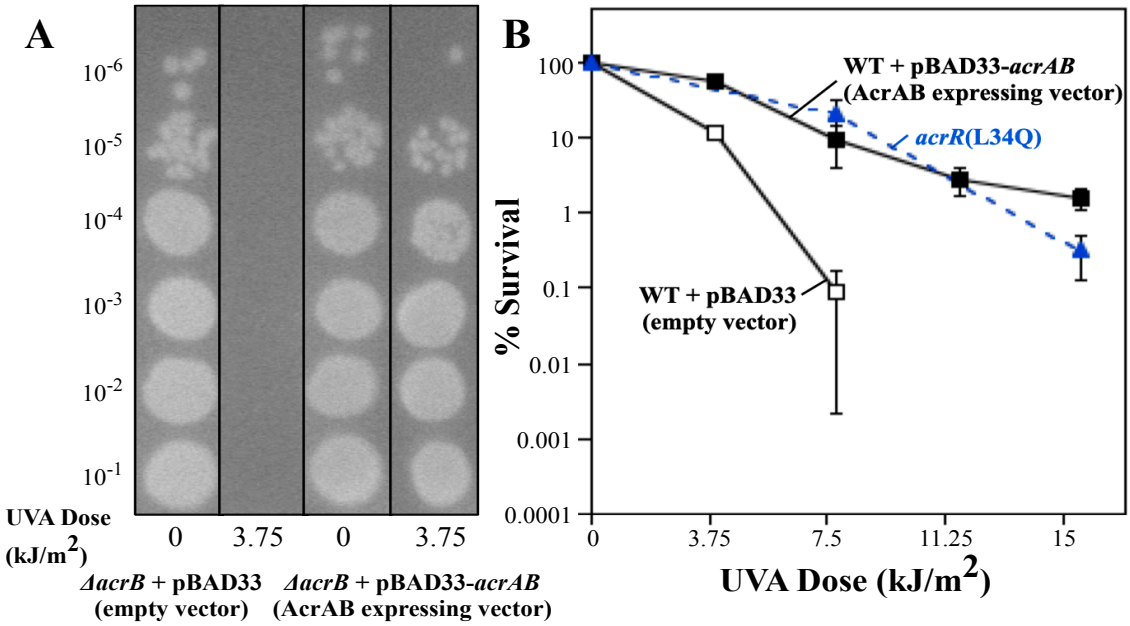

Fig. 2.11 Upregulation of the AcrAB efflux pump confers resistance to psoralen-UVA, similar to *acrR*(L34Q). (A) The expression of AcrAB functionally complements an *acrB* mutant. Ten-microliter drops of 10-fold serial dilutions are shown for the Δ*acrB* mutant containing the pBAD33 vector and the Δ*acrB* mutant containing pBAD33-AcrAB following UVA irradiation at the indicated doses in the presence of 20 μg/mL 8-methoxypsoralen. (B) The survival of BW25113 containing the pBAD33 vector (black open squares), BW25113 containing pBAD33-AcrAB (black filled squares), and the *acrR*(L34Q) mutant (blue filled triangles) in the presence of 20 μg/mL 8-methoxypsoralen at the indicated UVA doses is plotted. Plots represent the averages from two experiments. Error bars represent the standard errors of the means.

Discussion

Here, I demonstrate that gain-of-function mutations in *rpoA* and *acrR* can confer high-level resistance to psoralen-UVA treatment. Considering that psoralen-UVA cytotoxicity arises from the formation of interstrand cross-links (40, 57), I initially expected that mutations conferring resistance might upregulate or induce mechanisms for repairing these lesions. However, although high-level resistance was observed, both mutations appeared to operate through mechanisms that reduce or prevent the formation of DNA cross-links, rather than increase repair. This would be consistent with the results of a previous study by Cole et al., in which the researchers failed to observe significant contributions from the known repair enzymes and found that as few as 1 to 2 cross-links per genome were lethal to *E. coli* (39). Those observations led the researchers to conclude that mechanisms for the effective repair of cross-links may not exist and that resistance therefore would likely involve mechanisms that primarily reduce or prevent cross-link formation, as reported here.

The results reported here identify psoralen as a novel substrate of the AcrAB-TolC efflux pump. This is based on the observations that mutations in the efflux regulator *acrR* were present in all three psoralen-resistant strains. The deletion of AcrB, which inactivates the efflux system, hypersensitized cells to psoralen and completely reversed the resistance phenotype of the resistant isolates (Fig. 2.9 and 2.10). Additionally, mutations that upregulate or downregulate the *acrR* regulator correlate with the ability of psoralen to form cross-links in cells, as does the presence or absence of the transporter (Fig. 2.9 and 2.11). Notably, the AcrAB-TolC efflux pump is a tripartite

efflux system that was originally identified for its ability to transport acridine dyes (58–60), which are structurally similar to the three-ringed psoralen molecule (Fig. 2.12) (9, 61). AcrAB-TolC has since been shown to pump a broad variety of substrates, with many sharing the carbon ring structures associated with multidrug resistance in Gram-negative bacteria (62, 63).

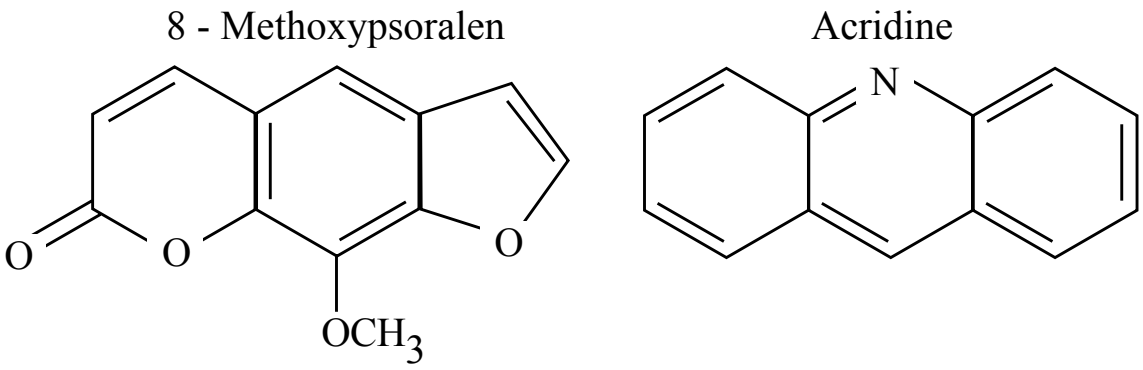

Fig. 2.12. Structures of 8 – methoxypsoralen and acridine are similar. (Left) 8 – methoxypsoralen. (Right) acridine.

The observation that point mutations in *acrR* confer resistance to psoralen, whereas its deletion renders the strains hypersensitive, argues that regulation by this protein is more complex than previously appreciated. AcrR belongs to the TetR family of transcriptional regulators and has generally been thought to function as a repressor of the efflux genes *acrA* and *acrB* (45). If AcrR functioned as a simple repressor, one would expect that the deletion of *acrR* would upregulate expression and increase resistance. However, the opposite was observed, implying that regulation by AcrR may involve both activation and repression under various conditions. Based on the correlation of the absence of the efflux pump with increased cross-link formation (Fig. 2.8), I infer that the *acrR* point mutations in the resistant isolates upregulate the expression of the pump. Other regulators in the TetR family have similarly been reported to be able to function as both activators and repressors (64–66). One early study found that changes in *acrAB* operon expression occurred even if *acrR* was deleted and proposed that *acrR* is a secondary modulator of AcrAB expression (45).

How the various mutations in *acrR* affect protein function is unclear. Based on the reported structure of AcrR (67, 68), the point mutation in isolate 2 alters a single amino acid in the DNA binding domain. Isolates 1 and 3 contain an IS*5* insertion and a frameshift, respectively, at the end of the DNA binding domain that effectively truncate AcrR after this region (Fig. 2.13A). Folding predictions suggest that the DNA binding domain could remain intact in all three mutants (Fig. 2.13B and C) (69), which I speculate may account for the gain-of-function phenotypes observed for these alleles.

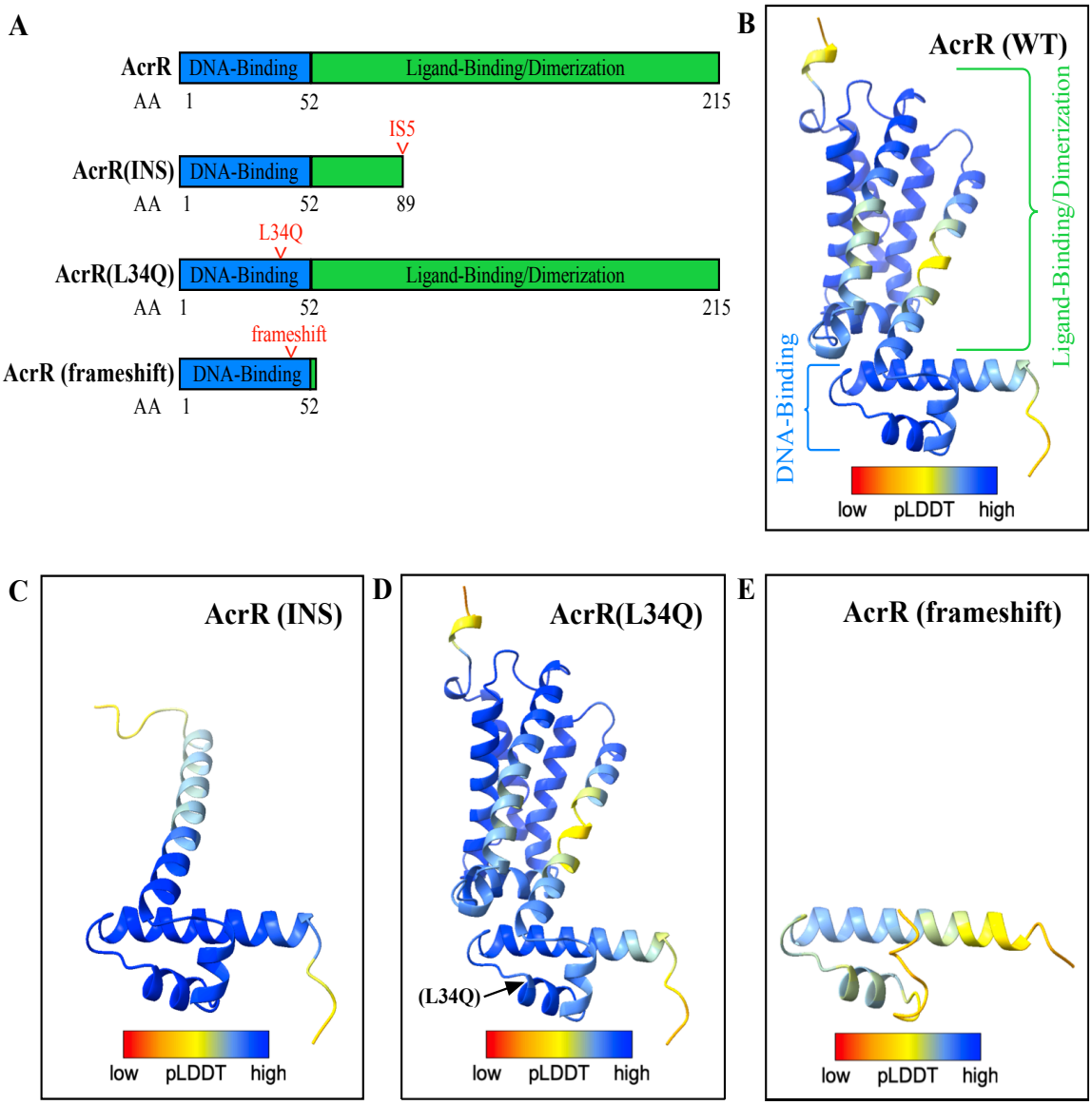

Fig. 2.13. DNA-binding domain is retained in *acrR* mutants. (A) Locations of mutations in *acrR* are shown relative to DNA-binding domain (blue) and ligand-binding/dimerization domain (green). AlphaFold predicted structures of (B) AcrR (WT); (C) AcrR (INS) from resistant isolate 1; (D) AcrR (L34Q) from resistant isolate 2; (E) AcrR (frameshift) from resistant isolate 3 are shown using ChimeraX. Colors on models correspond to pLDDT confidence score (Mariani et al 2013) from AlphaFold predictions (see included color key).

