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Review: Structure and transcriptional regulation of the Acr efflux pumps and their role in antibiotic resistance in *Escherichia coli*

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SUMMARY Multidrug resistance in Gram-negative bacterial pathogens is often mediated by efflux pumps that actively transport antibiotic compounds out of the cell. The AcrAB, AcrAD, and AcrEF proteins form such efflux pump complexes with the TolC protein, that confer resistance to a broad subset of antibiotics in *Escherichia coli*. In this review, we outline the genomic and protein structures and transcriptional regulation of AcrAB-TolC, AcrAD-TolC, and AcrEF-TolC. These tripartite systems are homologous, with differences in substrate specificity mainly attributed to the binding-site structure of their inner membrane transporters. On the genome, expression of the *acrAB* operon, *acrD* gene, and *acrEF* operon is modulated by multiple mechanisms, such as the local transcriptional repressors AcrS and AcrR, global transcriptional activators including regulators of the XylS/AraC family and H-NS, as well as transposable insertion elements IS186 and IS2. We conclude this review with a comparison of AcrS and AcrR regulation of the AcrAB, AcrAD, and AcrEF systems. We also discuss current knowledge gaps regarding the regulatory mechanisms that underlie Acr-mediated antibiotic resistance, particularly to kanamycin, that should be addressed in future studies.

INTRODUCTION

There is a global rise of infections caused by bacterial pathogens that are resistant to multiple classes of antibiotics (1). Historically, Gram-negative bacteria have been thought to have more intrinsic resistance against antimicrobial agents than Gram-positive bacteria, which is usually attributed to the presence of an outer membrane that slows influx of substances into the cells. Gram-negative bacteria such as *Escherichia coli* also have tripartite efflux pumps embedded in their double membranes that allow for extracellular transport of a variety of molecules from both the periplasmic space and the cytoplasm (2–4) . While a number of efflux pumps are specific to only one type of substrate, such as TetA in the transport of tetracycline, multidrug efflux pumps can transport numerous molecules that differ in structure (2, 5). AcrAB, for example, transports sodium dodecyl sulfate, metabolites, as well as a broad range of antibiotics and dyes (5). This plays a role in the increasing multidrug resistance (MDR) of bacterial agents in the clinical setting (6).

Efflux pumps can be categorized into six classes— the ATP-binding cassette (ABC) family, multidrug and toxic compound extrusion (MATE) family, major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance nodulation division (RND) family, and the recently identified proteobacterial antimicrobial compound efflux (PACE) family (7, 8). These families use distinct energy sources to transport substrates. More specifically, the ABC family uses ATP, the MATE family uses Na^+/H^+ antiporters, while the

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MFS, SMR, and RND families use proton-motive forces (9). Out of these transporter groups, the RND family is the most significant to intrinsic antibiotic resistance as they expel the widest range of antimicrobial agents (9).

The most well-characterized pump of the RND family is the acriflavine (Acr) system which includes the AcrAB-TolC, AcrAD-TolC, and AcrEF-TolC transporters, of which AcrAB-TolC is the most extensively studied (9). These multidrug efflux pumps are composed of three components: the outer membrane channel, TolC, a periplasmic bridge, and an inner membrane energy-transducer (10). In addition to antibiotic transport, these TolC-dependent Acr efflux pumps can expel metabolites and signaling molecules important for quorum sensing (7, 10). This review will present current knowledge on the Acr efflux system in *E. coli*, with focus on their structure, regulation, and implications in antibiotic resistance.

GENOMIC ORGANIZATION OF *acrAB, acrEF,* AND *acrD*

AcrA and AcrB are encoded by genes *acrA* (1194 bp) and *acrB* (3150 bp) from the constitutively expressed operon *acrAB* (Fig. 1) (11). The *acrR* gene, encoding a local repressor of *acrAB*, AcrR, is situated 141 bp upstream of *acrA* and is transcribed divergently (Fig. 1) (12, 13). Genes *acrE* (1260 bp) and *acrF* (4037 bp), of the *acrEF* operon, encode proteins AcrE and AcrF where *acrE* is localized 396 bp away from the divergently transcribed *acrS,* which encodes AcrS (Fig. 1) (14, 15). Both AcrR and AcrS proteins, members of the TetR family of repressors, recognize and bind a 24 bp palindromic sequence found in the promoter region of *acrAB* (13, 15). Furthermore, AcrD relies on AcrA for its activity, but the *acrD* gene (3114 bp) is independent of *acrAB*, located further downstream, and found upstream of *acrEF* on the *E. coli* chromosome (Fig. 1) (16).

STRUCTURE AND SUBSTRATE SPECIFICITY OF EFFLUX PUMPS AcrAB, AcrAD, AND AcrEF

The AcrAB-TolC transporter is composed of three components in a 1:2:1 ratio: an inner membrane active transporter, AcrB, a periplasmic adaptor, AcrA, and an outer membrane channel, TolC (Fig. 2) (10, 17, 18). The assembly of these components is required for energy-dependent multidrug efflux driven by a proton motive force (10). The active AcrB component is made up of three protomers organized in a ring-like manner as trimers spanning the inner membrane and forming a pore extending into the periplasm, where it can interact with AcrA (18, 19). AcrA interactions with AcrB form the AcrAB complex (10). The AcrA protein is composed of three domains: a β-barrel domain, a lipoyl domain and an α-hairpin domain (10, 19). Finally, the TolC component is anchored to the outer membrane by its β-barrel structure with an α-helical barrel extending into the periplasm (19, 20). Conserved motifs in these periplasmic TolC coiled-coil helical structures bind AcrA α-hairpins in a tip-to-tip manner forming the stabilized AcrAB-TolC complex (19, 20). This AcrA-TolC interaction is thought to induce conformational change in the α-helical barrel of TolC to allow diffusion of substrates across the outer membrane and into the external medium (19). Ligand interactions

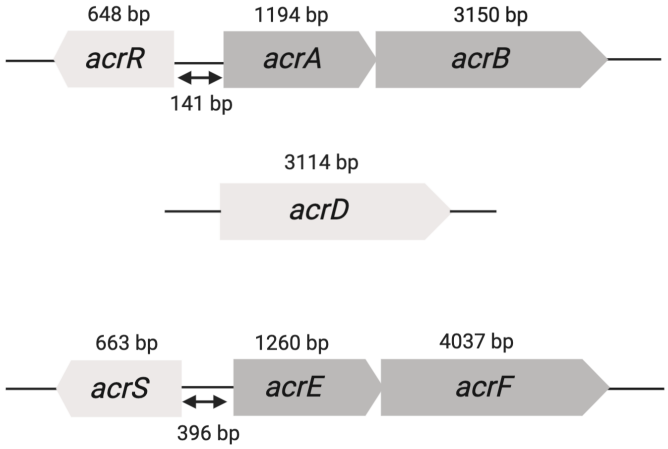


FIG. 1 Structure of the *acrAB* and *acrEF* operons. This schematic depicts the base pair (bp) lengths of the genes in the *acrAB* and *acrEF* operons, the *acrD* gene, and the distance in bp between the divergently transcribed *acrR* and *acrS* genes relative to the *acrAB* and *acrEF* operons, respectively. Genes are not to scale.

with puromycin have revealed that TolC presumes an open conformation which is thought to allow drug extrusion and maintenance of connectivity to AcrA (20).

