Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis 830 (2025) 111898

Contents lists available at ScienceDirect

Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/mut

The complex development of psoralen-interstrand crosslink resistance in *Escherichia coli* requires AcrR inactivation, retention of a *marbox* sequence, and one of three MarA, SoxS, or Rob global regulators

Travis K. Worley [*,1], Ayah H. Asal, Lo Cooper, Charmain T. Courcelle, Justin Courcelle

Department of Biology, Portland State University, Portland OR, United States

ARTICLE INFO

Keywords:
DNA interstrand crosslinks
Psoralen-UVA
Antimicrobial
Cancer therapeutic

ABSTRACT

Crosslinking agents, such as psoralen and UVA radiation, can be effectively used as antimicrobials and for treating several dysplastic conditions in humans, including some cancers. Yet, both cancer cells and bacteria can become resistant to these compounds, making it important to understand how resistance develops. Recently, several mutants were isolated that developed high levels of resistance to these compounds through upregulation of components of the AcrAB-TolC efflux pump. Here, we characterized these mutants and found that resistance specifically requires inactivating mutations of the *acrR* transcriptional repressor which also retain the *marbox* sequence found within this coding region. In addition, the presence of any one of three global regulators, MarA, SoxS, or Rob, is necessary and sufficient to bind to the *marbox* sequence and activate resistance. Notably, although psoralen is a substrate for the efflux pump, these regulators are not naturally responsive to this stress as neither psoralen, UVA, nor crosslink induction upregulates *acrAB* expression in the absence of mutation.

1. Introduction

Psoralen in the presence of UVA irradiation forms DNA interstrand crosslinks and 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 is attributed to its ability to form lethal lesions known as DNA interstrand crosslinks [3–6]. In *Escherichia coli*, a single DNA interstrand crosslink in the genome is sufficient to inactivate the cell [7,8]. However, the use of psoralen-UVA and other crosslinking agents as antimicrobials and chemotherapeutics can be compromised by the emergence of cells resistant to these drugs [9,10]. In *E. coli*, several highly resistant mutants to psoralen-UVA interstrand crosslinks have been isolated whose resistance is driven by increased expression of the AcrAB-TolC efflux pump, which protects the DNA and effectively prevents these lethal lesions from forming when psoralen is present in the media [11].

AcrAB-TolC belongs to a highly conserved RND (Resistance-Nodulation-Division) efflux pump family found in Gram-negative bacteria [12–16]. The efflux pump consists of a proton-driven transporter AcrB, a periplasmic adapter protein AcrA, and the TolC transmembrane channel [17–20]. AcrAB-TolC is capable of effluxing a wide variety of structurally dissimilar substrates, including many dyes, detergents, and antibiotics [21–25], making it a primary driver of multiple-antibiotic resistance [23].

The highly resistant mutants were each found to have mutations in the transcriptional regulator AcrR [11]. *acrR* encodes a TetR family transcriptional regulator that is located immediately upstream of *acrAB* and is divergently transcribed [26]. Based on *lacZ*-fusion and gel mobility shift assays, Ma et. al. demonstrated that AcrR functions as a repressor of *acrAB* that releases upon binding a recognized substrate [27]. Consistent with this, some substrates of the efflux pump, such as rhodamine, ethidium bromide, and proflavine, bind to AcrR [28,29] and this correlates with a loss of DNA binding activity in vitro [30].

Surprisingly however, deletion of *acrR*'s coding region does not increase resistance to psoralen interstrand crosslinks, suggesting a more complex mechanism of regulation than a simple repressor function is involved [11]. We noted that the first 20 nucleotides of *acrR*'s coding region contains a *marbox*-binding sequence for three closely related global stress regulators, MarA, SoxS and Rob [31]. These three regulators share approximately 50 % sequence identity [32,33] and regulate expression of approximately 50 genes, including *acrA* and *acrB*, in response to various environmental stressors and toxins (Fig. 1 and

* Corresponding author.
E-mail address: tworley2@wisc.edu (T.K. Worley).
[1] present address: Department of Microbiology, University of Wisconsin – Madison, Madison WI

https://doi.org/10.1016/j.mrfmmm.2025.111898
Received 26 November 2024; Received in revised form 22 January 2025; Accepted 22 January 2025
Available online 27 January 2025

[31–40]. Using a *lacZ*-reporter construct and gel mobility shift assays, several groups demonstrated that protein binding to the *marbox* upstream of *acrAB* correlated with its expression [27,40]. This led to a general model that these global stress activators drive *acrAB* expression, with AcrR serving as a secondary repressive modulator.

Given the importance of DNA interstrand crosslinks in antimicrobrial and chemotherapeutic therapies, here we sought to characterize the mechanism by which resistance was achieved in these mutants. We found that although the pump confers resistance to crosslinks, it is not naturally responsive or upregulated in their presence. Resistance relies on mutations that inactivate the AcrR repressor but retain the *marbox* sequence within the gene's coding region. The resistance can then be activated by the presence of any one of the three global activators, MarA, SoxS, or Rob.

2. Materials and methods

2.1. Bacterial strains

All strains utilized in this study were derived from BW25113, which is the parent strain of the Keio collection [41], from which the *marA*, *soxS*, and *rob* deletion mutants were obtained. The *acrR* deletion mutant was originally obtained from the Keio collection but was reconstructed by P1 phage transduction into wild-type BW25113. The *acrR*(L34Q) mutant was constructed in our previous study [11]. 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*. This process was repeated in the *acrR*(L34Q) mutant to generate the *acrR*(L34Q)*marAsoxSrob* quadruple mutant. 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. For the deletion of *acrR* past the *marbox* sequence, the Kan^R cassette was recombineered into BW25113 using primers 5'AGAAGCGCAAGAAACGCGCCAACACATCCTCGATGTGGCTCTACGT CTTTATGATTCCGGGGATCCGTCGACC3' and 5'CAGG AAAAATCCTG-GAGTCAGATTCAGGGTTATTCGTTAGTGGCAGGATT TGTAGGCTG-GAGCTGCTTCG3'. All strains used in this study are listed in Table 1.

2.2. 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/m^2/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 percent survival.

For overexpression of *acrAB* from expression vectors, 5 mL LB subcultures were inoculated with 50 μL of overnight cultures containing the expression plasmid, pBAD33-acrAB, or its parent vector, pBAD33, and grown in a 37°C shaking water bath to OD600 of 0.4. 1 mM L-arabinose was added to subcultures for the last 30 minutes of incubation before proceeding with survival assays as described above.

2.3. acrAB-lacZ expression

10-μL aliquots of 10-fold serial dilutions from overnight cultures containing pNN608 (*acrABp*-lacZ) or pNN387 (empty vector) 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 LB X-GaL and LB X-Gal + 20 μg/mL 8-methoxypsoralen were then exposed to 3.8 kJ/m^2 UVA radiation as described above for survival assays. Plates were then compared to unexposed plates and photographed.