It also remains unclear how the point mutations in *rpoA* confer resistance to cross-links. Both mutations map to the C-terminal domain of the RNA polymerase alpha subunit, which is known to interact with a variety of transcriptional regulators (70–75). In many cases, these regulators are associated with resistance to other cytotoxic agents (76–78). Thus, the *rpoA* mutations could confer resistance through the regulation of any number of operons. However, the finding that *rpoA*(E273G) conferred resistance to chloramphenicol suggests that resistance may involve the regulation of factors that may suppress membrane permeability or other importers or exporters that can accommodate psoralen. Finally, although *rpoA*(E273G) appears additive with *acrR*(L34Q), I cannot rule out the possibility that this occurs exclusively through the additional upregulation of the AcrAB-TolC efflux pump. Several alternative mechanisms for how the *rpoA* alleles affect interstrand cross-linking tolerance remain possible. In addition to its regulatory roles, RNA polymerase can function as an impediment to other cellular processes or as a sensor that recruits repair enzymes to specific lesions (79–81). It remains possible that mutations affecting these activities could alter cellular tolerance to cross-linking agents.

The resistant mutants isolated here are consistent with previous work that implied that the prevention, rather than the repair, of cross-links is the primary mechanism of survival for bacteria challenged with chemicals that form these lesions (39). These results demonstrate that this prevention in *E. coli* is dependent on the active efflux of the drug, a mechanism that has been shown to be responsible for multidrug resistance in both bacteria and human cancers (23, 24, 62, 63). These results also suggest that the regulation of efflux by *acrR* is more complicated than previously believed. Additionally, while it is

suspected that the mutations in *rpoA* likely confer resistance by modulating the RNA polymerase's interactions with a variety of transcriptional regulators, the actual mechanism remains uncharacterized. It will therefore be of interest to understand how the resistance observed in this study is regulated.

Materials and Methods

Bacterial strains.

SR108, a *thyA36 deoC2* derivative of W3110 (82), was used as the parent for the selection of psoralen-resistant strains. Thymine auxotrophy was used to confirm that the selected populations were derived from the parental population and were not contaminants. To characterize candidate mutations for their contribution to psoralen-UVA resistance, mutations were placed into the BW25113 background, which is the parental strain used for the Keio collection (49). Mutations present in psoralen-resistant strains were first linked to Kan^r cassettes approximately 25 kb away by P1 transduction of *ybaT*::Kan for *acrR*(L34Q) and *chiA*::Kan for *rpoA*(E273G) from strains JW0475 and JW3300, respectively, selecting for resistance to kanamycin and psoralen-UVA irradiation (49). For *rclA*(A368G), the Kan^r cassette was recombineered into *ecpD* using primers 5′-CAGCGGCCTCTCATCGTGGGCGGCGGTGACGCAGACAGGAGAAGAGAATGATTCCGGGGATCCGTCGACC-3′ and 5′-CCAGCATACAGACCGCTGTCAGCAGGGCCTTAGTTAATGTTACGCCACGTTGTAGGCTGGAGCTGCTTCG-3′ (Eurofins Genomics, Louisville, KY, USA) to amplify the Kan^r cassette from JW0318 and transformed into electrocompetent arabinose-induced

isolate 2 cells containing plasmid pKD46 as described previously (50). P1 transduction was then performed to cotransduce each target mutation and linked mini-Kan cassette into BW25113. In the case of *acrR*(L34Q) and *rpoA*(E273G), the linked genes were confirmed by psoralen-UVA resistance in the cotransductants. The presence of *rclA*(A368G) was confirmed by sequencing (Fig. 2.14). Strains CL5333 to CL5336 were constructed by transforming pBAD33 or pBAD33-AcrAB plasmids into electrocompetent BW25113 or JW0451 cells (83). A complete list of the strains used in this study is shown in Table 2.2.

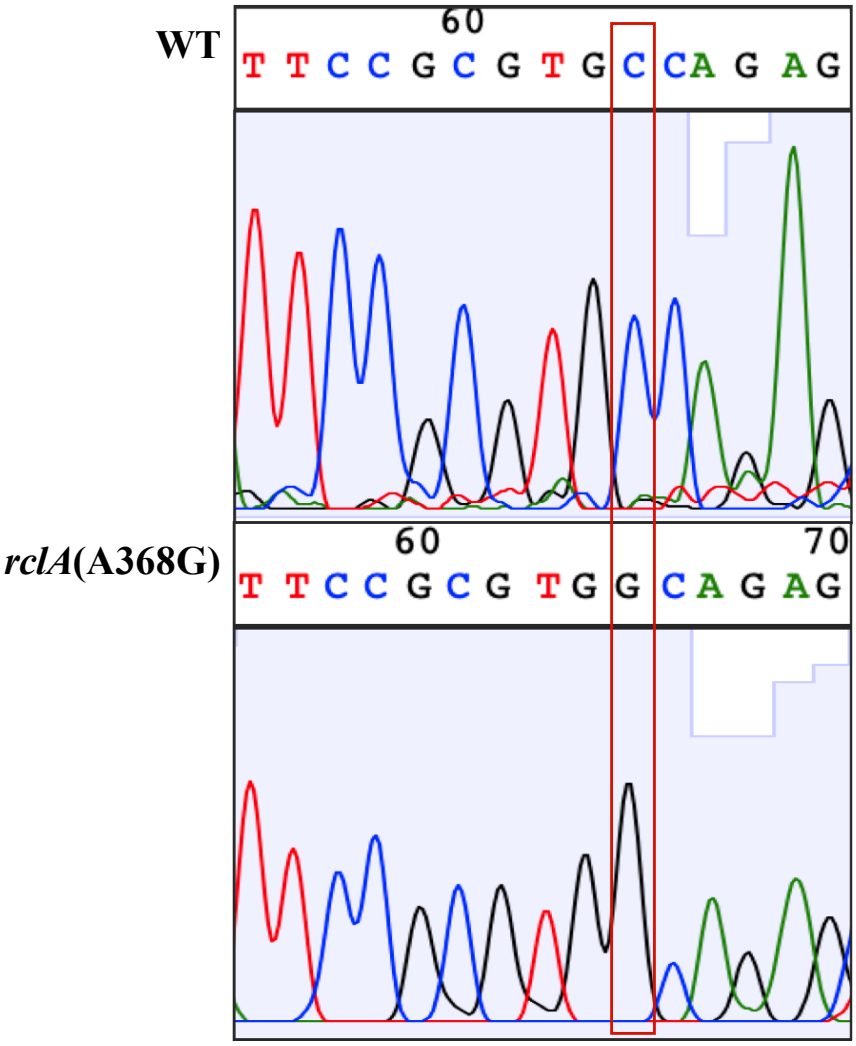

Fig. 2.14. Sequencing of *rclA* supports successful transduction of *rclA*(A368G) mutation into BW25113. (Top) Wild type *rclA*. (Bottom) *rclA*(A368G) mutation in CL5233. Red box indicates location of point mutation.

Table 2.2. List of strains used in this study.

Strain	Relevant Genotype	Source or Construction
SR108	*thyA deoC* IN(*rrnD-rrnE*)	(81)
BW25113	*lacIq rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78*	(50)
CL3844	Resistant Isolate 1	Mutagenized with psoralen and UVA
CL3845	Resistant Isolate 2	Mutagenized with psoralen and UVA
CL3846	Resistant Isolate 3	Mutagenized with psoralen and UVA
JW0453	*acrR*::FRT-minikan	(49)
JW5040	*rclA*::FRT-minikan	(49)
JW0298	*rclR*::FRT-minikan	(49)
JW5041	*ykgE*:FRT-minikan	(49)
JW0451	*acrB*::FRT-minikan	(49)
CL4426	Resistant Isolate 1 *acrB*::FRT-minikan	P1 transduction of *acrB*::FRT-minikan from JW0451 into Resistant Isolate 1
CL4427	Resistant Isolate 2 *acrB*::FRT-minikan	P1 transduction of *acrB*::FRT-minikan from JW0451 into Resistant Isolate 2
CL4428	Resistant Isolate 3 *acrB*::FRT-minikan	P1 transduction of *acrB*::FRT-minikan from JW0451 into Resistant Isolate 3
JW0475	*ybaT*::FRT-minikan	(49)
JW3300	*chiA*::FRT-minikan	(49)
JW0284	*ecpD*::FRT-minikan	(49)
CL5227	Resistant Isolate 2 *ybaT*::FRT-minikan	P1 transduction of *ybaT*::FRT-minikan from JW0475 into Resistant Isolate 2
CL5228	Resistant Isolate 2 *chiA*::FRT-minikan	P1 transduction of *chiA*::FRT-minikan from JW0475 into Resistant Isolate 2
CL5229	Resistant Isolate 2 *ecpD*::FRT-minikan	Recombineering to replace *ecpD* in Resistant Isolate 2 with FRT-minikan
CL5230	*acrR*(L34Q)	P1 cotransduction of *acrR*(L34Q) and *ybaT*::FRT-minikan from CL5227 into BW25113
CL5231	*rpoA*(E273G)	P1 cotransduction of *rpoA*(E273G) and *chiA*::FRT-minikan from CL5228 into BW25113
CL5232	*acrR*(L34Q)*rpoA*(E273G)	P1 cotransduction of *rpoA*(E273G) and *chiA*::FRT-minikan from CL5231 into CL5234
CL5233	*rclA*(A368G)	PI cotransduction of *ecpD*::FRT-minikan and *rclA*(A368G) from CL5229 into BW25113
CL5235	Resistant Isolate 2 *chiA*::FRT	Removal of minikan cassette from CL5227 via pCP20 expression of FLP recombinase
CL5333	pBAD33	Transformation of pBAD33 (82) into BW25113
CL5334	pBAD33-acrAB	Transformation of pBAD33-*acrAB* (82) into BW25113
CL5335	*acrB*::FRT-minikan pBAD33	Transformation of pBAD33 (82) into JW0451
CL5336	*acrB*::FRT-minikan pBAD33-acrAB	Transformation of pBAD33-*acrAB* (82) into JW0451

Selection for psoralen-UVA resistance.