The AcrAB-TolC complex is known to efflux lipophilic and amphiphilic substrates but can also pump out substrates which cannot spontaneously cross the cytoplasmic membrane, such as doxorubicin (Table 1). This indicates that high lipophilicity is not necessary for transport but contributes to more efficient export (19). Co-crystallization of AcrB with several ligands, such as minocycline or doxorubicin, show that ligands bind two periplasmic loops of AcrB through hydrophobic interactions (21, 22). It is thought that substrate binding in this periplasmic region induces AcrB loose, tight, and open conformational changes with Wang *et al*. demonstrating three conformational changes in AcrB upon binding to the known transport substrate, puromycin (19, 20). Such conformational changes are thought to mediate a functional rotatory pumping mechanism to allow proton/substrate antiport where substrates are effluxed to the TolC channel and eventually to the external medium (Fig. 2) (10, 19). The mechanism by which substrates move from AcrB to TolC, however, has not yet been determined (19). It has been proposed by Wang *et al*., however, that conformational changes in AcrB upon substrate binding causes AcrA repacking and a subsequent TolC open conformation to allow drug extrusion. The repacking of AcrA is proposed to prevent substrates from leaking back into the periplasm (20).

The AcrAB-TolC system is structurally similar to AcrAD-TolC, the only difference being the inner membrane transporter (Fig. 2). Yamamoto *et. al.* proposed that AcrAD-TolC is not assembled *de novo* but instead high *acrD* expression disrupts AcrB that is already complexed with AcrA and TolC (23). Indole induction of AcrD was demonstrated to interfere with AcrB in the absence of AcrB-specific substrates, with AcrD likely destabilizing and replacing AcrB at the cytoplasmic membrane to assemble the AcrAD-TolC complex. Compared to the AcrAB-TolC pump, AcrAD-TolC has a smaller range of substrates, primarily effluxing hydrophilic substrates that do not readily cross the cytoplasmic membrane (Table 1) (16). The trimeric inner membrane transporter AcrD is a close homolog of AcrB, sharing high sequence similarity but slightly differs structurally by two periplasmic loops that confer substrate selectivity (16, 24). The surface of the periplasmic domain in AcrD is abundant in oxygen atoms that may facilitate hydrophilic interactions, whereas in AcrB, the surface of this

TABLE. 1 Known substrates of RND efflux pumps AcrAB-TolC, AcrAD-TolC and AcrEF-TolC.

Efflux Pump	Substrates		References
	Antibiotics	Non-antibiotics	
AcrAB-TolC	Erythromycin, novobiocin, fusidic acid, tetracycline, β-lactams, chloramphenicol, mitomycin C, quinolones, fluoroquinolones, cefuroxime, trimethoprim, clindamycin, linezolid, tigecycline, nalidixic acid	Acriflavine, enterobactin, organic solvents, steroid hormones (bile acids, progesterone, estradiol, hydrocortisone), dyes, sodium dodecyl sulfate (SDS), Triton X-100, aminoacyl β-naphthylamides, quorum sensing autoinducers	(5, 27, 71–76)
AcrAD-TolC	Aminoglycosides (amikacin, gentamicin, neomycin, kanamycin, and tobramycin), novobiocin, anionic β-lactams (aztreonam, carbenicillin, and sulbenicillin)	Enterobactin, steroid hormones (bile acids, progesterone, estradiol, hydrocortisone)	(2, 16, 77)
AcrEF-TolC	Erythromycin, fusidic acid, tetracycline, mitomycin C, tigecycline, quinolones, chloramphenicol, fluoroquinolones, cefuroxime, trimethoprim, clindamycin, linezolid	Indole, dyes, SDS	(4, 15, 16, 19)

substrate-binding domain is hydrophobic (25). Specifically, the proximal binding pocket of this periplasmic domain determines the β-lactam specificity of AcrB and AcrD, with AcrD selectively recognizing anionic β-lactams (Table 1) (26). Primarily, AcrD is critical for the efflux of aminoglycosides, a highly hydrophilic class of antibiotics, with *acrD*-deletion mutants showing decreases in minimum inhibitory concentration (MIC) of either gentamicin, neomycin, kanamycin and tobramycin (16).

Unlike AcrAB-TolC and AcrAD-TolC, the structure of AcrEF-TolC has not been extensively characterized. However, AcrF and AcrE have 77.6% and 69.3% homology with AcrB and AcrA respectively; therefore, it is thought that the AcrEF-TolC complex has a similar structure and assembly as AcrAB-TolC (Fig. 2) (9). In addition, the substrate specificity of AcrEF-TolC has not been fully elucidated, although there is evidence indicating that its broad substrate specificity is similar to that of AcrAB-TolC (Table 1) (27).

STRUCTURE OF LOCAL REGULATORY PROTEINS AcrR AND AcrS

AcrR is a transcriptional repressor that is involved in the local regulation of *acrAB*. It is entirely helical, composed of 9 folded α-helices (28). The crystal structure of AcrR reveals a dimeric structure with two-domains, a DNA-binding domain with a helix-turn-helix motif at the N-terminus, and a larger ligand-binding domain at the C-terminus (28). The ligand-binding domain is lined with mostly hydrophobic residues with negative potential in the inner pockets. It is therefore thought that this repressor can bind neutral and positively charged ligands mediating DNA binding (28). Extensive characterization of AcrS, a putative local repressor of *acrEF*, has not yet been done. However, AcrS has a homologous structure to AcrR as both proteins have a helix-turn-helix motif close to their respective N-termini (15, 29, 30).

H-NS REGULATION OF *acrEF* EXPRESSION

The expression of *acrEF* is low and is attributed to repression by the global regulator H-NS, a histone-like nucleoid protein (15). Nishino and Yamaguchi showed that *ΔhnsΔacrAB E. coli* strains exhibit increased *acrEF* expression and increased resistance to antibiotics, antiseptics, dyes and detergents, relative to *ΔacrAB* alone (31). Subsequent deletion of *acrEF* reversed MDR (31). Hirakawa *et al.* further showed with LacZ reporter assays that *acrEF* promoter activity increases following *hns* deletion (15).

AcrR AND AcrS REGULATION OF *acrAB*

AcrR and AcrS are local transcriptional repressors thought to regulate *acrAB* and *acrEF* expression, respectively (Fig.3) (13, 15, 25, 26). AcrR is an autoregulated local repressor of *acrAB* that binds the *acrA* promoter region, presumably blocking RNA polymerase, such that inactivation of *acrR* causes increased *acrAB* expression and antibiotic resistance in clinical *E. coli* isolates (Fig. 3) (15, 32). The mechanism by which drug inducers bind to AcrR and cause *acrAB* expression is not well understood, but it is thought that ligand binding to the AcrR C-terminal domain induces conformational change in its N-terminal domain, releasing AcrR from the promoter region and causing subsequent *acrAB* expression (28). Ligand binding of AcrR has been characterized with ethidium (Et), proflavine (Pf) and rhodamine 6G (R6G) (Fig. 3) (33). Pf can bind apo-AcrR and AcrR-Et non-competitively, but competitive binding occurs between Et and R6G (33).