3. Results

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

In previous work, three mutations in the transcriptional repressor *acrR* were isolated and found to confer high-level resistance to psoralen-UVA through the upregulation of *acrA* and *–B*, encoding components of the AcrAB-TolC efflux pump (Fig. 2A and [11]). However, when we deleted the entire *acrR* coding region, we found that unlike the other *acrR* mutations, no resistance was conferred (Fig. 2A). The observation argues that the loss of the AcrR repressor is insufficient to confer resistance to psoralen interstrand crosslinks and a more complex mechanism is involved in the acquisition of resistance.

Common to all three of the resistance-conferring *acrR* mutants that were isolated is that they retain the initial third of *acrR*'s coding sequence but alter or remove the latter two-thirds of the protein. The

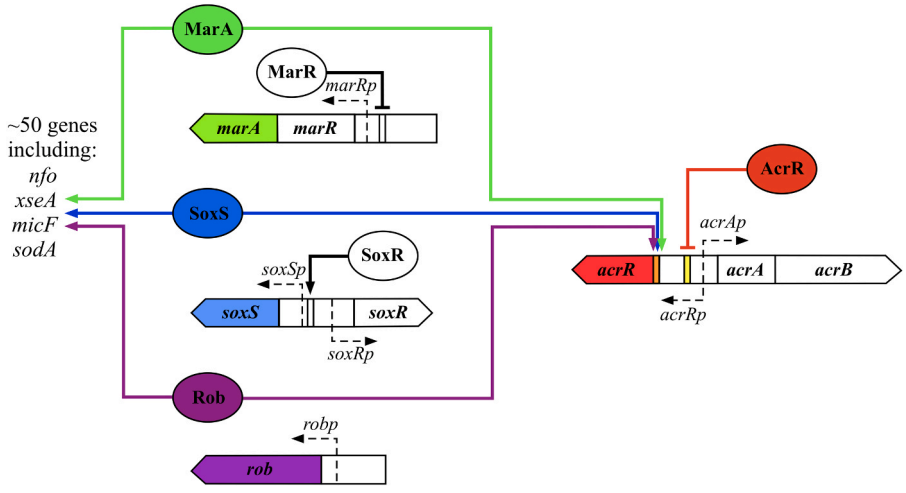

Fig. 1. Current model of MarA, SoxS, and Rob global gene regulation. Green, MarA; blue, SoxS; purple, Rob; red, AcrR; yellow, DNA binding sites; orange, Mar/Sox/Rob binding site (*marbox*). Arrows indicate activation, while interruption of the end of a line indicates repression (derived from models and data presented in [26,31,33,36,37,45]).

Table 1
List of strains used in this study.

Strain	Relevant Genotype	Source or Construction
BW25113	*lacIq rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78*	[60]
JW0453	*acrR*::FRT-minikan	[41]
JW5249	*marA*::FRT-minikan	[41]
JW4023	*soxS*::FRT-minikan	[41]
JW4359	*rob*::FRT-minikan	[41]
CL5312	*marA*::FRT	pCP20-mediated [61] 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 [61] 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
CL5433	*acrR*(L34Q) *marA*::FRT	pCP20-mediated [61] removal of minikan from CL5324
CL5436	*acrR*(L34Q) *marA*::FRT *soxS*::FRT-minikan	P1 transduction of *soxS*::FRT-minikan from JW4023 into CL5433
CL5438	*acrR*(L34Q) *marA*::FRT *soxS*::FRT	pCP20-mediated [61] removal of minikan from CL5436
CL5440	*acrR*(L34Q) *marA*::FRT *soxS*::FRT *rob*::FRT-minikan	P1 transduction of *rob*::FRT-minikan from JW4359 into CL5438
CL5442	*acrR* (aa 7 – 215)::FRT-minikan	Recombineering to replace amino acids 7 – 215 of *acrR* in BW25113 with FRT-minikan
CL5333	pBAD33	[11]
CL5334	pBAD33-acrAB	[11]
CL5415	*marA*::FRT-minikan pBAD33	Transformation of pBAD33 [62] into JW5249
CL5416	*marA*::FRT-minikan pBAD33-*acrAB*	Transformation of pBAD33-*acrAB* [62] into JW5249
CL5417	*soxS*::FRT-minikan pBAD33	Transformation of pBAD33 [62] into JW4023
CL5418	*soxS*::FRT-minikan pBAD33-acrAB	Transformation of pBAD33-*acrAB* [62] into JW4023
CL5419	*rob*::FRT-minikan pBAD33	Transformation of pBAD33 [62] into JW4359
CL5420	*rob*::FRT-minikan pBAD33-acrAB	Transformation of pBAD33-*acrAB* [62] into JW4359
DH7169	pNN387	[63]
CR6000	pNN608	[27]
CL5402	BW25113 + pNN387	Transformation of pNN387 [63] into BW25113
CL5403	BW25113 + pNN608	Transformation of pNN608 [27] into BW25113
CL5421	*acrR*(L34Q) + pNN387	Transformation of pNN387 [63] into CL5230
CL5422	*acrR*(L34Q) + pNN608	Transformation of pNN608 [27] into CL5230
CL5530	*marA*::FRT *soxS*::FRT *rob*::FRT-minikan + pNN387	Transformation of pNN387 [63] into CL5414
CL5531	*marA*::FRT *soxS*::FRT *rob*::FRT-minikan + pNN608	Transformation of pNN608 [27] into CL5414
CL5532	*acrR*(L34Q) *marA*::FRT *soxS*::FRT *rob*::FRT-minikan +pNN387	Transformation of pNN387 [63] into CL5440
CL5533	*acrR*(L34Q) *marA*::FRT *soxS*::FRT *rob*::FRT-minikan +pNN608	Transformation of pNN608 [27] into CL5440

first third of the gene encodes the DNA-binding domain for the AcrR regulator. However, this region also contains a MarA, SoxS, and Rob binding sequence, known as the *marbox*, which has been reported to positively regulate the *acrAB* operon [27,31]. Thus, it is possible that the mutations confer psoralen resistance either through altering AcrR's DNA binding properties or through activation of *acrAB* by MarA, SoxS, or Rob.

If the psoralen resistance is mediated through the *marbox*, then deletion of the *marA*, *soxS*, and *rob* genes would be expected to impair resistance in these strains. To test this possibility, we 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. Fig. 2B 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. 2 C). Given that MarA, SoxS, and Rob all share a single *marbox* binding sequence within *acrR*, it is unexpected and remains unclear why deleting of any one of these three proteins renders cells hypersensitive. However, the observation indicates that all three proteins are required to maintain resistance to psoralen-UVA interstrand crosslinks, despite sharing a single DNA binding sequence.