Aliquots (0.1 mL) of a fresh culture grown overnight in Luria-Bertani medium supplemented with 10 μg/mL thymine (LBthy) were spread onto LBthy agar plates supplemented with 20 μg/mL 8-methoxypsoralen and UVA irradiated using two 32-W UVA bulbs (peak emittance at 320 nm) at an incident dose of 5.05 J/m^2/s for increasing exposure times. Following incubation overnight at 37°C, 100 μL of bacteria was scraped and collected from the plate irradiated with the lowest dose where cell lethality was evident (i.e., almost, but not quite, a lawn of bacteria), resuspended in 1 mL of LBthy medium, and used to inoculate a new 5-mL culture grown overnight before the selection process was repeated. A portion of the culture from each successive selection passage was frozen in LBthy medium supplemented with 20% glycerol and stored at −80°C for future characterization. This process was used to generate three independently derived psoralen-UVA-resistant strains, designated resistant isolate 1, resistant isolate 2, and resistant isolate 3. Resistant isolate 1 was isolated after six rounds of selection, whereas resistant isolates 2 and 3 were isolated after seven rounds of selection.

Genomic DNA purification.

Genomic DNA was purified from 0.75 mL of the culture by the addition of 0.75 mL of ice-cold 2× NET buffer (100 mmol/L NaCl, 10 mmol/L Tris [pH 8.0], 10 mmol/L EDTA) before cells were pelleted and resuspended in 140 μL of TE (10 mmol/L Tris [pH 8.0], 10 mmol/L EDTA) containing 1 mg/mL of lysozyme (Thermo Scientific, Waltham, MA, USA) and 0.2 mg/mL of RNase A (MP Biomedicals, Irvine, CA, USA). The samples were then treated with 10 μL each of 10 mg/mL of proteinase K

(Thermo Scientific, Waltham, MA, USA) and 20% Sarkosyl (Thermo Scientific, Waltham, MA, USA) and incubated for 30 min at 37°C. Following incubation, samples were extracted with 4 volumes of a 1:1 mixture of phenol-chloroform. Finally, samples were dialyzed on 47-mm Whatman 0.05-μm-pore-size discs (Merck Millipore, Darmstadt, Germany) floating in 250-μL beakers of TE (10 mmol/L Tris [pH 8.0], 10 mmol/L EDTA).

Genome sequencing of resistant strains.

Purified genomic DNA from each strain was sequenced using seqWell (Beverly, MA, USA) library prep kits and 50-bp single-end Illumina (San Diego, CA, USA) NextSeq 2000 high-throughput DNA sequencing according to the manufacturers' instructions. Sequence reads were then aligned and compared to the SR108 parent genome using Breseq (84) to identify mutations that arose in the resistant strains.

Psoralen-UVA survival.

Ten-microliter aliquots of 10-fold serial dilutions from cultures grown overnight were spotted onto LBthy plates containing 20 μg/mL 8-methoxypsoralen. The plates were then exposed to UVA irradiation at an incident dose of 6.5 J/m^2/s for the indicated doses and incubated overnight at 37°C. The surviving colonies at each dose were then counted and compared to those on the nonexposed plates to calculate the percent survival.

For the overexpression of AcrAB from expression vectors, 5-mL LBthy subcultures were inoculated with 50 μL of cultures grown overnight containing the expression plasmid and grown in a 37°C shaking water bath to an optical density at 600 nm (OD_{600}) of 0.4. l-

Arabinose (1 mM) was added to the subcultures for the last 30 min of incubation before proceeding with the survival assay as described above.

UVC survival.

Ten-microliter aliquots of 10-fold serial dilutions from cultures grown overnight were spotted onto LBthy plates. The plates were then exposed to UVC irradiation at an incident dose of 0.8 J/m^2/s for the indicated doses and incubated overnight at 37°C. The surviving colonies at each dose were then counted and compared to those on the nonexposed plates to calculate the percent survival.

Chloramphenicol resistance.

Ten microliters of 5, 10, or 20 mg/mL of chloramphenicol in ethanol (EtOH) was spotted onto 7-mm Whatman paper discs and allowed to dry for 1 h. Discs treated with only EtOH served as controls. One hundred fifty microliters of the cultures grown overnight were spread onto Davis medium supplemented with 0.4% glucose, 0.2% Casamino acids, and 10 μg/mL thymine (DGCthy) with a cotton swab, antibiotic discs were placed onto the surfaces of the plates, and the plates were incubated overnight at 37°C. The diameters of the zones of inhibition were measured using ImageJ software (85).

***In vivo* detection of DNA interstrand cross-links.**

The detection of cross-linked DNA was performed as previously described (39, 56). Briefly, cultures containing the plasmid pBR322 were grown overnight in DGCthy medium supplemented with 50 μg/mL ampicillin at 37°C. A 0.1-mL aliquot of this culture was pelleted, resuspended in 10 mL DGCthy medium without ampicillin, and

grown in a 37°C shaking water bath to an OD_{600} of 0.4. The cultures were treated with 20 μg/mL 8-methoxypsoralen for 10 min at 37°C and subsequently irradiated with the indicated doses of UVA light. Aliquots (0.75 mL) were collected and transferred to an equal volume of ice-cold 4× NET buffer, and the genomic DNA was purified as described above. The purified DNA was digested with PvuII (New England BioLabs, Ipswich, MA, USA), which linearizes pBR322, before samples were electrophoresed on a 0.75% alkaline agarose gel in a solution containing 30 mM NaOH and 1 mM EDTA at 1 V/cm for 16 h. The DNA in the gels was transferred to Hybond N^+ nylon membranes (Cytiva, Marlborough, MA, USA), and the plasmid DNA was visualized by probing with ^{32}P-labeled pBR322 prepared using a Prime-It RmT labeling kit (Agilent Technologies, Santa Clara, CA, USA) with >6,000 Ci/mmol [α-^{32}P]dCTP (PerkinElmer, Waltham, MA, USA). Southern blots were visualized and quantitated using the Storm 840 phosphorimager and its associated ImageQuant analysis software (Cytiva, Marlborough, MA, USA).

Data availability.

The sequencing data for the parental and resistant isolates have been deposited in the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra/) under BioProject accession number PRJNA952657.

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Chapter III. AcrR and MarA/SoxS/Rob confer psoralen-UVA resistance independently through upregulation of AcrAB- TolC efflux pump.

Abstract

Compounds that form DNA interstrand crosslinks can be found in nature as defensive mechanisms produced by plants and bacteria and can be used to effectively treat a variety of dysplastic conditions including some cancers. However, resistance can emerge to these agents, despite a limited ability of cells to repair DNA interstrand crosslinks. In previous work, I found that resistance could be achieved through mutations in the AcrR regulator that result in upregulation of *acrAB*, which encodes components of the AcrAB-TolC efflux system. Expression of *acrAB* is also regulated by three, related global regulators of environmental stress- MarA, SoxS, and Rob. Here, I show that all three global regulators contribute to psoralen-UVA resistance. Their contribution is shown to occur primarily through direct upregulation of *acrAB* expression. I also show that AcrR confers psoralen-UVA resistance by direct upregulation of *acrAB* that does not depend on MarA, SoxS, Rob, or induction by exogenous stressors.

Introduction

Psoralen-UVA (PUVA) irradiation is used in the treatment of psoriasis and vitiligo, as well as in the treatment of cutaneous T-cell lymphoma (1, 2). The potency of this treatment, and similar therapeutics, in the destruction of abnormal cells, particularly cancerous cells, is attributed to its ability to form a particularly lethal form of DNA lesion known as DNA interstrand crosslinks (3–6). Studies have found that a single DNA interstrand crosslink in the genome of *E. coli*, the model system used in this study, is

sufficient to inactivate the cell (7, 8). However, the continued clinical use of psoralen-UVA and other crosslinking agents has been threatened by the emergence of resistance to these drugs (9, 10). Following previous work that showed that the capacity of cells to repair DNA interstrand crosslinks was limited (8), I sought to investigate how resistance to crosslinking agents arises. To that end, I characterized mutations in strains of *E. coli* that became highly resistant to psoralen-UVA irradiation following repeated exposures and found that resistance to psoralen-UVA could be driven through upregulation of the AcrAB-TolC efflux pump (11).

AcrAB-TolC is a member of the RND family of efflux pumps, a highly conserved family of transporters in Gram-negative bacteria (12–16). Characterization of this pump has involved multiple research groups over several decades (17), demonstrating that it consists of a proton-driven transporter AcrB, a periplasmic adapter protein AcrA, and the TolC transmembrane channel (17–20). Importantly, the AcrAB-TolC pump is capable of effluxing a wide variety of structurally dissimilar substrates, including many dyes, detergents, and antibiotics (21–25). Given this ability, it is not surprising that AcrAB-TolC is a primary driver of multiple-antibiotic resistance (23), making the regulation of this system of particular interest to researchers.

Following their initial characterization of *acrA* and *acrB*, Ma et al. demonstrated that the gene *acrR*, located upstream of *acrAB*, encodes a TetR family transcriptional regulator (26). Based on *lac*-fusion and gel mobility shift assays, they proposed that AcrR functioned as a repressor of AcrAB expression that would release from the promoter upon binding a recognized substrate (27). Since then, structural studies have confirmed

that some compounds known to be substrates of the efflux pump, such as ethidium bromide and proflavine, can bind to AcrR (28, 29) and that this binding appears to promote a conformational change in the DNA binding domain of AcrR that correlates with loss of DNA binding activity (30).

In previous work, I isolated several mutations in *acrR* that resulted in upregulation of the AcrAB-TolC efflux pump and correlated with psoralen-UVA resistance. I speculated that these mutations would confer a loss of AcrR function given the repressor model of AcrR's regulation of *acrAB* transcription (27). The model would predict that loss of AcrR would presumably derepress *acrAB* transcription, leading to elevated levels of the AcrAB-TolC pump and greater efflux of psoralen. However, I was surprised to find that, while the isolated mutations of *acrR* led to resistance to psoralen-UVA irradiation, a complete deletion of *acrR* caused modest hypersensitivity to psoralen-UVA. The observation led us to propose the *acrR* mutations represented a gain of function and that AcrR, like some other TetR family regulators (31, 32), may act as an activator under certain conditions.

Though the mechanism by which the isolated *acrR* mutations would confer a gain of function is unclear, all three mutations isolated affect the C-terminus of the protein but leave the N-terminal DNA binding domain of AcrR intact, an observation also supported by the current version of the structural prediction software, AlphaFold (33). Thus, one mechanism by which the resistance conferred by mutations in *acrR* could occur would be that they alter the protein to make it a constitutive DNA-binding activator. However, an alternative mechanism is that the 5' region that remains intact also contains a marbox

binding sequence for three closely related regulators of global stress responses occurs within the first 20 nucleotides of *acrR* (34). These three regulators, MarA, SoxS, and Rob, share approximately 50% sequence identity (35, 36) and regulate expression of approximately 50 genes, including *acrA* and *acrB*, in response to various environmental stressors and toxins (Fig. 3.1 and (34, 37–43).

Using a *lac*-reporter construct and gel mobility shift assays, Ma et al. demonstrated activation of *acrAB* expression correlated with protein binding to the upstream marbox sequence (27). Expression of *acrAB* was also upregulated by stress induced by ethanol or high osmolarity in the growth medium (27). The induction did not require MarA, SoxS, or AcrR, leading the researchers to propose that a third as yet unidentified protein could bind marbox sequences, subsequently revealed to be the right-*oriC* binding protein, Rob (36). Importantly, Ma et al. reported the induced expression of *acrAB* during stress was more robust than that produced by AcrR, leading them to propose these global stress activators were drivers of *acrAB* expression, with AcrR serving as a secondary modulator.