Hirakawa *et al.* also demonstrated AcrS regulation of *acrA* (15). Specifically, when *acrS* was overexpressed in wild-type (WT) *E. coli* W3104, antibiotic resistance decreased drastically, however in *ΔacrAB* mutants, antibiotic susceptibility for chloramphenicol, tetracycline, erythromycin, novobiocin, nalidixic acid and norfloxacin was not affected relative to normal *acrS* expression (15). Hirakawa *et al.* therefore argued that AcrS is a negative regulator of *acrAB* such that in *acrAB*-deficient *E. coli*, AcrS-mediated repression is diminished (Fig. 3) (15). To further corroborate AcrS in *acrA* repression, AcrS binds the same 24 bp palindromic sequence in the *acrA* promoter region as AcrR (Fig. 3) (15). This DNA-binding is thought to inhibit RNA polymerase binding as is characteristic of the TetR family

of repressors (34). The compounds that regulate AcrS, however, have not yet been identified (15).

Belmans *et al.* also demonstrated AcrS regulation of *acrA* through MIC analysis (35). It was shown that an *ΔacrSΔkan* BW25113 mutant exhibited relatively high kanamycin resistance, followed by decreased resistance for the WT *E. coli* strain, and the lowest resistance for *ΔacrAΔkan* mutant. Belmans *et al.* therefore argued that *acrS* deletion may cause de-repression of *acrA,* inducing increased *acrA* expression and eventually allowing increased kanamycin efflux (35).

AcrR REPRESSES *acrAB* TO A LESSER EXTENT THAN AcrS

Although *acrR* is transcribed closely upstream of the *acrAB* operon, the binding affinity of AcrR to the *acrAB* promoter region is weaker than that of AcrS, thus AcrS is thought to regulate *acrAB* more than AcrR (15, 28). Hirakawa *et al.* showed that in *acrEF*-overexpressing W3104 *E. coli* (*Δhns*), *acrR* overexpression decreased *acrA* expression less than *acrS* overexpressing counterparts (15). Additionally, *acrR* overexpression decreased MICs for fewer toxic compounds tested and caused a less pronounced decrease in AcrB protein levels than *acrS* overexpressing counterparts (15). This weaker AcrR repressor activity relative to AcrS can be explained in part by a potential dampening role of AcrR for *acrAB* as opposed to complete repression (36). For instance, Ma *et al.* showed that the presence of stress factors, such as ethanol and high osmolarity, increased both *acrAB* and *acrR* expression (36). Thus, Ma *et al.* argued that AcrR may be modulating *acrAB* overexpression, thus acting as a secondary modulator, explaining the simultaneous increase of *acrAB* and *acrR* transcript levels (36). This proposed modulatory regulation of *acrA* overexpression by AcrR may be necessary to offset toxic effects such as cell cycle defects, as observed in *acrEF* deficient *E. coli* made to overexpress *acrA* (37).

AcrS MAY NOT REGULATE *acrEF*

AcrS is thought to be a local transcriptional repressor of the *acrEF* operon (36, 37). Hirakawa *et al.*, however, showed that AcrS is a transcriptional repressor of the *acrAB* operon, as described previously, and not the *acrEF* operon (15). This is because *acrS* overexpression in *acrEF* overexpressing *E. coli* (*Δhns*) was shown to cause a larger fold decrease in *acrA* expression than *acrE* (15). Moreover, overexpression of *acrS* in *ΔhnsΔacrAB* strains did not affect antibiotic resistance. Hirakawa *et al.* therefore argued that *acrS* overexpression does not affect *acrEF*-mediated drug resistance (15). Additionally, *ΔhnsΔacrAB* mutants did not exhibit changes in antibiotic resistance with *acrS* deletion. Furthermore, β-galactosidase assays seemed to reveal that in *Δhns* MC4100 *E. coli*, both *acrE* and *acrS* promoter activity was high (15). This finding suggests that *acrS* is not regulating

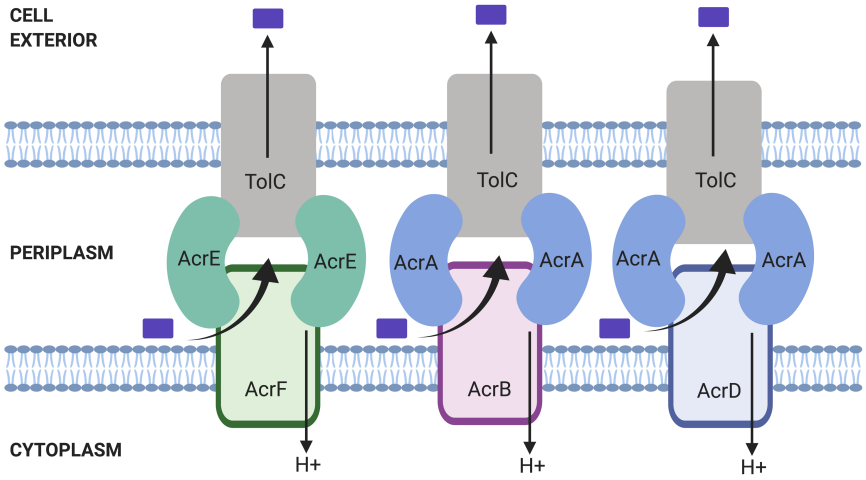


FIG. 2 . Structure and function of the efflux pumps AcrAB-TolC, AcrAD-TolC and AcrEF-TolC. Schematic depiction of the structure of AcrAB-TolC, AcrAD-TolC and AcrEF-TolC efflux pumps, as well as the active transport of substrates (purple rectangles) driven by proton/substrate antiport with subsequent substrate efflux through TolC.

acrE as high *acrS* expression did not decrease *acrE* expression. To further corroborate these findings, it has been shown that Δ*acrS*::*kan* mutants, of *Salmonella enterica* (*S. enterica*) serovar Typhimurium DT204 strain 102SA00, do not exhibit *acrF* overexpression (38).

Contrarily, Hay *et al.* showed that deleting *acrS* in *E. coli* BW25113 increased *acrE* expression but not *acrA,* implicating AcrS in *acrEF* repression and not *acrAB* (39). They also found that *acrS* deletion led to increased kanamycin resistance (39). Baeva *et al*., however, demonstrated that overexpression of *acrS* in an *ΔacrEΔkan E. coli* mutant decreased kanamycin resistance suggesting that AcrS is regulating expression beyond *acrE* (40). It is worth noting, however, that aminoglycosides such as kanamycin are not known to be substrates for AcrAB-TolC and AcrEF-TolC (Table 1). As shown by these contradictory findings, the effect of AcrS on *acrEF* and *acrAB* expression is yet to be completely elucidated.

MarA, SoxS, AND Rob REGULATION OF *acrAB*

Global regulators MarA, SoxS and Rob from the XylS/AraC family of regulators, are homologous transcriptional activators that positively regulate *acrAB* expression in response to environmental stimuli (15, 32, 41, 42). Transcriptional repression of *marA* and subsequent reduction in *acrAB* expression is mediated by the repressor MarR which binds *marO,* the operator region of the *marRAB* operon (Fig. 3) (43). The transcriptional repressor SoxR, binds the *soxS* promoter thereby repressing *soxS* expression. Oxidative stress, however, inactivates SoxR enabling *soxS* expression (Fig. 3) (43). Both MarA and SoxS are further regulated post-translationally by protein degradation from the ATP-dependent Lon protease such that mutations in Lon confers increased AcrAB efflux activity and MDR (43). The regulation of Rob proteins, on the other hand, has been shown to occur post-translationally (44).