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

MarA, SoxS, and Rob upregulate expression of approximately 50 genes in response to various cellular stresses [31]. Thus, although the results of Fig. 2 indicate that MarA, SoxS, and Rob are required for full resistance to psoralen, they do not establish if this contribution can be attributed to the upregulation of *acrAB* or if resistance is conferred by other *marbox*-regulated genes. To test this, we used an arabinose-inducible *acrAB* plasmid to overexpress *acrAB* in the *marA*, *soxS*, *rob* deletion mutants, which would result in upregulation of *acrAB*, but not any other *marbox*-regulated genes. Actively growing cultures containing the plasmid were incubated with arabinose for 30 minutes to induce *acrAB* expression prior to psoralen-UVA treatment. Fig. 3 shows that plasmids containing the *acrAB* sequence increase resistance in *marA*, *soxS*, and *rob* mutants to near wild-type levels. By contrast, 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.

3.3. MarA, SoxS, and Rob activation and AcrR derepression contribute additively to psoralen interstrand crosslink resistance

MarA-, SoxS-, and Rob-mediated upregulation of *acrAB* is required for full resistance to psoralen-UVA (Figs. 2 and 3). Since the highly resistant *acrR*(L34Q) mutant retains the *marbox* sequence, it is possible that the high level of resistance requires activation by MarA, SoxS, or Rob. If true, we would expect that deletion of *marA*, *soxS*, or *rob* would significantly reduce psoralen-UVA resistance in the *acrR*(L34Q) strain. As shown in Fig. 4, *acrR*(L34Q) mutants remained resistant to psoralen-UVA, when either *marA*, *soxS*, or *rob* was deleted. However, the loss of all three genes reduced the resistance of *acrR*(L34Q) mutants to levels similar to wild-type cells and the *acrR* deletion mutant. Taken together with the previous observations, the results support the idea that both derepression by AcrR and activation by MarA, SoxS, or Rob are required to achieve resistance to psoralen interstrand crosslinks.

To confirm these requirements directly, we used recombineering to generate a complete deletion of the *acrR* coding sequence with the exception of the first 21 nucleotides encoding the *marbox* sequence. Fig. 5 shows that the *marbox* sequence alone is sufficient to restore full

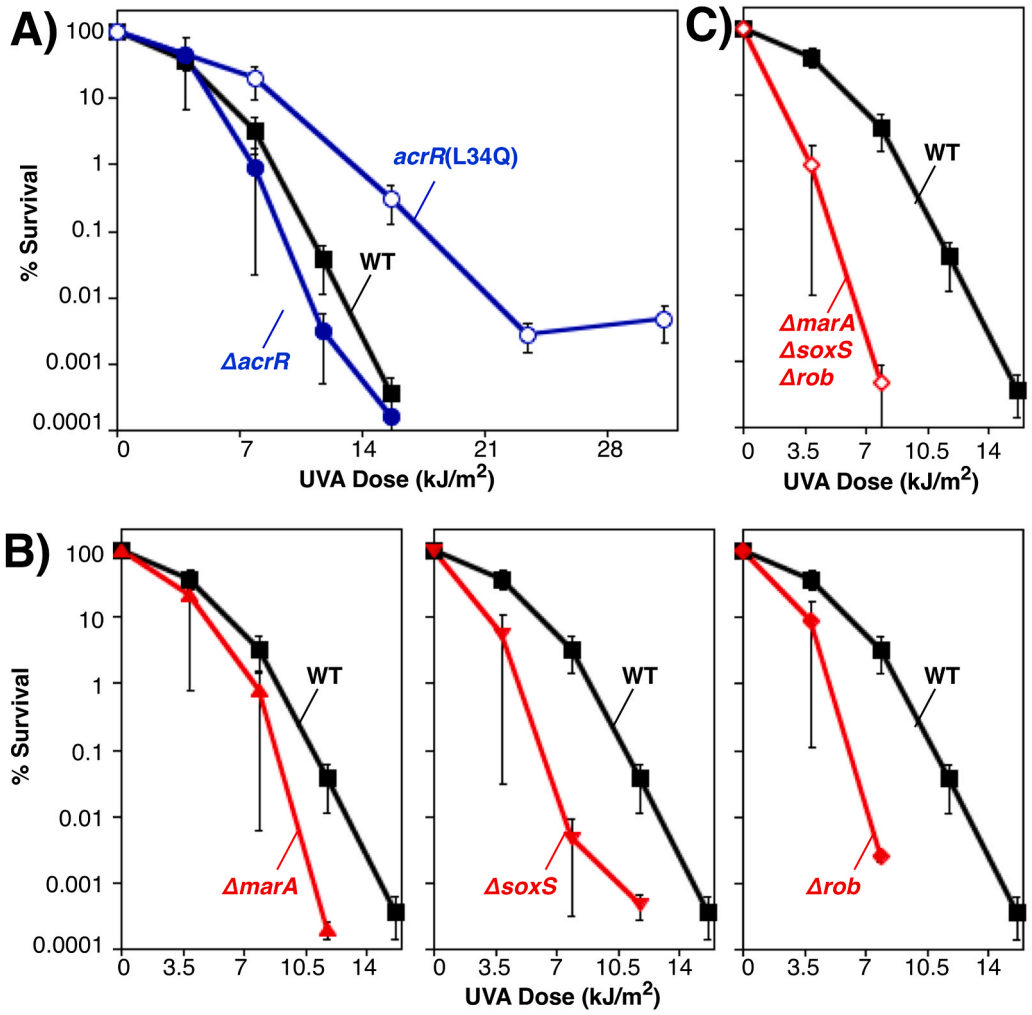

Fig. 2. *acrR*(L34Q), but not deletion of *acrR*, confers resistance to psoralen–UVA, while deletion of *marA*, *soxS*, or *rob* renders cells hypersensitive. A) The survival of wild-type cells (filled squares); Δ*acrR* (filled circles), and *acrR*(L34Q) mutants (open circles), B) Δ*marA* (filled triangles), Δ*soxS* mutant (filled inverted triangles), Δ*rob* mutants (filled diamonds) and C) Δ*marA* Δ*soxS* Δ*rob* mutants (open diamonds) is plotted following UVA irradiation at the indicated doses in the presence of 20 μg/mL 8-methoxypsoralen. Plots represent the average of at least two independent experiments. Error bars represent the standard error of the mean. Wild type is replotted in each graph for comparison.

resistance to *acrR* deletion mutants, mimicking the resistance seen in the *acrR*(L34Q) mutant.