Thus, in this work, I sought to differentiate between these two possibilities and to determine whether the resistance conferred by mutations in *acrR* was due to changes in global regulation by MarA, SoxS, and Rob or alteration of AcrR protein function.

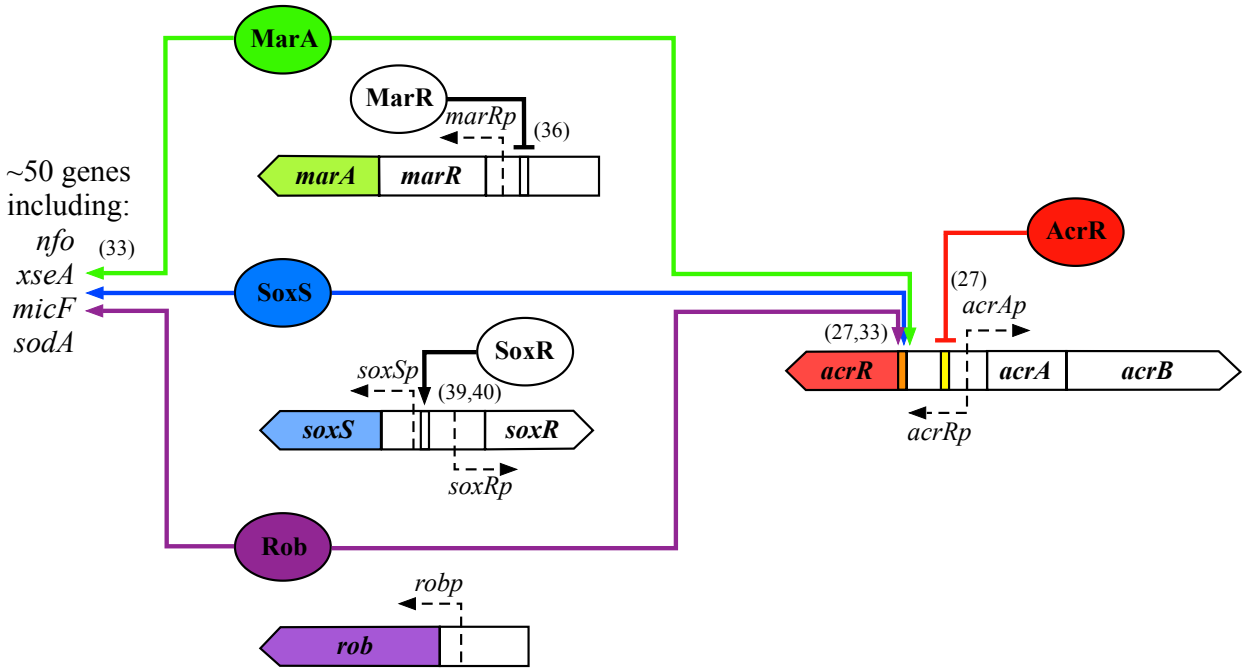

Figure 3.1. Psoralen-UVA resistance conferred by *acrR*(L34Q) may be dependent on activation by MarA, SoxS, and Rob. Green, MarA; blue, SoxS; purple, Rob; red, AcrR; yellow, DNA binding sites; orange, Mar/Sox/Rob binding site (marbox). Arrows indicate activation, while interruption of the end of a line indicates repression.

Results

Global effectors MarA, SoxS, and Rob are required for full resistance to psoralen-UVA.

We previously described an *acrR*(L34Q) mutation that confers resistance to psoralen-UVA (Fig. 3.2A and (11)). In contrast, deleting the entire *acrR* coding region renders cells hypersensitive (Fig. 3.2A and (11)), suggesting the point mutation represents a gain of function that results in upregulation of the AcrAB-TolC efflux pump. A total of three resistant *acrR* mutants were isolated and common to all is that they retain the initial third of *acrR*'s coding sequence but alter or remove the latter two thirds of the protein (11). The retained region contains both the MarA, SoxS, and Rob binding sequence, which positively regulates divergent *acrAB* (27, 34), and encodes for the DNA-binding domain for the AcrR regulator. Thus, it is possible that the mutations could confer psoralen resistance could through either of these effectors.

If resistance in the *acrR* point mutants is mediated through the marbox, then deletion of the *marA*, *soxS*, and *rob* regulators would be expected to impair resistance in these strains. To examine this possibility, I first examined the ability of mutants deleted for these genes to survive psoralen-UVA treatment. Ten-fold serial dilutions of an overnight culture were spotted on plates containing 20 μg/mL 8-methoxypsoralen and exposed to increasing doses of UVA. Following overnight incubation at 37 C, surviving colonies were counted and compared to the unexposed plate to determine percent survival. Figure 3.2B, C, and D shows that deletion of either *marA*, *soxS*, or *rob* renders cells more sensitive than WT to psoralen-UVA irradiation, indicating that all three of

these genes are important for psoralen-UVA resistance. Notably, the contribution of each was not additive, as the absence of any single regulator resulted in hypersensitivity that was similar to the *marA soxS rob* triple mutant (Fig. 3.2E). The observation indicates that all three proteins are required to maintain psoralen-UVA resistance, despite their having a shared binding sequence.

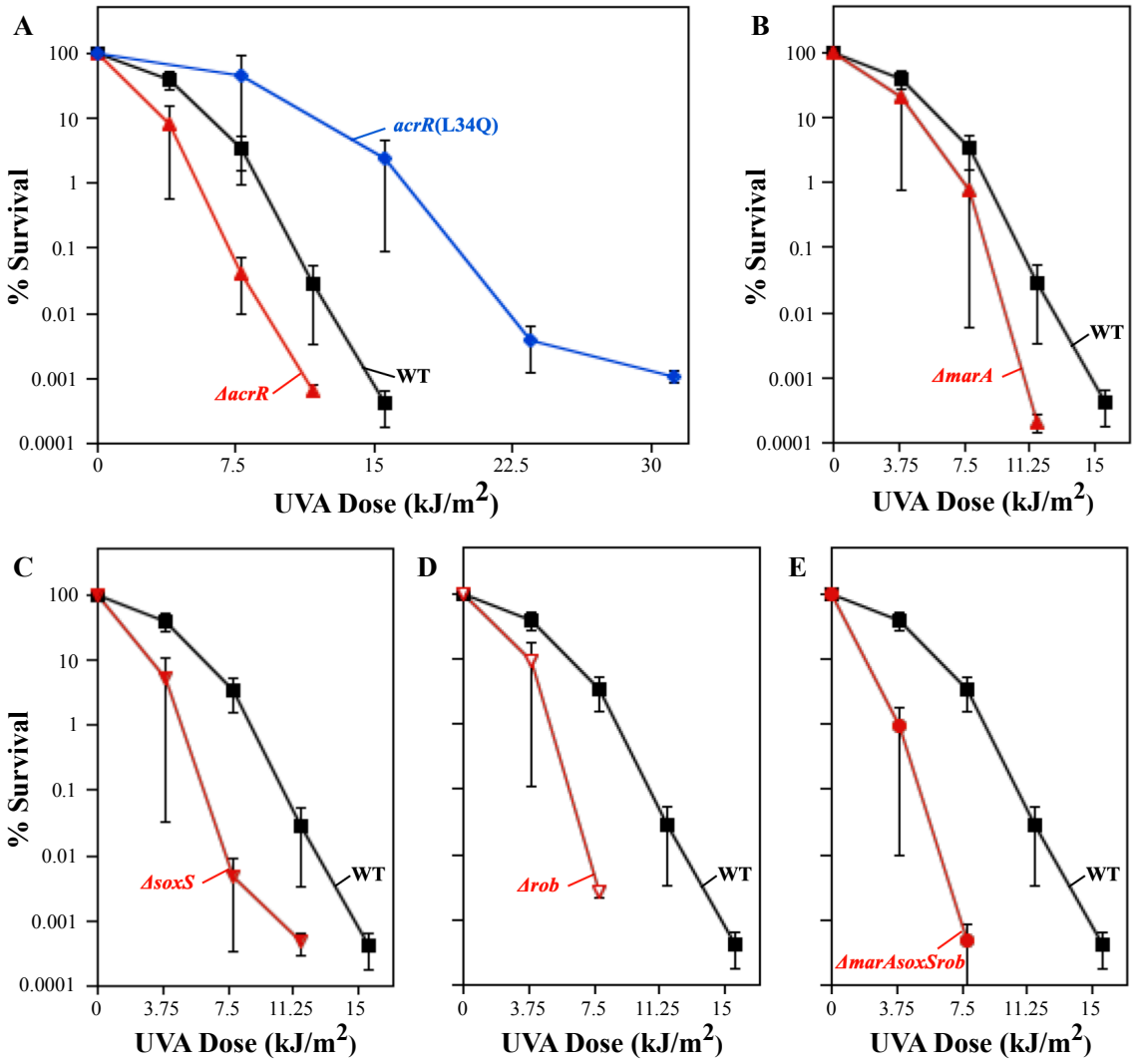

Figure 3.2. *acrR*(L34Q), but not *acrR* deletion mutant, confers resistance to psoralen – UVA. MarA, SoxS, and Rob are all required for full resistance to psoralen-UVA**.** The survival of BW25113 wild type (black, filled square); (A) *acrR* deletion mutant (red, filled triangle), *acrR*(L34Q) mutant (blue, filled diamond); (B) *marA* deletion mutant (red, open triangle); (C) *soxS* deletion mutant (red, inverted open triangle); (D) rob deletion mutants (red, inverted filled triangle); (E) *marA soxS rob* triple mutant (red, filled circle) in the presence of 20 µg/mL 8-methoxypsoralen at the indicated UVA doses is plotted. Plots represent the average of at least two independent experiments. Error bars represent the standard error of the mean.

MarA, SoxS, and Rob contribute to psoralen-UVA resistance primarily through upregulation of *acrAB*.

MarA, SoxS, and Rob upregulate the expression of approximately 50 genes in response to various cellular stresses (34). Thus, although the results of Fig. 3.2 indicate that MarA, SoxS, and Rob are required for full resistance to psoralen, they do not establish if this contribution can be attributed directly to the upregulation of *acrAB* or if resistance is conferred by other marbox-regulated genes. To test this, I used an arabinose-inducible *acrAB* plasmid to overexpress *acrAB* in the *marA*, *soxS*, *rob* and *marA soxS rob* deletion mutants, which would result in the upregulation of *acrAB*, but none of the other marbox-regulated genes. To this end, wild type or mutant cells containing the plasmid were grown to mid-log phase before arabinose was added for 30 minutes to induce *acrAB* expression prior to psoralen-UVA treatment. Figure 3.3 shows that the presence of the *acrAB* expression plasmid increases the resistance of *marA*, *soxS*, and *rob* mutants to near wild type levels. By contrast, these mutants containing an identical plasmid lacking the *acrAB* sequence remain hypersensitive to psoralen-UVA treatment. The results indicate that MarA, SoxS, and Rob contribute to psoralen-UVA resistance primarily through upregulation of *acrAB* expression and support the idea that loss of this upregulation in the *acrR* deletion mutant could be responsible for its inability to confer resistance.