De-repression of *marA* and subsequent *acrAB* promoter binding and expression activation by MarA is mediated by ligand binding to MarR of phenolic compounds such as sodium salicylate, which is thought to inhibit MarR DNA binding to the operator region of *marRAB* (Fig. 3) (43). Mutations in *marR* have been shown to increase *marA* expression, broad-spectrum antibiotic resistance, and *acrAB* expression in WT *E. coli* K-12 but not in *ΔacrAB* counterparts (27, 42). Transcriptional regulation of *soxS* and subsequent *acrAB* promoter binding and expression activation by SoxS is mediated by oxidative stress, whereby iron-sulfur clusters in SoxR become oxidized thus inactivating SoxR and allowing *soxS* expression activation (44). Rob activation of *acrAB* expression can occur by binding of Rob to the *acrAB* promoter region (45). Ligand binding to Rob of lipophilic unconjugated bile salts, such as deoxycholate and chenodeoxycholate, medium-chain fatty acids such as decanoate, and cationic peptides are thought to induce *acrAB* expression in a Rob-dependent manner (Fig. 3) (45). These ligands are thus thought to make *E. coli* more resistant to lipophilic antibiotics (43, 45). Additionally 2,2'-dipyridyl, a metal chelator, can activate Rob allowing downstream *acrAB* expression (46). In addition to *acrAB* regulation, MarA, SoxS and Rob activate *tolC* expression and thus these global regulators can regulate the complete AcrAB-TolC complex (Fig. 3) (47). Interestingly, antibiotic resistant phenotypes are typically influenced by combinatory effects where both *marA* and *soxS* overexpression mediate increased antibiotic resistance (43).

BaeSR- AND CpxAR-MEDIATED INDUCTION OF *acrD* EXPRESSION

Unlike *acrAB*, which is constitutively expressed, *acrD* is mainly expressed due to environmental stressors that trigger microbial survival signals, such as indole (48). In *E. coli*, indole, a tryptophan-derived metabolite, triggers transcription of various efflux pump genes, such as *acrD* and *acrE* (49). Dual two-component systems, BaeSR and CpxAR, transduce intracellular signals like indole and function together to increase *acrD* expression (Fig. 3). Both BaeR and CpxR directly interact with the *acrD* promoter in different regions (Fig. 3). BaeSR, however, acts as the main two-component system for inducing *acrD* expression, since a greater reduction in *acrD* expression is seen in a *baeSR*-deletion mutant compared to a *cpxAR*-deletion mutant (49). BaeR binds to the proximal sites of the *acrD* promoter whereas CpxR binds to distal regions, thus CpxR is thought to modulate and likely enhance the BaeR

activity on the *acrD* promoter site. Studies focused on kanamycin adaptive resistance have highlighted the role of BaeSR and potential assistance from CpxAR on *acrD* induction. The induction of *acrD* expression after kanamycin pre-treatment has been shown to depend on BaeSR, since *acrD* expression levels and kanamycin adaptive resistance is lower in *baeS* and *baeR* single-deletion strains (50, 51).

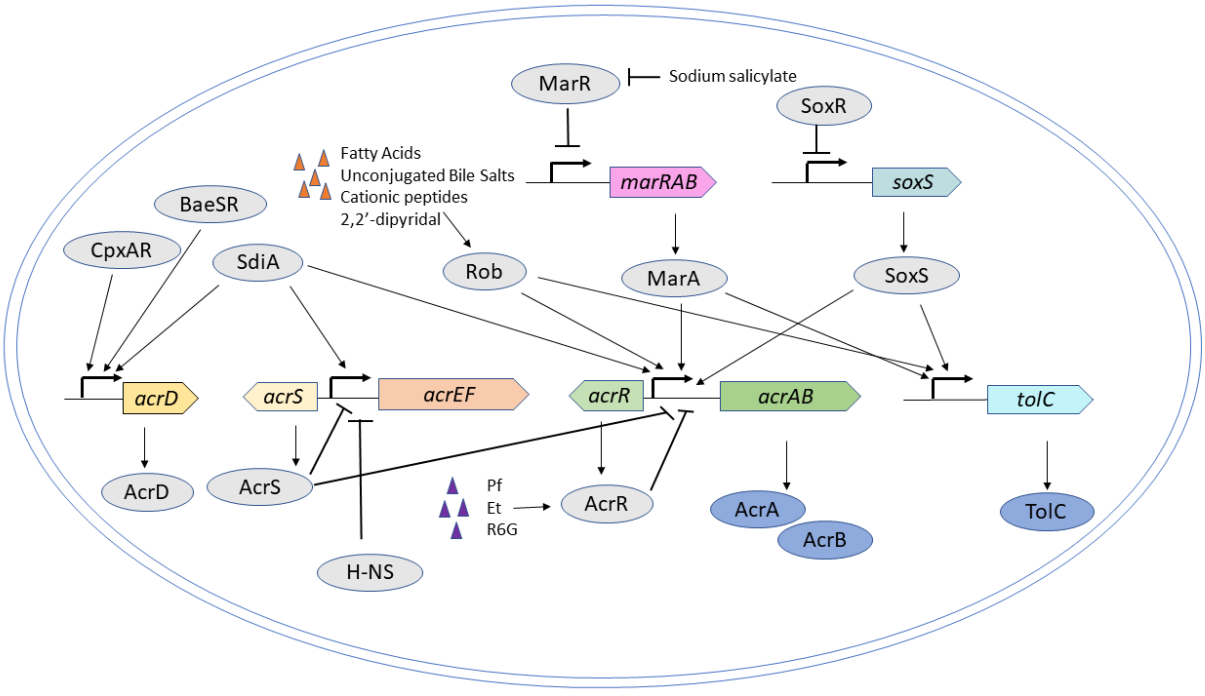
AcrS BUT NOT AcrR MAY REGULATE AcrD IN KANAMYCIN ADAPTIVE RESISTANCE

Studies employing *E. coli* BW25113 deletion mutants have highlighted the role of AcrD in kanamycin adaptive resistance. After pre-treatment with sub-inhibitory doses of kanamycin, Sidhu *et al.* observed increased kanamycin MIC in the WT strain but not in an *acrD*-deletion strain (52). Since increased *acrD* expression had also been observed in the pre-treated BW25113 WT strain, AcrD was thought to promote kanamycin adaptive resistance (50, 52). Although AcrD-mediated kanamycin adaptive resistance is thought to be primarily regulated by BaeSR (50, 51), Emami *et al.* proposed AcrS as a positive regulator of *acrD* (53). Emami *et al.* observed *acrD* expression was absent in a kanamycin pre-treated *acrS*-deletion mutant but present in a pre-treated WT strain, based on an RT-PCR electrophoresis gel (53). Additionally, the *acrS*-deletion mutant showed higher kanamycin adaptive resistance. Meanwhile, Dick *et al.* observed that a pre-treated *acrR*-deletion mutant showed similar *acrD* and *acrA* expression to the pre-treated WT, implying that AcrR has no regulatory effect on AcrAD-mediated kanamycin adaptive resistance (54).