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

The results above demonstrate that AcrR derepression and activation by either MarA, SoxS, or Rob are required to upregulate *acrAB* and confer to crosslink resistance. However, how *acrAB* is regulated in wild-type cells during challenge with psoralen-UVA is unknown. Previous studies have shown that exposure to other stressors and agents, including ethidium bromide, cadaverine, ethanol, or high osmolarity, can induce expression of *acrAB* to increase resistance [27,42]. To examine if *acrAB* expression is responsive to psoralen-UVA treatments, we used a plasmid that contained the *acrAB* promotor region fused to *lacZ*. The cloned promoter region contains both the AcrR binding site as well as the first 102 nucleotides of *acrR* coding sequence which contains the *marbox* binding site. To test if psoralen, UVA irradiation, or the presence of interstrand crosslinks can serve to induce *acrAB* expression, cultures 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 Fig. 6 A, in the presence of the *acrAB-p-lacZ* reporter, the parental strain detectably expressed the *acrAB* genes as indicated by the partially blue colonies, relative to the control plasmid. As controls, we also examined the *acrR*(L34Q) resistant mutant and the sensitive *marA soxS rob* deletion mutant. As expected, *acrAB* expression was elevated in *acrR*(L34Q) mutant as indicated by the intensely blue colonies, correlating with the increased expression of *acrAB* and resistance in this strain. Similarly, colonies were noticeably less blue in the *marA soxS rob* deletion background which correlates with reduced *acrAB* expression and hypersensitivity (Fig. 6B).

Notably however, expression did not increase in the presence of either psoralen, UVA, or psoralen plus UVA treatments. The results imply that psoralen, UVA, or the combination do not generate substrates that activate *acrAB* and suggest these regulators are not normally responsive to this challenge, in the absence of mutation.

4. Discussion

The results demonstrate that all three of the related global regulators MarA, SoxS, and Rob have a significant role in psoralen-UVA resistance. However, regulation by these activators was found to be complex. Deletion of any single global effector gene in wild-type cells had a similar impact on psoralen-UVA resistance as deleting all three genes. This result is unexpected for several reasons. First, although *rob* is expressed constitutively, *marA* and *soxS* are expressed at relatively low

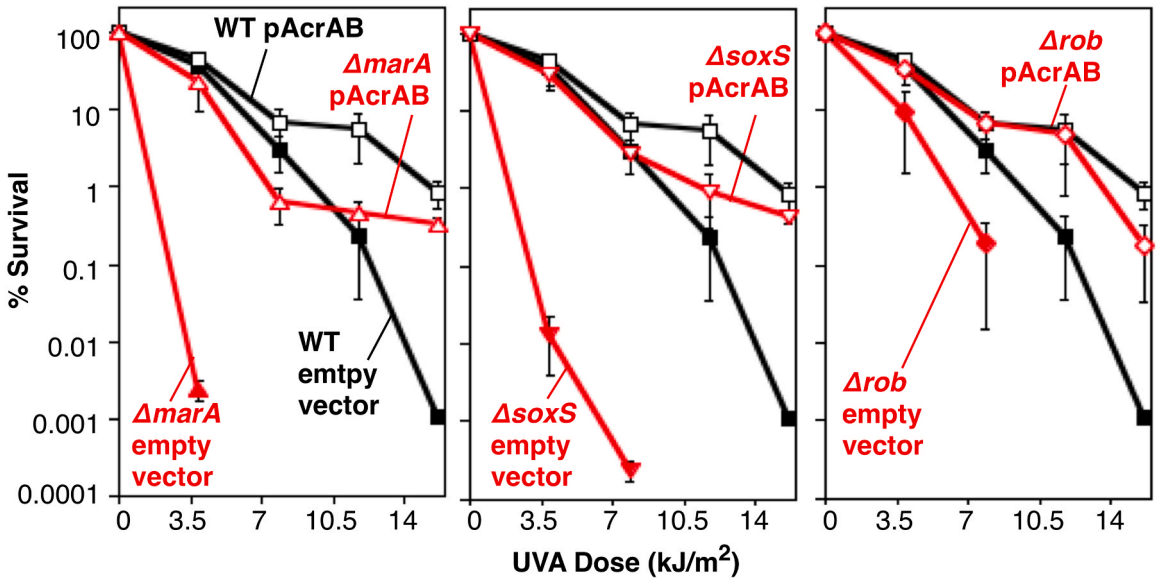

Fig. 3. Overexpression of AcrAB alone is sufficient to restore psoralen-UVA resistance in Δ*marA*, Δ*soxS*, and Δ*rob* mutants. The survival of wild type (squares), Δ*marA* (triangles), Δ*soxS* (inverted triangles), and Δ*rob* (diamonds) containing either an empty pBAD33 expression vector (filled symbols) or an AcrAB expression vector (open symbols) is plotted following UVA irradiation at the indicated doses in the presence of 20 μg/mL 8-methoxypsoralen. Plots represent the average of at least two independent experiments. Error bars represent the standard error of the mean. Wild type is replotted in each graph for comparison.

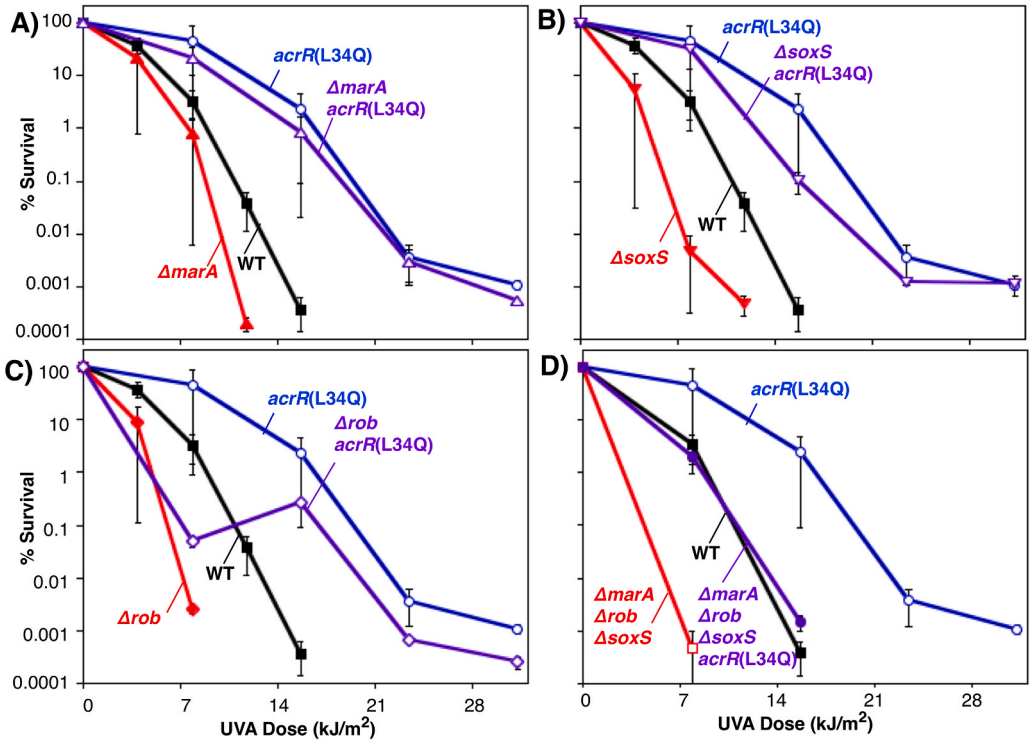

Fig. 4. *acrR*(L34Q) psoralen-UVA resistance requires MarA, SoxS, and Rob activation. The survival of wild-type cells (filled squares), *acrR*(L34Q) (open circles), Δ*marA* (closed triangles), *acrR*(L34Q) Δ*marA* (open triangles); (B) Δ*soxS* (filled inverted triangles), *acrR*(L34Q) Δ*soxS* (open inverted triangles); (C) Δ*rob* (filled diamonds), and *acrR*(L34Q) Δ*rob* (open diamonds); (D) Δ*marA* Δ*soxS* Δ*rob* mutants (open squares), *acrR*(L34Q) Δ*marA*Δ*soxS*Δ*rob* (filled circles) 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. Wild type is replotted in each graph for comparison.