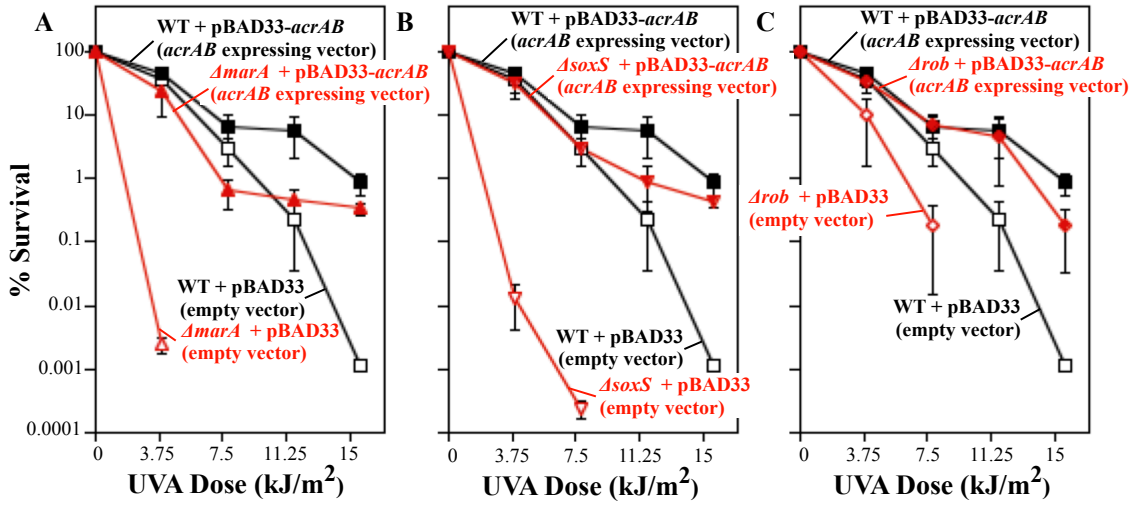

Figure 3.3 Overexpression of *acrAB* rescues sensitivity of *marA*, soxS, *rob* deletion mutants to psoralen-UVA irradiation. Survival of BW25113 + pBAD33 vector (black, open square), BW25113 + pBAD33-*acrAB* (black, filled square); (A) *marA* + pBAD33 vector (red, open triangle) *marA* + pBAD33-*acrAB* (red, filled triangle); (B) *soxS* + pBAD33 vector (red, inverted open triangle), *soxS* + pBAD33-*acrAB* (red, inverted filled triangle); (C) *rob* + pBAD33 vector (red, open diamond), and *rob* + pBAD33-*acrAB* (red, filled diamond) in the presence of 20 μg/ml 8-methoxy-psoralen at the indicated UVA doses is plotted. Plots represent average of at least two independent experiments. Error bars represent the standard error of the mean.

AcrR confers psoralen-UVA resistance even in the absence of MarA, SoxS, and Rob.

Considering that MarA, SoxS, and Rob are required for full resistance to psoralen-UVA (Fig. 3.2B, C, D), and that this resistance appears to be driven by upregulation of *acrAB* (Fig. 3.3), I hypothesized that the higher resistance of the *acrR*(L34Q) mutant relative to the *acrR* deletion could be the result of additional upregulation by MarA, SoxS, or Rob. As mentioned earlier, complete deletion of *acrR* removes the marbox upstream of *acrAB*, which would be expected to prevent upregulation by MarA, SoxS, and Rob. If true, I would expect that deletion of *marA*, *soxS*, or *rob* would significantly reduce the level of psoralen-UVA resistance conferred by *acrR*(L34Q) to a similar level exhibited by that of the *acrR* deletion. To test this possibility, I examined how survival of *acrR*(L34Q) mutants were affected by the presence or absence of MarA, SoxS, or Rob. As shown in Fig. 3.4, unlike in the wildtype background, *acrR*(L34Q) mutants remained resistant, even when *marA*, *soxS*, or *rob* were deleted. The results are consistent with a mechanism in which *acrR*(L34Q) confers psoralen-UVA resistance by directly derepressing *acrAB* expression and that additional upregulation by MarA, SoxS, and Rob is not required for this resistance. That deletion of *marA*, *soxS*, and *rob* again had minimal impact on the resistance of cells overexpressing *acrAB* provides further support for the role of AcrAB-TolC as the primary driver of psoralen-UVA resistance.

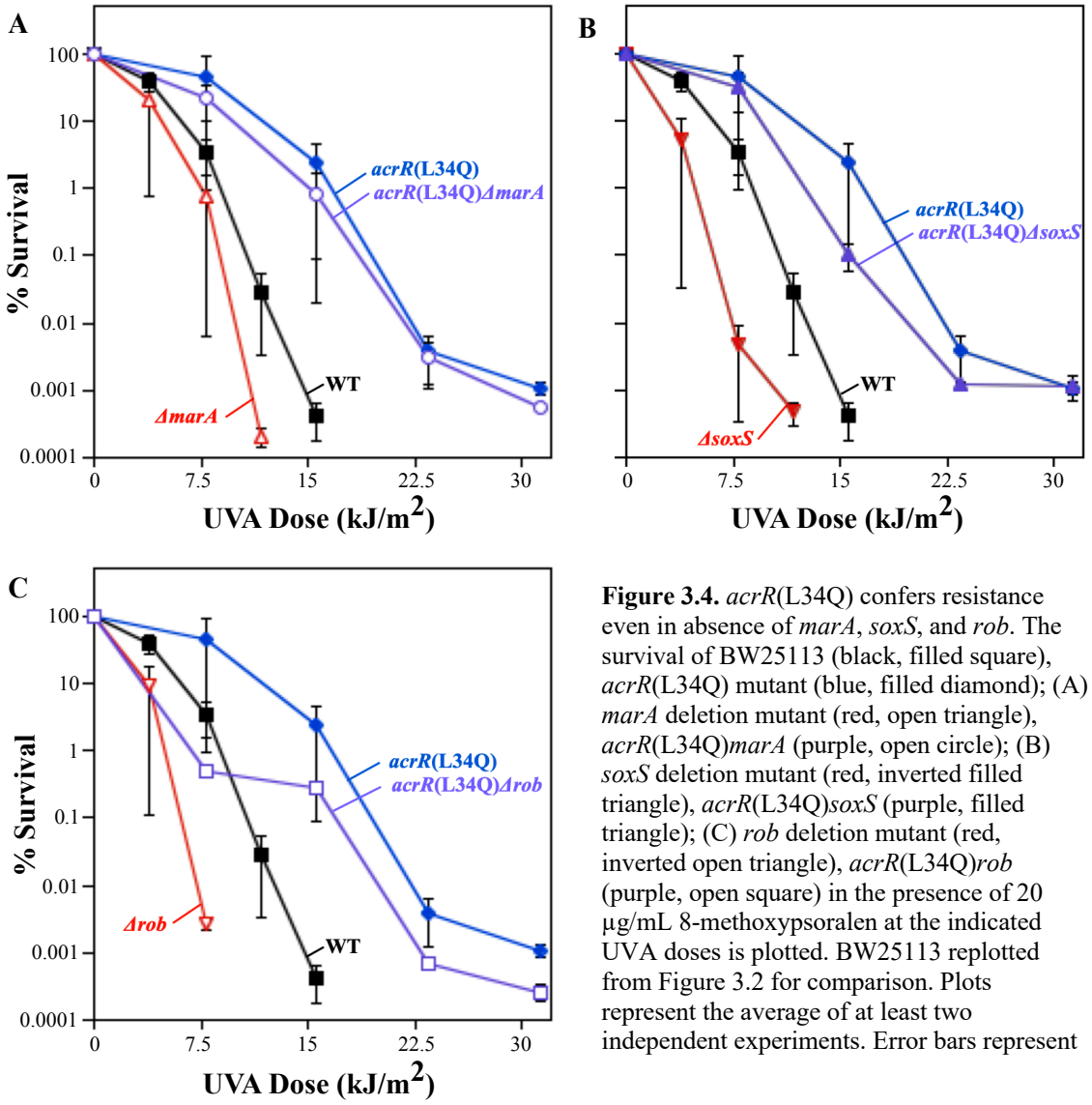

Figure 3.4. *acrR*(L34Q) confers resistance even in absence of *marA*, *soxS*, and *rob*. The survival of BW25113 (black, filled square), *acrR*(L34Q) mutant (blue, filled diamond); (A) *marA* deletion mutant (red, open triangle), *acrR*(L34Q)*marA* (purple, open circle); (B) *soxS* deletion mutant (red, inverted filled triangle), *acrR*(L34Q)*soxS* (purple, filled triangle); (C) *rob* deletion mutant (red, inverted open triangle), *acrR*(L34Q)*rob* (purple, open square) in the presence of 20 µg/mL 8-methoxypsoralen at the indicated UVA doses is plotted. BW25113 replotted from Figure 3.2 for comparison. Plots represent the average of at least two independent experiments. Error bars represent

***acrAB* expression is not induced by psoralen, UVA, or psoralen-UVA irradiation.**

The results suggest psoralen-UVA resistance is mediated primarily through *acrAB* expression and that *acrR*(L34Q) confers psoralen-UVA resistance through upregulation of *acrAB*. Expression of *acrAB* can be induced by exposure to ethidium bromide and cellular metabolites such as cadaverine (44), as well as stress induced by ethanol or high osmolarity (27). However, it remains unclear if the wild type *acrAB* allele can be induced in response to psoralen-UVA as would be expected if some component of the treatment acts as a substrate for the AcrR protein. One possibility is that psoralen binds AcrR directly to release it from the *acrAB* promoter, thus upregulating expression of the pump. Alternatively, UVA radiation alone or reactive oxygen species that result from UVA irradiation could act as the signal for upregulation. A third possibility is that that the DNA adducts created by psoralen-UVA irradiation could be recognized by the cell and result in upregulation of the efflux pump. To differentiate between these possibilities, I used a LacZ-reporter plasmid that contained an *acrABp-lacZ* fusion. The promoter region on this plasmid retained both the AcrR binding site as well as the first 102 nt of the *acrR* coding sequence which contains the marbox binding site. To examine if components of psoralen-UVA treatment can serve as substrates for AcrR and induce *acrAB* expression, cultures of both the parental strain and the *acrR*(L34Q) mutant containing the plasmid were spotted in 10 µL serial dilutions on X-Gal plates that were left untreated or exposed to either psoralen, UVA, or psoralen-UVA. As shown in Figure 3.5, neither the parental nor *acrR*(L34Q) showed any LacZ expression under any conditions in the presence of the control plasmid. In the presence of the *acrAB-lacZ* reporter, the parental strain detectably

expressed the *acrAB* genes as indicated by the partially blue colonies. *acrAB* expression was noticeably higher in the presence of the *acrR*(L34Q) mutation, which correlates with the level of resistance shown in each strain. Unexpectedly, the expression remained similar under all conditions examined. Neither psoralen, UVA, nor the combination of the two altered the expression of the *acrAB-lacZ* reporter in either parent or *acrR*(L34Q) mutant. The results would suggest that psoralen, UVA, or the combination do not generate substrates that can bind and serve to inactivate the AcrR repressor. Instead, the elevated level of *acrAB* generated in all conditions in the presence of *acrR*(L34Q) support the idea that the mutant results in a constitutively derepressed state. However, I cannot rule out the possibility that the reporter construct is missing sequences that are critical for this regulation to occur. Additionally, it remains possible that the conditions for induction are responsive to other factors than UVA and or psoralen. Irrespective of the inducibility, reporter expression appears to correlate with both overall cellular resistance to psoralen-UVA treatment and the expression of *acrAB*.