SdiA REGULATION OF *acrABDEF* EXPRESSION

DNA microarray and RT-qPCR based studies have revealed that SdiA—a positive activator of *ftsQAZ*, which is involved in cell septation—increases the transcript levels of *acrABDEF* (Fig. 3). More specifically, RT-qPCR-based assays reveal that overexpression of

FIG. 3 Schematic overview of the transcriptional regulation of *acrAB*, *acrEF*, and *acrD* efflux pump genes in *Escherichia coli*. Efflux genes *acrAB, acrEF, acrD, tolC* and global regulator genes *soxS* and *marRAB* are depicted. Protein products are represented as ovals. Black bar-headed arrows depict repression while black point-headed arrows depict activation of the respective genes at the promoter regions or proteins upon ligand binding. Pf (proflavine), Et (ethidium), R6G (rhodamine 6G).



sdiA causes a large increase in *acrE* expression followed by *acrA, acrD*, *acrF* and *acrB* expression (55). Interestingly, AcrEF has been implicated in septation and is thought to play a role in cell division much like SdiA (37, 55). Furthermore, indole, which is effluxed by AcrEF-TolC, can induce the sensor of SdiA and is known to increase expression of *acrE* and *acrD,* as previously mentioned (49, 56, 57). The mechanism by which SdiA increases *acrABDEF* expression remains to be elucidated and warrants further exploration.

TRANSPOSABLE ELEMENT INSERT IS186 INCREASES *acrB* EXPRESSION UPON *marRAB* DELETION

Mutations in DNA replication genes DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) have been associated with increased MDR (27). Such phenotypes co-occur with increased *marA* and *soxS* expression plus accompanying point mutations in *marR* (32). These downstream mutations seem to affect MDR phenotypes by causing upregulation of *acrAB* and other drug efflux pumps (32). Furthermore, Jellen-Ritter and Kern showed that *marRAB*-deletion mutants of *E. coli* K-12 AG100 which harbour *gyrA* point mutations possess insertions of the transposable element IS186 within the *acrR* gene (27). This mutant was then associated with increased MDR and *acrB* expression possibly due to disruption of the *acrAB* repressor gene, *acrR* (27). In other words, additional mutations in the form of transposable elements have been shown to accommodate the absence of an *acrAB* transcriptional activator, MarA.

TRANSPOSABLE ELEMENT INSERT IS2 INCREASES *acrEF* EXPRESSION UPON *acrB* DELETION

Transposable insertion element IS2 upstream the *acrEF* operon has been implicated in increased *acrEF* expression (11, 27). *E. coli ΔacrAB* K-12 strains with *gyrA* point mutations exhibited enhanced drug efflux, restoring multi-drug resistance, with associated increase in *acrEF* expression (27). IS2 inserts were found upstream the *acrEF* operon in these mutants and the inserts were thought to enhance the *acrEF* promoter region (27). Similarly, *E. coli* OST5500, which has an inactivated *acrB*, was shown to elevate *acrEF* levels with accompanied IS1 and IS2 insertions upstream *acrE*, ultimately restoring organic solvent resistance (58). A similar phenomena was observed in *S. enterica Serovar Typhimurium DT204 acrB* mutants where IS1 and IS10 inserts were found disrupting the *acrS* gene or disrupting the *acrEF* promoter, respectively, with associated increase in *acrF* expression and restoration of fluoroquinolone resistance (38)**.**

DELETION OF *acrB* AND REPEATED SUBLETHAL ANTIBIOTIC EXPOSURE INCREASES OFF-TARGET MUTAGENESIS WITH EFFECTS ON REGULATION

Repeated exposure to sublethal doses of antibiotics over time can induce mutagenesis. Cudkowicz and Schuldiner showed that chloramphenicol-exposed BW25113 *E. coli* have a single point mutation in *acrB* and MFS transporter gene *mdfA,* with two nucleotide mutations upstream of *marR* (59). Deletion of *acrB,* however, increased these mutational events. Such mutations consisted of transposon insertions upstream of the gene *uspC,* a universal stress protein, and point mutations in *marR, mdfA, gyrB* and *sapC*, genes implicated in antibiotic resistance (59). Point mutations in *marR* and *mdfA* caused decreased and increased expression of the respective genes, in both WT and *ΔacrB* strains (59). Decrease in *marR* expression accompanied an increase in *marA* expression, allowing regulation of multiple genes involved in antibiotic resistance including *acrAB* (59). These findings support the idea of bacterial cells adapting to their environment to maintain drug homeostasis.

CROSSTALK BETWEEN MULTIPLE DRUG TRANSPORTERS MAINTAINS MULTI-DRUG RESISTANCE IN *acrB* DEFICIENT STRAINS

MDR is thought to be maintained in *acrAB* deficient *E. coli* by upregulation of other drug transporters (59). More specifically, in *ΔacrB* BW25113 *E. coli*, Shuster *et al.* showed that

norfloxacin resistance, after repeated sublethal exposure, seemed to reach WT levels with associated increase in *acrEF* expression (60). Furthermore, *acrB* deletion of BW25113 *E. coli* without antibiotic exposure was shown to cause higher TolC-dependent transporter gene expression (*acrF, acrD*, *macB, mdtF*) relative to WT with expression levels increasing upon sublethal chloramphenicol exposure (59). With this chloramphenicol exposure, accompanying cross resistance to nalidixic acid, ofloxacin, norfloxacin and erythromycin was observed followed by smaller effects on kanamycin and fosfomycin (59). Furthermore, Hirakawa *et al*. proposed that AcrS mediates the switch between *acrAB* expression and *acrEF* expression by decreasing *acrAB* expression upon high *acrEF* expression (15). This is a proposed mechanism that accounts for conservation of cell resources as upregulation of both AcrAB and AcrEF pumps could be considered redundant.

AcrAB-TolC ACTIVITY INHIBITION SEEMS TO INCREASE *acrAB* PROMOTER ACTIVITY

Ruiz and Levy showed that deletion of *acrB* or *tolC* increased *acrAB* promoter activity in *E. coli* (61). Deletion of *acrA* also increased *acrAB* promoter activity but to a lesser extent. Moreover, in the absence of *acrAB* deletions, *acrAB* promoter activity increased with the efflux pump inhibitor, phenylalanine-arginine-β-naphthylamide (PAβN) but PAβN did not induce additional effects in Δ*acrB* strains. With this, Ruiz and Levy argued that a lack of AcrAB-TolC activity induces *acrAB* expression (61).

INTERPLAY OF AcrR, SoxRS and MarRA MAY INCREASE *acrAB* PROMOTER ACTIVITY IN *ΔacrB* MUTANTS

Ruiz and Levy showed that relative to WT, *ΔacrB E. coli* exhibited increased *acrAB* global activator *marA* and *soxS* expression levels, but only in the presence of functional repressor genes *marR* and *soxR* (61). Expression of *acrR* with *acrB*-deletion, however, was unchanged. The promoter activity of *acrAB* was not affected in *ΔacrBΔacrR* double mutants relative to *ΔacrR* mutants. Therefore, Ruiz and Levy argued that in *ΔacrB* strains a decrease in *acrR* activity, but not expression, may mediate observed increases in *acrAB* promoter activity (61). Similarly, *acrAB* promoter activity was not affected in *ΔacrBΔsoxS* double mutants relative to *ΔsoxS* mutants leading to the similar argument that *ΔacrB* mediates *acrAB* induction with *soxS*. Furthermore, *acrAB* promoter activity ratios of *ΔacrBΔmarA* to *ΔmarA* and *ΔacrBΔsoxR* to *ΔsoxR* were smaller than *marA*(+) and *soxR*(+) counterparts, respectively. This led Ruiz and Levy to suggest that *marA* and *soxR* also mediate *acrAB* induction in *ΔacrB* mutants. With the aforementioned expression data, Ruiz and Levy suggest that these findings are likely due to *ΔacrB* effects on *marR* causing increased *marA* expression, and *ΔacrB* effects on *soxR* causing *soxS* expression. Combined effects of *acrR*, *marA* and *soxS* may also explain *ΔacrB*-mediated *acrAB* induction (61).