levels until a specific stressor induces their expression [31,32,34,35,39,43]. Additionally, the activity of Rob has been shown to be responsive to its recognition of various substrates and its release from sites of sequestration in the cell [40,44]. Yet despite these regulators responding to different stressors, Fig. 2 demonstrates that no single effector is responsible for initiating the stress response to psoralen-UVA irradiation. Second, given the high level of homology between MarA, SoxS, and Rob, and their ability to bind the same *marbox* sites across the genome, one might expect that loss of one regulator could be offset by the presence of the other two [31,32,34,35,39,45]. Yet this is not observed in

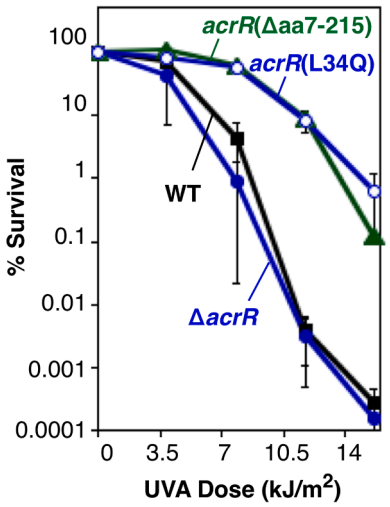

Fig. 5. The *marbox* sequence is necessary and sufficient to induce resistance to psoralen interstrand crosslinks in the absence of the AcrR repressor. The survival of wild-type cells (filled squares), Δ*acrR* (filled circles), *acrR*(L34Q) (open circles), and Δ*acrR* (aa7–215) (filled triangles) in 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.

wild-type cells. On the other hand, if no redundancy existed, one might expect that deleting *marA*, *soxS*, and *rob* would have an additive effect on psoralen-UVA sensitivity. This is also not observed (Fig. 2). Thus, the apparent any-and-all requirement for MarA, SoxS, and Rob could suggest that crosstalk between these activators is particularly important in psoralen-UVA resistance. Alternatively, it is possible that the sensitivity of this assay to distinguish phenotypic differences decreases as the limits of detectability are approached.

Irrespective of the crosstalk, the AcrAB-TolC transporter appears to be the causal target for MarA, SoxS, and Rob generating psoralen crosslink resistance, since the hypersensitive phenotype of these mutants can be suppressed by overexpression of AcrAB alone and does not require any of the other approximately 50 genes under their regulation ([31–40] and Fig. 3). Previous studies have presented evidence consistent with the idea that psoralen is acting as a substrate the AcrAB-TolC efflux pump. The resistant *acrRL34Q* mutation prevents the accumulation of cellular psoralen adducts in an AcrB-dependent manner without altering the DNA repair capacity. This suggests psoralen efflux, rather than repair as a mechanism of resistance [11]. Recently, Green et al. [46] demonstrated that either *tolC* deletion or treatment with the efflux inhibitor phenylalanine-arginine b-naphthylamide (PAβN) sensitized *E. coli* to both amotosalen and 8-methoxypsoralen in a TolC-dependent manner [46]. This implies that efflux pump activity, not just expression, is important for psoralen resistance. They also used fluorescence polarization assays to show amotosalen bound directly to the AcrB homolog of *Acinetobacter* and reported that docking analyses predicted 8-methoxysporalen would bind in a similar manner. In future work, similar biochemical methods or membrane eversion assays could provide definitive support for this mechanism in *E. coli* [47].

The results of our current study may also suggest a more complex mechanism of regulation by AcrR than that of a simple repressor. The *acrR*(L34Q) point mutation is resistant to psoralen-UVA treatment and retains the *marbox* activation sequence, yet a deletion of the *acrR* open reading frame that deletes the *marbox* activation sequence renders cells sensitive. If AcrR acts as a basic repressor, then the simplest model would be that *acrR*(L34Q) is a null mutation, and that upregulating *acrAB* expression enough to provide full resistance requires both removal of the AcrR repressor and activation by MarA, SoxS, and Rob. The finding that removal of all three proteins renders the *acrR*(L34Q) mutant similar in resistance to the *acrR* deletion supports this model (Fig. 4D). In contrast to what was seen in wild-type cells (Fig. 2), deletion of *marA*, *soxS*, or *rob* individually was insufficient to noticeably reduce resistance in *acrR*(L34Q). As mentioned above, one possible explanation would be that the sensitivity of the survival assays used in this study are insufficient to distinguish small differences between hypersensitive strains, as their numbers approach the lower end of detectability. If true, increasing the background level of resistance through de-repression of *acrAB* expression as in Fig. 4 could make the differences between individual *marA*, *soxS*, and *rob* deletion mutants and the *marAsoxSrob* triple mutant more detectable.

The most prominent model for AcrR repressor function is that it releases upon binding a recognized substrate [27–29,48,49]. These initial studies used both LacZ reporter constructs and gel-shift binding assays to provide strong evidence that AcrR can repress expression of *acrAB* when