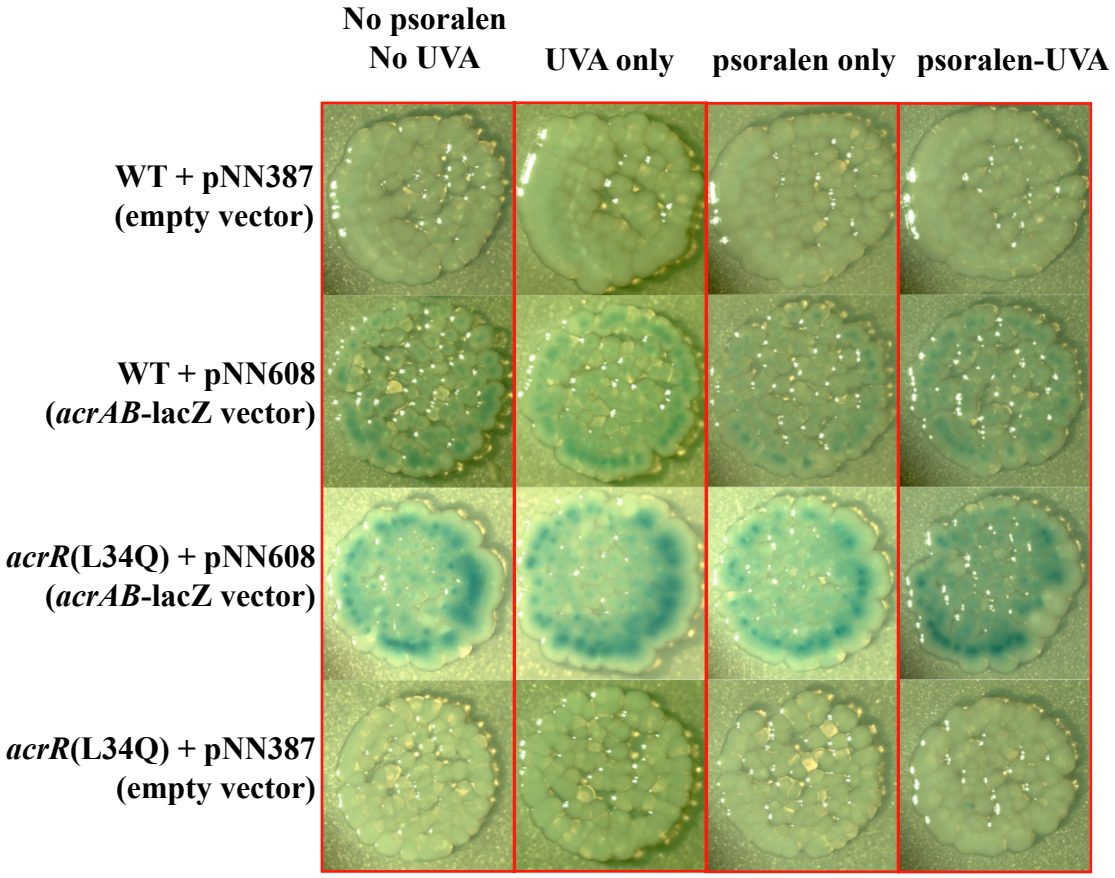

Figure 3.5. Expression of *acrAB* is not induced by psoralen, UVA, or psoralen-UVA. Representative images of 10 µL spots of 10-fold serial dilutions plated on LBthy X-Gal with or without 20 µg/mL 8-methoxypsoralen. UVA only and psoralen-UVA plates were exposed to 3.8 kJ/m^2 UVA radiation. WT + pNN387 and *acrR*(L34Q) + pNN387 serve as negative controls. AcrAB expression from pNN608 plasmid is indicated by blue color in colonies.

Discussion

The results described above demonstrate that all three of the global effectors MarA, SoxS, and Rob have a significant role in the resistance of cells to psoralen-UVA resistance (Fig. 3.2B). Given the global nature of the Mar/Sox/Rob stress pathway and its ability to respond to a wide variety of stressors, this result was not in itself surprising (34, 37–39, 42). However, the fact that deletion of any one of the genes for the global effectors had a similar impact on psoralen-UVA resistance to deletion of another, or even all three genes, argues not only that MarA, SoxS, and Rob contribute similarly to psoralen-UVA resistance, but that all three proteins are required to maintain this resistance. This result is unexpected for several reasons. First, while *rob* is believed to be expressed constitutively, *marA* and *soxS* are expressed at relatively low levels until recognition of a stressor by their local regulators *marR* or *soxR* induces expression, thereby activating the global Mar/Sox/Rob stress response (Fig. 3.1 and (34, 35, 37, 38, 42, 45). While MarA, SoxS, and Rob are all apparently necessary for psoralen-UVA resistance, the results of Fig. 3.2B, C, D do not suggest that a single effector is solely responsible for initiation of a global response to psoralen-UVA irradiation. Furthermore, given the high level of homology between MarA, SoxS, and Rob and the resulting ability of these proteins to bind to the same sites across the genome, albeit with differential affinity (46), it would be expected that loss of one regulator could potentially be offset by binding of the other two (34, 35, 37, 38, 42). If such redundancy truly existed, one would again expect that deletion of *marA*, *soxS*, and *rob* together would have a significantly greater impact on psoralen-UVA resistance than deletion of any of these genes

individually, which was not observed in this study (Fig. 3.2E). Finally, the similar sensitivity of the *marA*, *soxS*, and *rob* deletion mutants argues that their differential affinity for binding sites across the genome (46) has a relatively small impact on psoralen-UVA resistance.

The majority of the hypersensitivity of the *marA*, *soxS*, and *rob* deletion mutants was rescued by overexpression of *acrAB,* suggesting that the main cause of this hypersensitivity was loss of activation of *acrAB* by the Mar/Sox/Rob pathway. Based on these results, and the results of previous work describing how psoralen-UVA resistance develops (11), I infer that psoralen export by the AcrAB-TolC efflux pump is the primary mechanism by which *E. coli* resists psoralen-UVA irradiation. With the confirmation that MarA, SoxS, and Rob all have a substantial contribution to psoralen-UVA resistance (Fig. 3.2B, C, and D) and evidence that this contribution is primarily mediated through upregulation of *acrAB* (Fig. 3.3), it seemed increasingly likely that activation by these proteins could be responsible for some of the resistance of the *acrR*(L34Q) mutant. In contrast, the loss of the marbox residing within *acrR* upon deletion of the entire gene would preclude upregulation of *acrAB* expression by these proteins. I anticipated that demonstration of this dependence on the presence of an intact marbox could explain how mutations in AcrR can lead to a gain of function in terms of psoralen-UVA resistance, while deletion of *acrR* does not (Fig. 3.2A), despite strong evidence that AcrR represses expression of *acrAB* (27). Furthermore, in the initial characterization of AcrR, Ma et al. concluded that AcrR had a secondary role in the regulation of *acrAB*, with MarA, SoxS, and Rob serving as the primary regulators (27). However, I interpret the results of Fig.

3.4 to demonstrate that the *acrR*(L34Q) mutation does not depend on the presence of MarA, SoxS, or Rob to confer psoralen-UVA resistance. Upregulation of *acrAB* by *acrR*(L34Q) was also sufficient to compensate for the loss of MarA, SoxS, or Rob, providing further evidence that the hypersensitivity of the *marA*, *soxS*, and rob deletion mutants is caused by downregulation of *acrAB*.

Finally, in seeking to better understand how upregulation of *acrAB* by *acrR*(L34Q) occurs, I found evidence to suggest that induction of the operon by an exogenous stressor is not required in the response to psoralen-UVA. Local control of *acrAB* is believed to be achieved by the binding of AcrR to the promoter, with AcrR functioning as a repressor of *acrAB* expression (27, 44, 47). Several of the known substrates of the AcrAB-TolC efflux pump have been shown to act as ligands for AcrR (28–30, 44, 47). Binding of a ligand induces a conformational change in AcrR that leads to its release from the *acrAB* promoter, thus leading to upregulation of the efflux pump (28–30, 44, 47). While many studies have provided evidence for this mechanism in AcrR and other TetR family transcriptional regulators (28–30, 44, 47), relatively few ligands of AcrR have been characterized compared to the large number of known substrates of AcrAB-TolC (44, 47). The fact that the presence of psoralen, with or without UVA, did not detectably induce expression of AcrAB-TolC suggests that psoralen may not be recognized by AcrR, despite clear evidence that it is a substrate of the AcrAB-TolC efflux pump (11). Additionally, the lack of induction under all tested conditions further argues against the dependence of *acrAB* on detection of a signal and subsequent upregulation by the Mar/Sox/Rob pathway in the response to psoralen-UVA irradiation.

Through this study, I have demonstrated that the *acrR*(L34Q) mutation confers resistance to psoralen-UVA irradiation by upregulating expression of *acrAB* and that this upregulation does not depend on the Mar/Sox/Rob pathway or on induction by an exogenous stressor. Our results indicate that while psoralen is a substrate of the AcrAB-TolC efflux pump, it does not appear to be a ligand of AcrR and does not appear to induce expression of *acrAB* through any other means. It will be of interest to determine whether other known substrates of AcrAB-TolC demonstrate a similar lack of induction. I have also shown that MarA, SoxS, and Rob contribute similarly to psoralen-UVA resistance and are all required to provide full resistance in wild type cells. Additionally, I provided evidence that the primary mechanism by which the Mar/Sox/Rob global pathway confers resistance to psoralen-UVA irradiation is through upregulation of efflux by AcrAB-TolC, though it will be of value to determine whether other operons regulated by this pathway have minor contributions to resistance. Finally, I conclude that efflux of psoralen by the AcrAB-TolC efflux pump is the main driver of resistance to psoralen-UVA in *E. coli* and that this provides further evidence to support our previous assertion that prevention, rather than repair, is the strategy by which cells respond to challenge with DNA interstrand crosslinks (11).

Materials and Methods

Bacterial Strains.

All strains utilized in this study were derived from BW25113, which is the parent strain of the Keio collection (48), from which the *acrR*, *marA*, *soxS*, and *rob* deletion mutants were obtained. The *acrR*(L34Q) mutant was constructed in our previous study.

The *marA*, *soxS*, and *rob* deletions were transduced into *acrR*(L34Q) using a standard PI phage transduction. The *marAsoxSrob* triple mutant was constructed by using FLP recombinase expression from the pCP20 plasmid to remove the kan^R cassette from the *marA* deletion mutant, transducing the *soxS* deletion into the *marA* deletion mutant, and then repeating the above process to also delete *rob*. The presence of all three deletions was confirmed using PCR. Strains CL5415 - CL5422 were constructed by transforming pBAD33, pBAD33-acrAB, pNN387, or pNN608 plasmids into electrocompetent JW5249, JW4023, JW4359. All strains used in this study are listed in Table 3.1.

Psoralen-UVA (PUVA) survival.

10-µL aliquots of 10-fold serial dilutions from overnight cultures were spotted onto LBthy plates containing 20 µg/mL 8-methoxypsoralen. Plates were then exposed to UVA irradiation at an incident dose of 6.5 J/m2/s for the indicated dose and incubated overnight at 37°C. Surviving colonies at each dose were then counted and compared to the non-exposed plates to calculate a percent survival.