DELETIONS OF VARIOUS GENES FROM DIFFERENT METABOLIC PATHWAYS SEEM TO INCREASE *acrAB* PROMOTER ACTIVITY IN *ΔacrB* MUTANTS

It has previously been demonstrated by Helling *et al*. that metabolism-related gene mutations for isocitrate dehydrogenase (*icdA*), purine rich element binding protein B (*purB*), cysteine biosynthesis gene (*cysH*) and methionine biosynthesis gene (*metE*) demonstrate reduced resistance to nalidixic acid and increased *acrAB* expression in *E. coli* isolates (62). Furthermore, Rosner and Martin proposed that metabolite accumulation upon metabolic gene mutations may be inducing MarA, SoxS and Rob upregulation with the eventual outcome of pumping out metabolites to homeostatic levels using TolC-dependent efflux pumps (47).

To further the above arguments, Ruiz and Levy showed that metabolism-related gene deletions for enterobactin biosynthesis, (*entA* and *entE),* tryptophan biosynthesis (*trpE), metE*, gene for the tricarboxylic acid cycle *acnB,* glycerol-3-phosphate dehydrogenase subunits B and C (*glpB* and *glpC), or* gluconeogenesis gene *glpX ,* increased *acrAB* expression (61). Moreover, deletions of *entA*, *entE*, *cysH, purA* (gene in purine biosynthesis)

and *glpX* decreased *acrAB* promoter activity to a lesser extent in *ΔacrB* strains relative to *acrB*(+) strains, compared to no metabolic gene deletions. This implicated these metabolic genes in effecting *ΔacrB*-mediated *acrAB* induction. Ruiz and Levy therefore argue that deletion of *acrB* may be negatively affecting metabolic pathways potentially causing accumulation of metabolites and subsequent *acrAB* induction (61). To corroborate this observation, WT *E. coli* grown with metabolite 2,3-dihydroxybenzoate (DHB), which accumulates in *ΔentE* mutants and binds and represses MarR, thus inducing *marA* expression, exhibited increased *acrAB* promoter activity (61, 63). However, this DHB-mediated *acrAB* expression was mitigated completely with *marA* inactivation and partially with *marR* inactivation. Biologically speaking, accumulated metabolites are known to be effluxed out of the cell by AcrAB-TolC pumps so the relationship of metabolic gene deletion and metabolite accumulation leading to increased AcrAB-TolC levels is thought to have biological relevance (61).

DISCUSSION

The RND Acr efflux pumps are largely responsible for antibiotic resistance in *E. coli* due to their broad antibiotic substrate specificity. As a result of protein structure, AcrAB-TolC and AcrEF-TolC recognize lipophilic and amphiphilic compounds, while AcrAD-TolC transports hydrophilic and amphiphilic molecules. The complex regulation of these transporters has been demonstrated in this review (Fig. 3). On one hand, regulatory proteins that are part of the Acr system, such as AcrS and AcrR have been shown to repress *acrAB* expression while global regulators MarA, SoxS, and Rob activate *acrAB* expression (Fig. 3). Moreover, the global regulator H-NS represses *acrEF* expression while two-component systems BaeSR and CpxAR are known to be transcriptional activators of *acrD* (Fig. 3). Finally, SdiA seemingly increases transcription levels of all Acr efflux pump genes (Fig. 3). In addition to these regulatory elements, factors such as point mutations, transposable element insertions, gene deletions and accumulation of metabolites from metabolic pathways have also been shown to affect the expression of *acr* genes.

Regarding Acr protein-mediated transcriptional regulation, specifically, it was previously assumed that AcrS primarily represses *acrEF* expression as the *acrS* gene is encoded adjacent to the *acrEF* operon (Fig. 1) (15). Likewise, AcrR was presumed to be the primary repressor of *acrAB* since *acrR* is encoded proximal to *acrAB* (Fig. 1). However, Hirakawa *et al.* provided evidence that demonstrates AcrS as the primary repressor of *acrAB* and was shown to be more efficient in repression than AcrR (15). Moreover, Belmans *et al.* showed that an *ΔacrSΔkan E. coli* exhibits a relatively high MIC for kanamycin followed by lower levels for the BW25113 WT *E. coli*, and even lower for *ΔacrAΔkan* (35).

These findings served to corroborate findings by Hirakawa *et al.* in which AcrS was shown to be a negative regulator of *acrA*. Since kanamycin, an aminoglycoside antibiotic, is not considered a substrate for the AcrAB-TolC pump but is a substrate for AcrAD-TolC, we hypothesize that loss of *acrS*-mediated repression of *acrA* upregulated AcrAD-TolC, conferring increased kanamycin resistance. An increase in kanamycin resistance upon *acrS* deletion was also observed by Hay *et al.* (39). Unfortunately, Belmans *et al.* did not perform transcriptional analysis, so effects of *ΔacrS* on *acrD* (and *acrA*) transcript levels could not be elucidated to support AcrAD-TolC mediated kanamycin resistance in *ΔacrSΔkan* mutants.

Our suggestion that *ΔacrS* would present increased expression of AcrAD-TolC, however, seems to contradict data by Emami *et al*. in which *acrS* is proposed to be a positive regulator of AcrD (53). Emami *et al.* found that while a WT strain expressed *acrD* after pre-treatment with sub-inhibitory kanamycin, an *acrS*-deletion mutant did not (53). However, this finding was based on highly qualitative observations based on the presence or absence of the *acrD* band on an RT-PCR electrophoresis gel, which appeared faint for the pre-treated WT strain. Further experiments using quantitative methods should be conducted to validate that *acrD* expression is absent in pre-treated *acrS*-deletion strains. Moreover, the *acrS*-deletion strain had increased kanamycin adaptive resistance. Given that AcrS is a putative activator of *acrD*, which encodes for a key inner membrane protein of the efflux of aminoglycosides, deletion of *acrS* presumably should have decreased kanamycin resistance. Thus, it is unclear how AcrS, a negative regulator of *acrA* but a potential positive regulator of AcrD, impacts AcrAD-TolC mediated kanamycin resistance. Perhaps, deletion of *acrS* led to decreased AcrAD-TolC

mediated resistance but also increased kanamycin resistance through other efflux pumps. For instance, it is likely that the loss of this transcriptional repressor facilitated higher activity levels of AcrAB and AcrEF. However, since kanamycin is not a substrate for AcrAB or AcrEF pumps, increased expression of the associated genes may not explain the increased kanamycin resistance. Having said this, it is possible that AcrS plays a minimal role on *acrD* transcriptional activation relative to positive regulators BaeR and CpxR, such that deletion of *acrS* does not greatly offset AcrD protein levels. Hence, *acrS* deletion can facilitate increased kanamycin resistance due to upregulation of AcrA proteins more so than downregulation of AcrD proteins. Given that there is a 1:2 ratio of AcrD to AcrA in the AcrAD-TolC complex (Fig.2), effects on *acrA* expression by *acrS* deletion may be more pronounced for this reason. Indeed, Alian *et al.* found that deleting *acrA* or *tolC* resulted in loss of kanamycin adaptive resistance (64).