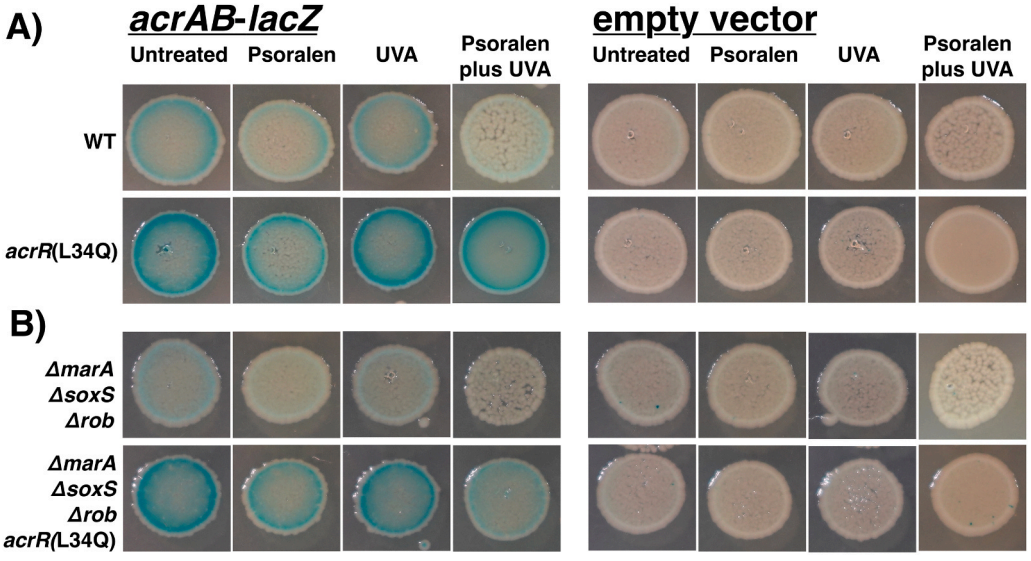

Fig. 6. *acrAB* expression is not upregulated by psoralen, UVA, or psoralen-UVA. 10 μL spots of 10^4 cells of wild type, *acrR*(L34Q), or Δ*marA* Δ*soxS* Δ*rob*, mutants containing a LacZ reporter plasmid fused with the *acrAB* promoter region (p-*acrAB-lacZ*) or no promoter region (empty vector) were plated on LB plates containing X-Gal. Plates contained 20 μg/mL 8-methoxypsoralen and were UVA irradiated with 3.8 kJ/m2 as indicated. LacZ expression from the plasmids is indicated by blue color in colonies.

bound to its promoter [27]. However, other aspects of this study also suggested more complexity in its function. Transcription of the *acrR* repressor was also induced by the same stressors that upregulated *acrAB* transporter expression, and *acrAB* upregulation during ethanol stress or growth phase occurred independently of MarA and SoxS, similar to what we observe in the presence of psoralen [27]. The repressor model of AcrR activity is based on its similarity to other TetR family transcriptional regulators. It proposes that upon ligand binding, AcrR releases from DNA to allow transcription. However the AcrAB-TolC transporter is active on a wide range of structurally divergent substrates [42,49], making it unclear how the protein could effectively recognize this diverse range of toxic substrates. The few substrates which have been examined and shown to promote AcrR release from oligos *in vitro* have been DNA intercalators [28,29,48] which makes it difficult to determine if release is due to ligand binding or changes to the DNA structure of the oligos used. Further, studies looking for AcrR-dependent induction of *acrAB* following treatments with known substrates of the pump have seen modest to no effect [42,49]. Thus, the mechanism of regulation and natural substrates for AcrR derepression of the efflux pump genes remain unclear.

This lack of mechanistic clarity also holds true for psoralen interstrand crosslink resistance. Despite evidence suggesting that psoralen is a substrate for the AcrAB-TolC efflux pump, AcrR does not appear to be naturally responsive to psoralen-UVA stress, as neither psoralen, UVA, nor crosslink induction upregulates *acrAB* expression (Fig. 6). While this finding may seem counterintuitive given the requirement of AcrAB for psoralen-UVA resistance, other examples of efflux pump substrates which fail to induce *acrAB* expression have been reported. No significant induction of *acrAB* expression was observed in *Salmonella enterica* treated with several antibiotics that are canonical substrates of AcrAB-TolC, including chloramphenicol and ciprofloxacin, echoing similar previous findings regarding tetracyclines [26,50]. Nevertheless, null mutations in *acrR* confer resistance to these antibiotics [51]. We hypothesize that a similar situation may exist in the case of psoralen, with derepression of *acrAB* by AcrR in the presence of psoralen-UVA only being possible through null mutations.

It is also notable that the three global stress regulators respond to compounds that do not appear to be ligands of AcrR and they have been proposed to be the primary regulators of *acrAB* expression with AcrR providing a secondary dampening function to counter overexpression [27,31–40]. Our inability to detect altered *acrAB* expression wild type cells (Fig. 6) argues that the overall influence of psoralens on *acrAB* activation is limited. However, psoralens can cause pleiotropic effects on cell membranes, and it remains possible that other stress pathways could contribute to the response to psoralen-UVA without detectably activating *acrAB* [52,53].

Importantly, we show here that full resistance is only achieved when *acrR* null mutations preserve the *marbox* sequence (Fig. 5), as in *acrR* (L34Q). This is particularly relevant to the emergence of multi-drug resistance in Gram-negative bacteria, as mutations in *acrR* are known to drive multi-drug resistance and are commonly found in clinical isolates [54–58]. Additionally, the original studies that characterized AcrR used insertion mutants that disrupted the protein after the *marbox*, while later studies used the complete deletion mutant of *acrR* from the Keio collection [26,27,41,42,49]. Our results demonstrate that it will be important in the future to consider the impact of *acrR* mutations on the *marbox* when assessing their phenotypic effects. Finally, it is also notable that the resistance to psoralen interstrand crosslinks is achieved by preventing this drug from forming this lesion. No mutations upregulating repair pathways or proteins were observed in the initial screen, consistent with previous studies that found cells lack effective repair mechanisms for this form of damage [6–8,59].

Ethical approval

Not required.

Funding

This study was supported by grant MCB1916625 from the National Science Foundation, R21ES034880 from the NIH National Institute of Environmental Health Science, and R16GM14554 from the NIH National Institute of General Medical Sciences.

CRediT authorship contribution statement

Cooper Lo: Investigation. **Asal Ayah H.:** Investigation. **Courcelle Charmain T.:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Courcelle Justin:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Worley Travis K.:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors have no competing interests to declare.

Acknowledgement

We thank C. Ruiz Rueda for generously sharing the pNN387 and pNN608 plasmids.

Data availability

Data will be made available on request.