For overexpression of *acrAB* from expression vectors, 5 mL LBthy subcultures were inoculated with 50 µL of overnight cultures containing the expression plasmid and grown in a 37°C shaking water bath to OD600 of 0.4. 1 mM L-arabinose was added to subcultures for last 30 minutes of incubation before proceeding with survival assay as described above.

acrAB-lacZ expression.

10-µL aliquots of 10-fold serial dilutions from overnight cultures were spotted onto LBthy plates supplemented with 120 µg/mL 5-Bromo-4-Chloro-3-Indolyl β-D-

Galactopyranoside (X-Gal) either with or without 20 µg/mL 8-methoxypsoralen. Two plates each of LBthy X-GaL and LBthy X-Gal + 20 µg/mL 8-methoxypsoralen were then exposed to 3.8 kJ/m^2 UVA radiation as described above for survival assay. Plates were then compared to unexposed plates and photographed.

Table 3.1. List of strains used in this study.

Strain	Relevant Genotype	Source or Construction
BW25113	*lacIq rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78*	(49)
JW0453	*acrR*::FRT-minikan	(48)
JW5249	*marA*::FRT-minikan	(48)
JW4023	*soxS*::FRT-minikan	(48)
JW4359	*rob:*:FRT-minikan	(48)
CL5312	*marA*::FRT	pCP20 mediated removal of minikan from JW5249
CL5317	*marA*::FRT soxS::FRT-minikan	P1 transduction of *soxS*::FRT-minikan from JW4023 into CL5312
CL5322	*marA*::FRT *soxS*::FRT	pCP20 mediated removal of minikan from CL5317
CL5414	*marA*::FRT *soxS*::FRT *rob:*:FRT-minikan	P1 transduction of *rob*::FRT-minikan from JW4359 into CL5322
CL5230	*acrR*(L34Q)	(11)
CL5323	*acrR*(L34Q) *soxS*::FRT-minikan	P1 transduction of *soxS*::FRT-minikan from JW4023 into CL5230
CL5324	*acrR*(L34Q) *marA*::FRT-minikan	P1 transduction of *marA*::FRT-minikan from JW5249 into CL5230
CL5325	*acrR*(L34Q) *rob:*:FRT-minikan	P1 transduction of *rob*::FRT-minikan from JW4359 into CL5230
CL5333	pBAD33	(11)
CL5334	pBAD33-acrAB	(11)
CL5415	*marA*::FRT-minikan pBAD33	Transformation of pBAD33 (50) into JW5249
CL5416	*marA*::FRT-minikan pBAD33-*acrAB*	Transformation of pBAD33-*acrAB* (50) into JW5249
CL5417	*soxS*::FRT-minikan pBAD33	Transformation of pBAD33 (50) into JW4023
CL5418	*soxS*::FRT-minikan pBAD33-acrAB	Transformation of pBAD33-*acrAB* (50) into JW4023
CL5419	*rob:*:FRT-minikan pBAD33	Transformation of pBAD33 (50) into JW4359
CL5420	*rob:*:FRT-minikan pBAD33-acrAB	Transformation of pBAD33-*acrAB* (50) into JW4359
DH7169	pNN387	(51)
CR6000	pNN608	(27)
CL5421	*acrR*(L34Q) + pNN387	Transformation of pNN387 (51) into CL5230
CL5422	*acrR*(L34Q) + pNN608	Transformation of pNN608 (27) into CL5230

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Chapter IV: Conclusions

Drugs that form DNA interstrand crosslinks are clinically important for the treatment of a variety of dysplastic conditions and are often first- and second-line chemotherapeutics (1–7). Resistance to crosslinking agents has been observed in cancer cells (8, 9), but little is understood about how this resistance arises. Previously, many researchers proposed that DNA interstrand crosslinks could be repaired by the cell through the sequential operation of multiple independent repair pathways (10–16). However, existing studies have demonstrated that these pathways are ineffective in promoting repair of interstrand crosslinks (17), resulting in a single crosslink being lethal in *E. coli* (17, 18). Given the suggestion that effective repair of DNA interstrand crosslinks may not exist in cells, and the importance of crosslinking agents in the treatment of cancers, I sought through this study to understand how cells process DNA interstrand crosslinks and become resistant.

To this end, I first generated three independent strains of *E. coli* that became highly resistant to crosslinking by psoralen-UVA irradiation following repeated treatments. Genomic sequences of these strains showed numerous mutations, including some that were present in multiple strains. Importantly, no mutations were present in genes for DNA repair, further suggesting that repair of interstrand crosslinks may not be responsible for resistance to these lesions. Through reconstruction and characterization of the mutations common between the resistant strains, I identified that resistance to psoralen-UVA irradiation developed through mutations in the transcriptional regulator *acrR* and the alpha subunit of RNA polymerase *rpoA*. These mutations had an additive

contribution to psoralen-UVA, suggesting that they may be acting through separate mechanisms to confer resistance. Therefore, I next investigated more directly how these mutations confer psoralen-UVA resistance.

Following the observation that the resistant isolates appeared to be growing somewhat slower than wild-type cells, I first considered whether the *acrR* and *rpoA* mutants could be providing resistance by altering the growth rate of the cells, thereby reducing the frequency at which DNA damage is encountered during replication and allowing more time for repair. Growth curve analysis of these mutants showed that neither had a significant effect on the growth rate of cells, indicating that such a mechanism was not responsible for resistance. Further testing also showed that the resistant isolates and resistance mutants were not resistant to UVC irradiation, which forms bulky DNA adducts, indicating that removal of DNA adducts was not increased in these mutants. In contrast, these strains all showed cross-resistance to the antibiotic chloramphenicol, suggesting the involvement of more general drug resistance mechanisms. Additionally, Southern Blots showed that the resistant isolates and double mutants for *acrR* and *rpoA* reduced the accumulation of crosslinks in the DNA. Finally, I showed that the AcrAB-TolC multi-drug efflux pump, which is regulated by AcrR (19), was essential for resistance to crosslinks and that overexpression of AcrAB from a plasmid conferred resistance similar to that seen in the *acrR*(L34Q) point mutant. Taken together, these results suggested that resistance to psoralen-UVA irradiation was mediated through upregulation of the AcrAB-TolC efflux pump.

In the process of characterizing the contribution of *acrR* mutations to psoralen-UVA resistance, I noticed that deletion of *acrR*, which is believed to act as a repressor of AcrAB expression (19), did not confer resistance to psoralen, unlike mutations in *acrR*. This result was particularly confusing given the significant body of literature suggesting that AcrR binds to the promoter of AcrAB and represses expression until the binding of a recognized ligand by AcrR causes a conformational change that allows AcrR to release from the promoter and depress AcrAB (20–24). Closer investigation revealed that deletion of *acrR* removed the operon's binding site for MarA, SoxS, and Rob, which have been shown to activate expression of AcrAB (19, 25). As such, I decided to first characterize the involvement of MarA, SoxS, and Rob in psoralen-UVA resistance and then investigate in detail the regulation of AcrR as it relates to psoralen-UVA irradiation.

Through this study, I found that the global effectors MarA, SoxS, and Rob are important in resistance to psoralen-UVA irradiation. Interestingly, I also found that the sensitivity to psoralen-UVA that results from inactivation of MarA, SoxS, and Rob is not additive, indicating that all three proteins confer resistance through the same mechanism. I then showed that independent overexpression of *acrAB* by the *acrR*(L34Q) mutant or from an expression plasmid was sufficient to rescue the sensitivity of *marA*, *soxS*, and *rob* deletion mutants, indicating both that all three genes contribute to psoralen-UVA resistance primarily through additional upregulation of the AcrAB-TolC efflux pump and that derepression of AcrAB by mutations in AcrR can confer resistance even in the absence of MarA, SoxS, and Rob. Finally, I showed that while upregulation of *acrAB*

expression by AcrR is sufficient to produce psoralen-UVA resistance, this upregulation was not dependent on induction by psoralen, UVA, or the combination of the two.

The finding that resistance to psoralen-UVA irradiation occurs through upregulation of efflux by AcrAB-TolC adds to the body of evidence that prevention of crosslink formation, rather than repair of crosslinks, is the primary strategy by which cells respond to challenge by DNA interstrand crosslinks. This has significant relevance to the clinical use of crosslinking agents. In humans, multi-drug resistance often arises in cancers through upregulation of efflux by P-glycoprotein (27–30). The results shown here suggest that resistance to DNA interstrand crosslinks develops through selection for multi-drug resistance, as appears to be the case with the resistant strains of *E. coli* generated in this work, rather than through mechanisms specific to DNA interstrand crosslinks. As such, countering the specific mechanisms that lead to multi-drug resistance, such as drug efflux, may be the most important factor in maintaining the effectiveness of crosslinking agents in the treatment of cancers.

Besides the need to establish whether these predictions hold true for cancer cells, there are a number of questions illustrated by this work that remain to be answered. In particular, it will be important in future studies to more completely characterize the exact mechanisms by which the mutations in *acrR* and *rpoA* described in this thesis confer resistance to psoralen-UVA and other drugs, like chloramphenicol and tetracycline. These multi-drug resistance mechanisms, even where specific to *E. coli* and related bacteria, are clinically relevant to our ability to combat multi-drug resistant infections by pathogenic bacteria. For example, though the results of Chapter II implied that the

rpoA(E273G) mutation conferred psoralen-UVA resistance through a mechanism similar to that of the *acrR* mutations, more direct experimental evidence is needed to determine whether this mutation results in increased efflux of psoralen. One approach to testing this would be to monitor the efflux of the fluorescent dye ethidium bromide, another substrate of the AcrAB-TolC pump (24), to determine whether the efflux capacity is increased in the *rpoA*(E273G) mutant.

Additionally, it is clear that much remains to be learned about how the AcrAB-TolC efflux system is regulated, especially in regards to the local regulator AcrR. That complete loss of AcrR does not confer resistance to psoralen-UVA, in contrast to AcrR mutants and other strains that overexpress *acrAB*, suggests that its function extends beyond that of a traditional local repressor. Though AlphaFold predictions suggested that DNA binding activity may be retained in the various AcrR mutants, I have not been able to confirm this experimentally. As such, it would be useful to test the ability of these mutants to bind to their cognate DNA using electrophoretic mobility shift assays (EMSA) or similar techniques. The results of Chapter III also suggested that psoralen may not be a ligand of AcrR, despite it being a substrate of the AcrAB-TolC efflux pump. Quantitation of the *acrAB-lacZ* expression under the conditions tested in Chapter III would provide more definitive evidence of this phenomenon. If it is confirmed that psoralen does not act as a ligand for AcrR, it will be important to investigate whether other substrates of AcrAB-TolC show a similar lack of interaction with AcrR.

Finally, though I have found evidence that MarA, SoxS, and Rob contribute to psoralen-UVA resistance primarily through upregulation of AcrAB-TolC, Appendix B

shows preliminary data that supports the role of other operons regulated by the Mar/Sox/Rob in resistance. Of particular interest is the role of *sodA*, which encodes superoxide dismutase. Appendix A shows that deletion of *sodA* renders cells moderately hypersensitive to psoralen-UVA. This indicates not only that superoxides are produced by psoralen-UVA, as one study previously suggested, but that superoxide dismutase may contribute to psoralen-UVA resistance to some extent. The presence of superoxides could also hypothetically act as a signal not only for the Mar/Sox/Rob global stress response (31), but other stress responses in cancer cells that could promote resistance to psoralen-UVA. As such, it will be important to establish the role of superoxides produced by psoralen-UVA in both the efficacy of the treatment, and in possible signaling of stress responses.