Furthermore, Dick *et al*. showed that *acrA* expression increased in the kanamycin pre-treated WT but was unchanged in the pre-treated *acrR*-deletion mutant (54). However, before pre-treatment, *acrA* levels were already higher in the *acrR*-deletion mutant compared to the WT. This supports previous data which show that *acrR* inactivation causes increased *acrAB* expression, an observation in agreement with AcrR repressor activity on *acrA*. Possibly, kanamycin pre-treatment normally affects *acrA* regulation by lowering the repressive activity of AcrR, accounting for the observed increase in *acrA* expression in pre-treated WT. Thus, pre-treatment has no effect on *acrA* levels in *acrR* deficient *E. coli*. It is unclear, though, how sub-inhibitory exposure to kanamycin could suppress AcrR activity to upregulate *acrA*. Perhaps kanamycin modulates AcrR via ligand-binding, which may explain the potential loss of AcrR-mediated repression of *acrA* as characterized with Pf, R6G and Et. Interestingly, in addition to *acrA*, kanamycin pre-treatment also increases *acrD* expression, as observed by Emami *et al*. Considering that *acrD* expression is primarily regulated by two-component systems that respond to environmental stress signals, perhaps sub-inhibitory exposure to an antibiotic that is a substrate of AcrAD led to upregulated expression *acrD* by means of the two-component systems. Overall, given that kanamycin is a substrate for AcrAD-TolC, this phenomenon suggests substrate-induced regulation of AcrAD-TolC.

Finally, we presented data on compensatory expression of *acrF, acrD* and other drug transporter genes such as *macB, mdtF*, and transposon insertions upstream *acrEF* upon *acrB* deletion. We also reviewed the occurrence of mutations upon sublethal antibiotic treatment, which seems to increase with *acrB*-deletion, and the effects of *parC* and *gyrA* point mutations in causing downstream mutations conferring increased expression of *acrAB*. Taken together, these findings suggest an internal mechanism by which *E. coli* maintains drug homeostasis when a major efflux pump, AcrAB-TolC, is compromised due to sublethal antibiotic stress or with mutagenesis.

Given the complexities of regulation for the Acr systems, the mechanisms that affect efflux pump expression remain to be fully elucidated. Beyond transcriptional mechanisms like AcrS and AcrR, the expression of antibiotic resistance systems can be regulated by a variety of post-transcriptional or post-translational modulators, such as small RNAs (sRNAs) (65). In *E. coli,* SdsR is an sRNA, functioning with the chaperone protein Hfq to repress *tolC* expression (66). Post-translationally, the functional range of AcrAB-TolC mediated antibiotic resistance is speculated to depend on an association between AcrB with the small inner membrane protein AcrZ (67, 68). Moreover, interlinking of the various regulatory mechanisms may also influence Acr-mediated MDR. The CpxAR two-component system, for example, has been shown to activate *mar* transcription, indirectly stimulating *acrAB* expression (69). As such, the regulation of the Acr efflux pumps is complex and goes beyond transcriptional regulation.

Conclusions The tripartite efflux pumps AcrAB-TolC, AcrAD-TolC, and AcrEF-TolC together confer resistance to various antibiotics in *E. coli*. Prior work on elucidating the protein structure of these three pumps, particularly their respective inner membrane transporters, have identified subtle differences in their periplasmic binding sites. These differences determine the broad substrate specificity for both AcrAB-TolC and AcrEF-TolC, and narrower substrate specificity, mainly to aminoglycosides, for AcrAD-TolC. The interplay of multiple factors regulating Acr systems, as reviewed in this article, makes

regulation analysis highly variable if not adequately controlled. Notably, the transcriptional regulators AcrS and AcrR have been shown to have varying effects on the expression of the *acrAB, acrEF* and *acrD* genes. Given the wide antibiotic substrate specificity of Acr efflux pumps, researching and targeting relevant structures play a key role in combating MDR infections in the clinical setting, especially given the lack of mammalian homologs for these Acr structures (7). Understanding the regulatory mechanisms of the Acr efflux pumps could aid in generating novel strategies to repress efflux activity at the transcriptional level, with the ultimate outcome of attenuating the rise of antibiotic-resistant bacteria.

Future Directions Further understanding of the AcrAB-TolC, AcrEF-TolC and AcrAD-TolC efflux pumps can still be elucidated with future prospects focusing on key findings reviewed in this paper. To further investigate whether AcrS decreases *acrAB* levels more than *acrEF*, as proposed by Hirakawa *et al*., future studies may consider using reporter plasmids encoding a luciferase cassette downstream of the promoter sequences of either *acrAB* or *acrEF,* as designed by O'Neill *et al.* (70). A potential experiment can involve the induction of *acrS* overexpression, using the *acrS*-overexpressing plasmid employed by Baeva *et al.*, and compare *acrAB* or *acrEF* repression based on decreases in luciferase-derived light intensity. We do not, however, recommend kanamycin assays with respect to *acrE* mutants as kanamycin is not a substrate for AcrEF-TolC (or AcrAB-TolC).

Given that kanamycin is not a substrate for the *E. coli* AcrAB or AcrEF systems, investigating other classes of antibiotics for these systems may elucidate more clinically relevant findings. The inconsistencies presented in this review on the role of AcrS for *acrA* between Hay *et al*., Baeva *et al.* and Hirakawa *et al.* may simply be due to the inability of AcrAB and AcrEF systems to effectively efflux aminoglycosides. As such we recommend single mutant MIC analysis of *ΔacrA* and *ΔacrE* with an established antibiotic that is a substrate for both the AcrAB-TolC and AcrEF-TolC pumps, such as erythromycin (Table 1). A double mutant *ΔacrAΔacrE* may also aid in elucidating alternative pathways involved in antibiotic resistance upon *acrS*-overexpression.

Furthermore, we propose that increased kanamycin resistance upon *acrS*-deletion may be mediated by a mechanism employing the AcrAD-TolC efflux pump. We also suggest that deletion of *acrS*, encoding a regulator thought to activate *acrD* expression, plays a minimal role on *acrD* transcriptional levels so as to not offset AcrD requirements for the AcrAD-TolC pump. To investigate this further, we recommend future researchers first corroborate the activator activity of AcrS and then look into *acrD* and *acrA* transcript levels in WT and *ΔacrSΔkan E. coli* with and without kanamycin treatment. It is important to note that Emami *et al.* based their *acrD* expression data on the presence or absence of an *acrD* band, therefore more robust quantitative testing may be required to confirm AcrS activity on *acrD*. We also recommend supplementing *acrS*-overexpression MIC of kanamycin assays with an *ΔacrD* mutant. Protein analysis of AcrA and AcrD in an *ΔacrS* background is also encouraged to corroborate findings.