References

[1] Y. Huang, L. Li, DNA crosslinking damage and cancer - a tale of friend and foe, Transl. Cancer Res. 2 (2013) 144–154.
[2] W. Liszewski, D.G. Naym, E. Biskup, R. Gniadecki, Psoralen with ultraviolet A-induced apoptosis of cutaneous lymphoma cell lines is augmented by type I interferons via the JAK1-STAT1 pathway, Photodermatol Photoimmunol Photo 33 (2017) 164–171.
[3] M.A. PATHAK, Mechanism of psoralen photosensitization and in vivo biological action spectrum of 8-methoxypsoralen, J. Invest Dermatol. 37 (1961) 397–407.
[4] R.S. Cole, Light-induced cross-linking of DNA in the presence of a furocoumarin (psoralen) Studies with phage lambda, Escherichia coli, and mouse leukemia cells, Biochim Biophys. Acta 217 (1970) 30–39.
[5] J.E. Hearst, Psoralen photochemistry, Annu Rev. Biophys. Bioeng. 10 (1981) 69–86.
[6] F. Dall'Acqua, S. Marciani, L. Ciavatta, G. Rodighiero, Formation of inter-strand cross-linkings in the photoreactions between furocoumarins and DNA, Z. Nat. B 26 (1971) 561–569.
[7] W. SZYBALSKI, V.N. IYER, Crosslinking of dna by enzymatically or chemically activated mitomycins and porfiromycins, bifunctionally "alkylating" antibiotics, Fed. Proc. 23 (1964) 946–957.
[8] J.M. Cole, J.D. Acott, C.T. Courcelle, J. Courcelle, Limited capacity or involvement of excision repair, double-strand breaks, or translesion synthesis for psoralen cross-link repair in, Genetics 210 (2018) 99–112.
[9] S.W. Johnson, D. Shen, I. Pastan, M.M. Gottesman, T.C. Hamilton, Cross-resistance, cisplatin accumulation, and platinum-DNA adduct formation and removal in cisplatin-sensitive and -resistant human hepatoma cell lines, Exp. Cell Res. 226 (1996) 133–139.
[10] A.V.H. Lengert, L.D.N.B. Pereira, E.R.M. Cabral, I.N.F. Gomes, L.M. Jesus, M.F. S. Gonçalves, A.O.D. Rocha, T.A. Tassinari, L.S.D. Silva, A.C. Laus, D.O. Vidal, M. T. Pinto, R.M. Reis, L.F. Lopes, Potential new therapeutic approaches for cisplatin-resistant testicular germ cell tumors, Front. Biosci. 27 (2022) 245.
[11] T.K. Worley, E.A. Weber, J.D. Acott, R.S. Shimpi, J.M. Cole, C.T. Courcelle, J. Courcelle, Mutations in AcrR and RNA polymerase confer high-level resistance to psoralen-UVA irradiation, J. Bacteriol. 205 (2023) e0012623.
[12] L.J. Piddock, D.G. White, K. Gensberg, L. Pumbwe, D.J. Griggs, Evidence for an efflux pump mediating multiple antibiotic resistance in Salmonella enterica serovar Typhimurium, Antimicrob. Agents Chemother. 44 (2000) 3118–3121.
[13] A. Burse, H. Weingart, M.S. Ullrich, The phytoalexin-inducible multidrug efflux pump AcrAB contributes to virulence in the fire blight pathogen, Erwinia amylovora, Mol. Plant Microbe Inter. 17 (2004) 43–54.
[14] X.Z. Li, K. Poole, H. Nikaido, Contributions of MexAB-OprM and an EmrE homolog to intrinsic resistance of Pseudomonas aeruginosa to aminoglycosides and dyes, Antimicrob. Agents Chemother. 47 (2003) 27–33.
[15] X.Z. Li, H. Nikaido, Efflux-mediated drug resistance in bacteria, Drugs 64 (2004) 159–204.