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Appendix: OmpC, SodA, and OxyR contribute to psoralen-UVA resistance.

Introduction

Following early results of Chapter III that demonstrated that MarA, SoxS, and Rob are required for full resistance to psoralen-UVA resistance, I decided to test the contribution of key stress resistance operons regulated by the Mar/Sox/Rob pathway. Though MarA, SoxS, and Rob regulate approximately 50 genes throughout the genome (1), I chose to focus on genes in the pathway that represent major non-efflux strategies by which the cell responds to toxic compounds. These strategies are discussed in greater detail in Chapter I, but can be summarized as decreased membrane permeability, metabolic degradation of toxins, and repair of DNA damage. Examples of these strategies are found in the Mar/Sox/Rob pathway's downregulation of the membrane porin OmpF (2, 3), upregulation of superoxide dismutase (3), and upregulation of endonuclease IV (3) and exonuclease VII (4).

Importantly, though DNA interstrand crosslinks have been shown to be the main driver of psoralen-UVA's lethality (5, 6), some studies have proposed that reactive oxygen species produced by psoralen-UVA treatment may also contribute to toxicity (7–9). One study specifically suggested that 8-methoxypsoralen could produce superoxides (10), which would be directly relevant to the Mar/Sox/Rob pathway in that SoxR upregulates SoxS in response to the presence of superoxides (11, 12). Upregulation of SoxS significantly activates expression of *sodA* (3), which encodes a superoxide dismutase that detoxifies superoxides (13), and *nfo* (3), which encodes an AP endonuclease that participates in the repair of a variety of oxidative DNA lesions (14).

Finally, the Mar/Sox/Rob pathway also upregulates *xseA* (4), which encodes a subunit of exonuclease VII (15). Relatively little is known about exonuclease VII, but it has been suggested that it may be involved in processing some UV lesions (16) as well as in the repair of DNA damage caused by fluoroquinolones (4).

OmpC is required for full resistance to psoralen-UVA.

Given the various lines of evidence presented in Chapter II that resistance to psoralen-UVA is driven by prevention of crosslink formation, I decided to first investigate the role of the major outer membrane porins OmpF and OmpC in membrane permeability to psoralen. Previous work has shown that the Mar/Sox/Rob pathway activates expression of an antisense mRNA, *micF*, to reduce expression of OmpF and decrease membrane permeability to certain antibiotics (2, 17). Under normal conditions, both the larger OmpF porin and smaller OmpC porin (reviewed in (18)) are regulated individually by a two-component system consisting of the sensor histidine kinase EnvZ (19)and transcriptional regulator OmpR (20). I therefore elected to test OmpC, EnvZ, and OmpR in addition to OmpF. To determine if these proteins contribute to psoralen-UVA resistance, I tested the survival of *ompF*, *ompC*, *envZ*, and *ompR* deletion mutants as described in Chapters II and III. If psoralen passes through OmpF or OmpC, one would expect that deletion of these membrane porins would decrease permeability of the membrane to psoralen and increase psoralen-UVA resistance. Figure A.1A shows that deletion of neither *ompF* nor *ompC* led to an increase in psoralen-UVA resistance relative to wild type cells, indicating that the membrane porins do not significantly contribute to permeability of the outer membrane to psoralen. Surprisingly, deletion of *ompC* (Fig.

A.1A) and *envZ* (Fig. A.1B) rendered cells hypersensitive. Previous studies of *envZ* deletion mutants have indicated that such mutants are deficient in OmpC, but not OmpF (19, 21). Therefore, it appears that OmpC is required for full resistance to psoralen-UVA, though it is currently unclear what role this porin serves in resistance.

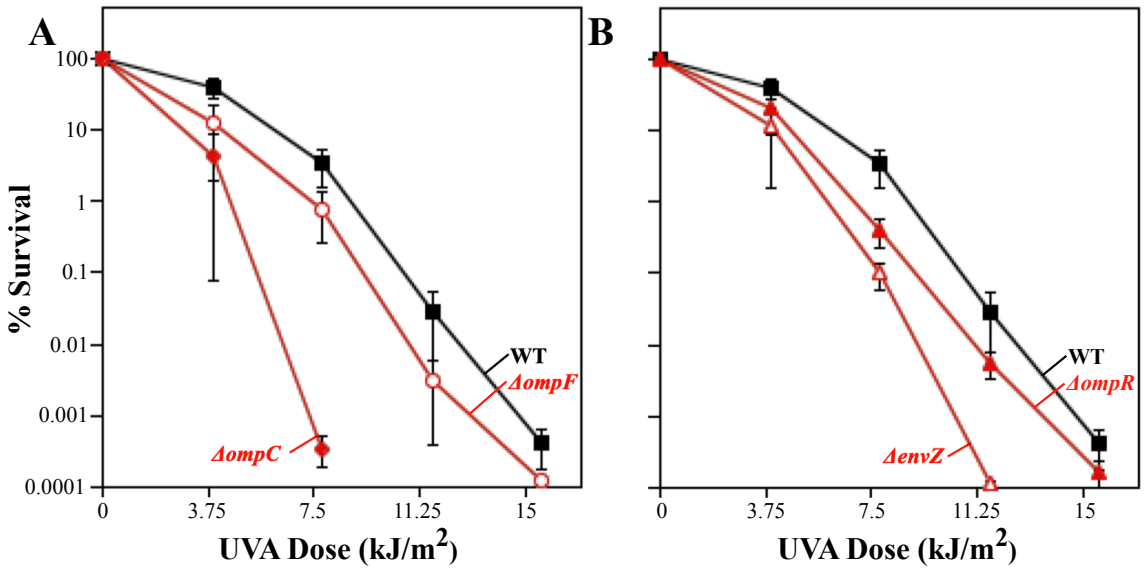

Figure A.1. *ompC* is required for full resistance to psoralen-UVA. The survival of BW25113 wild type (black, filled square); (A) *ompF* deletion mutant (red, open circle), *ompC* deletion mutant (red, filled diamond); (B) *ompR* deletion mutant (red, filled triangle), and *envZ* deletion mutant (red, open triangle) in the presence of 20 µg/mL 8-methoxypsoralen at the indicated UVA doses is plotted. Plots represent the average of at least two independent experiments. Error bars represent the standard error of the mean.

SodA and OxyR confer resistance to superoxides produced by psoralen-UVA.

As it seemed feasible that the products of *sodA*, *nfo*, and *xseA* could contribute to the psoralen-UVA resistance provided by the Mar/Sox/Rob pathway, I decided to test the survival of deletion mutants for these genes as before. Figure A.2A shows that deletion of *sodA* results in moderate hypersensitivity to psoralen-UVA, indicating both that psoralen produces superoxides and that the activity of superoxide dismutase contributes to tolerance to psoralen-UVA. Similar results were obtained by deletion of the oxidative stress response regulator *oxyR*, which regulates the response to peroxide stress (22). This provides additional support for the presence of superoxides in that dismutation of superoxides produces hydrogen peroxide (23). In contrast to these results, deletion of *xseA* and *nfo* had little effect on psoralen-UVA resistance, suggesting that the nucleases encoded by these genes do not have a significant role in repairing DNA damage induced by psoralen-UVA.

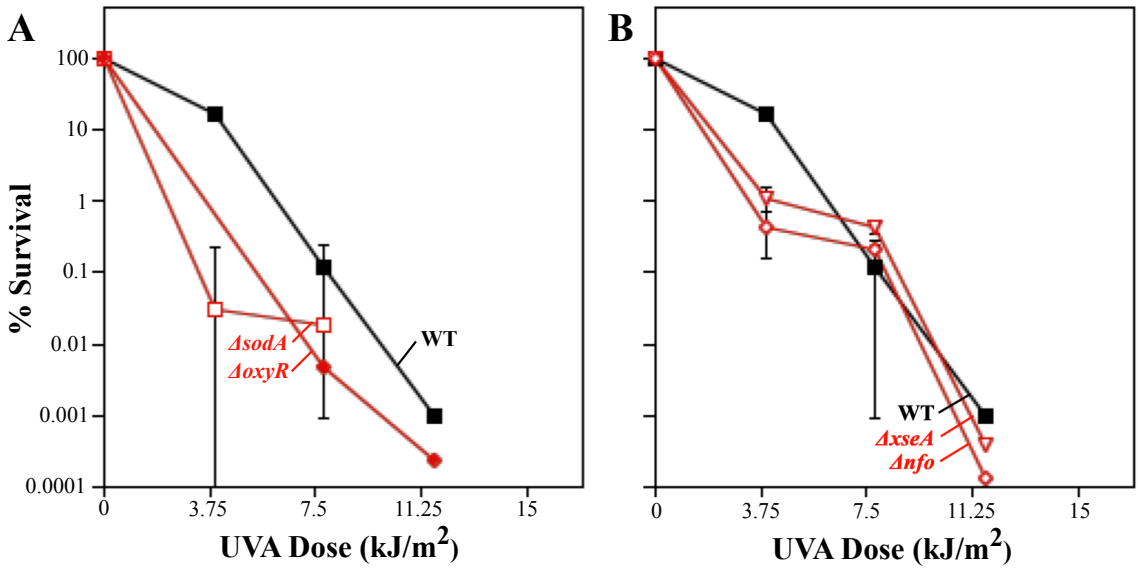

Figure A.2. *sodA* and *oxyR* are required for full resistance to psoralen-UVA. The survival of BW25113 wild type (black, filled square); (A) *sodA* deletion mutant (red, open square), *oxyR* deletion mutant (red, filled diamond); (B) *nfo* deletion mutant (red, open diamond), and *xseA* deletion mutant (red, inverted open triangle) in the presence of 20 µg/mL 8-methoxypsoralen at the indicated UVA doses is plotted. Plots represent the average of at least two independent experiments. Error bars represent the standard error of the mean.

Discussion

Though the results shown here demonstrate that another gene directly upregulated by the Mar/Sox/Rob pathway, *sodA*, is involved in psoralen-UVA resistance, it remains unclear to what extent *sodA* would be upregulated above its normal expression in response to psoralen-UVA. Furthermore, multiple lines of evidence presented in Chapters II and III support the idea that efflux of psoralen by AcrAB-TolC is the primary driver of psoralen-UVA resistance, implying that that superoxide dismutase has a smaller, supporting role. This seems particularly likely given that DNA interstrand crosslinks have been shown to be responsible for the vast majority of psoralen-UVA's cytotoxicity (5, 6), and efflux of psoralen would therefore be expected to have a greater impact on survival than detoxification of reactive oxygen species. Nevertheless, the findings of Fig. A.2 support earlier evidence that reactive oxygen species, including superoxides, are produced by psoralen-UVA (7–10), which could be important in understanding the signaling of global stress responses such as the Mar/Sox/Rob pathway.

Finally, while it is currently unclear why OmpC would be important for psoralen-UVA resistance, one possible explanation is that OmpC appears to be a key component of the Mla phospholipid trafficking system (24). The Mla pathway maintains lipid asymmetry in the outer membrane (25), which has been implicated in supporting barrier function of the membrane against some antibiotics (4). As such, OmpC's role in psoralen-UVA resistance may involve barrier function of the outer membrane against psoralen entry into the cell. It will therefore be important to investigate the role of the Mla system in psoralen-UVA resistance.

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