[16] H. Nikaido, Multidrug efflux pumps of gram-negative bacteria, J. Bacteriol. 178 (1996) 5853–5859.
[17] M. Chen, X. Shi, Z. Yu, G. Fan, I.I. Serysheva, M.L. Baker, B.F. Luisi, S.J. Ludtke, Z. Wang, In situ structure of the AcrAB-TolC efflux pump at subnanometer resolution, Structure 30 (2022) 107–113, e3.
[18] H. Nakamura, A. Suganuma, Membrane mutation associated with sensitivity to acriflavine in Escherichia coli, J. Bacteriol. 110 (1972) 329–335.
[19] D. Ma, D.N. Cook, M. Alberti, N.G. Pon, H. Nikaido, J.E. Hearst, Molecular cloning and characterization of acrA and acrE genes of *Escherichia coli*, J. Bacteriol. 175 (1993) 6299–6313.
[20] J.A. Fralick, Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of Escherichia coli, J. Bacteriol. 178 (1996) 5803–5805.
[21] H. Nakamura, Gene-controlled resistance to acriflavine and other basic dyes in Escherichia coli, J. Bacteriol. 90 (1965) 8–14.
[22] Y. Takatsuka, C. Chen, H. Nikaido, Mechanism of recognition of compounds of diverse structures by the multidrug efflux pump AcrB of Escherichia coli, Proc. Natl. Acad. Sci. USA 107 (2010) 6559–6565.
[23] H. Okusu, D. Ma, H. Nikaido, AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants, J. Bacteriol. 178 (1996) 306–308.
[24] L.M. McMurry, M. Oethinger, S.B. Levy, Overexpression of marA, soxS, or acrAB produces resistance to triclosan in laboratory and clinical strains of Escherichia coli, FEMS Microbiol Lett. 166 (1998) 305–309.
[25] H. Nikaido, Y. Takatsuka, Mechanisms of RND multidrug efflux pumps, Biochim Biophys. Acta 1794 (2009) 769–781.
[26] D. Ma, D.N. Cook, M. Alberti, N.G. Pon, H. Nikaido, J.E. Hearst, Genes acrA and acrB encode a stress-induced efflux system of Escherichia coli, Mol. Microbiol 16 (1995) 45–55.
[27] D. Ma, M. Alberti, C. Lynch, H. Nikaido, J.E. Hearst, The local repressor AcrR plays a modulating role in the regulation of acrAB genes of Escherichia coli by global stress signals, Mol. Microbiol 19 (1996) 101–112.
[28] M. Li, R. Gu, C.C. Su, M.D. Routh, K.C. Harris, E.S. Jewell, G. McDermott, E.W. Yu, Crystal structure of the transcriptional regulator AcrR from Escherichia coli, J. Mol. Biol. 374 (2007) 591–603.
[29] M.D. Routh, C.C. Su, Q. Zhang, E.W. Yu, Structures of AcrR and CmeR: insight into the mechanisms of transcriptional repression and multi-drug recognition in the TetR family of regulators, Biochim Biophys. Acta 1794 (2009) 844–851.
[30] W. Deng, C. Li, J. Xie, The underling mechanism of bacterial TetR/AcrR family transcriptional repressors, Cell Signal 25 (2013) 1608–1613.
[31] R.G. Martin, J.L. Rosner, Genomics of the marA/soxS/rob regulon of Escherichia coli: identification of directly activated promoters by application of molecular genetics and informatics to microarray data, Mol. Microbiol 44 (2002) 1611–1624.
[32] B. Demple, Regulation of bacterial oxidative stress genes, Annu Rev. Genet 25 (1991) 315–337.
[33] K. Skarstad, B. Thöny, D.S. Hwang, A. Kornberg, A novel binding protein of the origin of the Escherichia coli chromosome, J. Biol. Chem. 268 (1993) 5365–5370.
[34] S.P. Cohen, L.M. McMurry, S.B. Levy, marA locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of Escherichia coli, J. Bacteriol. 170 (1988) 5416–5422.
[35] L.M. McMurry, A.M. George, S.B. Levy, Active efflux of chloramphenicol in susceptible Escherichia coli strains and in multiple-antibiotic-resistant (Mar) mutants, Antimicrob. Agents Chemother. 38 (1994) 542–546.
[36] J.T. Greenberg, P. Monach, J.H. Chou, P.D. Josephy, B. Demple, Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in Escherichia coli, Proc. Natl. Acad. Sci. USA 87 (1990) 6181–6185.
[37] T. Nunoshiba, Two-stage gene regulation of the superoxide stress response soxRS system in Escherichia coli, Crit. Rev. Eukaryot. Gene Expr. 6 (1996) 377–389.
[38] J. Wu, B. Weiss, Two-stage induction of the soxRS (superoxide response) regulon of Escherichia coli, J. Bacteriol. 174 (1992) 3915–3920.
[39] R.R. Ariza, Z. Li, N. Ringstad, B. Demple, Activation of multiple antibiotic resistance and binding of stress-inducible promoters by Escherichia coli Rob protein, J. Bacteriol. 177 (1995) 1655–1661.
[40] E.Y. Rosenberg, D. Bertenthal, M.L. Nilles, K.P. Bertrand, H. Nikaido, Bile salts and fatty acids induce the expression of Escherichia coli AcrAB multidrug efflux pump through their interaction with Rob regulatory protein, Mol. Microbiol. 48 (2003) 1609–1619.
[41] T. Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K.A. Datsenko, M. Tomita, B.L. Wanner, H. Mori, Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection, Mol. Syst. Biol. 2 (2006) 2006.0008.
[42] D.E. Harmon, C. Ruiz, The multidrug efflux regulator AcrR of escherichia coli responds to exogenous and endogenous ligands to regulate efflux and detoxification, mSphere 7 (2022) e0047422.
[43] K.L. Griffith, I.M. Shah, R.E. Wolf, Proteolytic degradation of Escherichia coli transcription activators SoxS and MarA as the mechanism for reversing the induction of the superoxide (SoxRS) and multiple antibiotic resistance (Mar) regulons, Mol. Microbiol 51 (2004) 1801–1816.
[44] K.L. Griffith, M.M. Fitzpatrick, E.F. Keen, R.E. Wolf, Two functions of the C-terminal domain of Escherichia coli Rob: mediating “sequestration-dispersal” as a novel off-on switch for regulating Rob’s activity as a transcription activator and preventing degradation of Rob by Lon protease, J. Mol. Biol. 388 (2009) 415–430.
[45] R.G. Martin, W.K. Gillette, J.L. Rosner, Promoter discrimination by the related transcriptional activators MarA and SoxS: differential regulation by differential binding, Mol. Microbiol 35 (2000) 623–634.
[46] A.B. Green, L. Chiaraviglio, K.A. Truelson, K.E. Zulauf, M. Cui, Z. Zhang, M. P. Ware, W.A. Flegel, R.L. Haspel, E.W. Yu, J.E. Kirby, RND pump-mediated efflux of amotosalen, a compound used in pathogen inactivation technology to enhance safety of blood transfusion products, may compromise its gram-negative anti-bacterial activity, mSphere 8 (2023) e0067322.
[47] H.I. Zgurskaya, H. Nikaido, Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of Escherichia coli, Proc. Natl. Acad. Sci. USA 96 (1999) 7190–7195.
[48] C.C. Su, D.J. Rutherford, E.W. Yu, Characterization of the multidrug efflux regulator AcrR from Escherichia coli, Biochem Biophys. Res. Commun. 361 (2007) 85–90.
[49] C. Ruiz, S.B. Levy, Regulation of acrAB expression by cellular metabolites in Escherichia coli, J. Antimicrob. Chemother. 69 (2014) 390–399.
[50] V. Ricci, J. Kaur, J. Stone, L.J.V. Piddock, Antibiotics do not induce expression of acrAB directly but via a RamA-dependent pathway, Antimicrob. Agents Chemother. 67 (2023) e0062023.
[51] M.A. Webber, A. Talukder, L.J. Piddock, Contribution of mutation at amino acid 45 of AcrR to acrB expression and ciprofloxacin resistance in clinical and veterinary Escherichia coli isolates, Antimicrob. Agents Chemother. 49 (2005) 4390–4392.
[52] B. Van Aelst, R. Devloo, P. Zachée, R. t’Kindt, K. Sandra, P. Vandekerckhove, V. Compernolle, H.B. Feys, Psoralen and ultraviolet a light treatment directly affects phosphatidylinositol 3-kinase signal transduction by altering plasma membrane packing, J. Biol. Chem. 291 (2016) 24364–24376.
[53] D.J. dos Santos, L.A. Eriksson, Permeability of psoralen derivatives in lipid membranes, Biophys. J. 91 (2006) 2464–2474.
[54] L. Xian-Zhi, P. Patrick, N. Hiroshi, The challenge of efflux-mediated antibiotic resistance in gram-negative bacteria, Clin. Microbiol. Rev. 28 (2015) 337–418.
[55] H. Wang, J.L. Dzink-Fox, M. Chen, S.B. Levy, Genetic characterization of highly fluoroquinolone-resistant clinical Escherichia coli strains from China: role of acrR mutations, Antimicrob. Agents Chemother. 45 (2001) 1515–1521.
[56] T. Schneiders, S.G. Amyes, S.B. Levy, Role of AcrR and ramA in fluoroquinolone resistance in clinical Klebsiella pneumoniae isolates from Singapore, Antimicrob. Agents Chemother. 47 (2003) 2831–2837.
[57] M.A. Webber, L.J. Piddock, Absence of mutations in marRAB or soxRS in acrB-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of Escherichia coli, Antimicrob. Agents Chemother. 45 (2001) 1550–1552.
[58] P. Komp Lindgren, A. Karlsson, D. Hughes, Mutation rate and evolution of fluoroquinolone resistance in Escherichia coli isolates from patients with urinary tract infections, Antimicrob. Agents Chemother. 47 (2003) 3222–3232.
[59] P.F. Lin, E. Bardwell, P. Howard-Flanders, Initiation of genetic exchanges in lambda phage–prophage crosses, Proc. Natl. Acad. Sci. USA 74 (1977) 291–295.
[60] K.A. Datsenko, B.L. Wanner, One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products, Proc. Natl. Acad. Sci. USA 97 (2000) 6640–6645.
[61] P.P. Cherepanov, W. Wackernagel, Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant, Gene 158 (1995) 9–14.
[62] P. Rajapaksha, I. Ojo, L. Yang, A. Pandeya, T. Abeywansha, Y. Wei, Insight into the AcrAB-TolC complex assembly process learned from competition studies, Antibiot. (Basel) 10 (2021).
[63] S.J. Elledge, R.W. Davis, Position and density effects on repression by stationary and mobile DNA-binding proteins, Genes Dev. 3 (1989) 185–197.