Finally, given that compensatory expression of *acrF, acrD* and other drug transporter genes (*macB, mdtF*) are thought to occur in *ΔacrB* backgrounds, experimental designs based on *acrB* and possibly *acrA* deletions, may require further testing beyond MIC experiments on single mutants to adequately control confounding effects by other drug transporters. As such performing RT-qPCR of *acrA*, *acrE* and an array of other drug transporter genes such as those for MacB, MdtF alongside single mutant MIC analysis is encouraged to corroborate findings and eliminate the occurrences of compensatory overexpression. Similarly, transposon insertions upstream of *acrEF* may interfere with *acrS* based overexpression assays in which *acrB*, or possibly *acrA*, is deleted. As such, sequencing of *acrEF* promoter and gene regions may be useful to eliminate the potential occurrence of transposon inserts interfering with data analysis. Whole genome sequencing of experimental strains before beginning experiments is also encouraged to ensure no additional point mutations in *gyrA* or *parC*, for example, as these mutations have been shown to prime *E. coli* for *marR* mutations and transposon insertions.

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CONTRIBUTIONS

Harjot Bhandol worked on the regulation, discussion, future directions and conclusion sections and created Figure 3. Jairah Alindogan wrote the abstract and compiled the AcrD information, plus contributed to the discussion section and conclusion. Antyrah de Guzman wrote the introduction and acknowledgements, compiled data for Table 1, plus contributed to the discussion section and conclusion. Rebecca Lim worked on the structure section, created Figures 1 and 2, and contributed to the future directions section. The whole team contributed to overall edits and the compilation of references.

REFERENCES

1. **Rossolini GM, Arena F, Pecile P, Pollini S.** 2014. Update on the antibiotic resistance crisis. Curr Opin Pharmacol **18**:56–60.
2. **Nikaido H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J Bacteriol **178**:5853–5859.
3. **Blanco P, Hernando-Amado S, Reales-Calderon J, Corona F, Lira F, Alcalde-Rico M, Bernardini A, Sanchez M, Martinez J.** 2016. Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants. Microorganisms **4**:14.
4. **Sun J, Deng Z, Yan A.** 2014. Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. Biochem Biophys Res Commun **453**:254–267.
5. **Nikaido H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264**:382–388.
6. **Li X-Z, Nikaido H.** 2009. Efflux-Mediated Drug Resistance in Bacteria: An Update. Drugs **69**:1555–1623.
7. **Blair JM, Richmond GE, Piddock LJ.** 2014. Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. Future Microbiol **9**:1165–1177.
8. **Hassan KA, Liu Q, Henderson PJF, Paulsen IT.** 2015. Homologs of the Acinetobacter baumannii AceI Transporter Represent a New Family of Bacterial Multidrug Efflux Systems. mBio **6**:e01982-14.
9. **Anes J, McCusker MP, Fanning S, Martins M.** 2015. The ins and outs of RND efflux pumps in *Escherichia coli.* Front Microbiol **6**:1 - 14
10. **Symmons MF, Bokma E, Koronakis E, Hughes C, Koronakis V.** 2009. The assembled structure of a complete tripartite bacterial multidrug efflux pump. Proc Natl Acad Sci **106**:7173–7178.
11. **Klein JR, Henrich B, Plapp R.** 1991. Molecular analysis and nucleotide sequence of the envCD operon of *Escherichia coli.* Mol Gen Genet MGG **230**:230–240.
12. **Viveiros M, Dupont M, Rodrigues L, Couto I, Davin-Regli A, Martins M, Pagès J-M, Amaral L.** 2007. Antibiotic Stress, Genetic Response and Altered Permeability of *E. coli.* PLoS ONE **2**:e365.
13. **Pourahmad Jaktaji R, Jazayeri N.** 2013. Expression of *acrA* and *acrB* Genes in *Escherichia coli* Mutants with or without *marR* or *acrR* Mutations. Iran J Basic Med Sci **16**:1254–1258.
14. **Bernhardt TG, De Boer PAJ**. 2004. Screening for synthetic lethal mutants in *Escherichia coli* and identification of EnvC (YibP) as a periplasmic septal ring factor with murein hydrolase activity: *E. coli* synthetic lethal screen. Mol Microbiol **52**:1255–1269.
15. **Hirakawa H, Takumi-Kobayashi A, Theisen U, Hirata T, Nishino K, Yamaguchi A**. 2008. AcrS/EnvR Represses Expression of the *acrAB* Multidrug Efflux Genes in *Escherichia coli*. J Bacteriol **190**:6276–6279.
16. **Rosenberg EY, Ma D, Nikaido H.** 2000. AcrD of *Escherichia coli* Is an Aminoglycoside Efflux Pump. J Bacteriol **182**:1754–1756.
17. **Kim, Jin-Sik, Jeong, Hyeongseop, Song, Saemee, Kim, Hye-Yeon, Lee, Kang-seok, Hyun, Jaekyung, Ha, Nam-Chul.** 2015. Structure of the Tripartite Multidrug Efflux Pump AcrAB-TolC Suggests an Alternative Assembly Mode. Mol Cells **38**:180–186.
18. **Murakami S, Nakashima R, Yamashita E, Yamaguchi A.** 2002. Crystal structure of bacterial multidrug efflux transporter AcrB. Nature **419**:587–593.
19. **Hinchliffe P, Symmons MF, Hughes C, Koronakis V.** 2013. Structure and Operation of Bacterial Tripartite Pumps. Annu Rev Microbiol **67**:221–242.

20. **Wang Z, Fan G, Hryc CF, Blaza JN, Serysheva II, Schmid MF, Chiu W, Luisi BF, Du D.** 2017. An allosteric transport mechanism for the AcrAB-TolC multidrug efflux pump. eLife **6**:e24905.
21. **Yu EW.** 2003. Structural Basis of Multiple Drug-Binding Capacity of the AcrB Multidrug Efflux Pump. Science **300**:976–980.
22. **Eicher T, Cha H -j., Seeger MA, Brandstatter L, El-Delik J, Bohnert JA, Kern WV, Verrey F, Grutter MG, Diederichs K, Pos KM.** 2012. Transport of drugs by the multidrug transporter AcrB involves an access and a deep binding pocket that are separated by a switch-loop. Proc Natl Acad Sci **109**:5687–5692.
23. **Yamamoto K, Tamai R, Yamazaki M, Inaba T, Sowa Y, Kawagishi I.** 2016. Substrate-dependent dynamics of the multidrug efflux transporter AcrB of *Escherichia coli.* Sci Rep **6**:21909.
24. **Elkins CA, Nikaido H.** 2002. Substrate Specificity of the RND-Type Multidrug Efflux Pumps AcrB and AcrD of *Escherichia coli* Is Determined Predominately by Two Large Periplasmic Loops. J Bacteriol **184**:6490–6498.
25. **Nikaido H, Takatsuka Y.** 2009. Mechanisms of RND multidrug efflux pumps. Biochim Biophys Acta BBA - Proteins Proteomics **1794**:769–781.
26. **Kobayashi N, Tamura N, van Veen HW, Yamaguchi A, Murakami S.** 2014. β-Lactam selectivity of multidrug transporters AcrB and AcrD resides in the proximal binding pocket. J Biol Chem **289**:10680–10690.
27. **Jellen-Ritter AS, Kern WV.** 2001. Enhanced expression of the multidrug efflux pumps AcrAB and AcrEF associated with insertion element transposition in *Escherichia coli* mutants Selected with a fluoroquinolone. Antimicrob Agents Chemother **45**:1467–1472.
28. **Li M, Gu R, Su C-C, Routh MD, Harris KC, Jewell ES, McDermott G, Yu EW.** 2007. Crystal Structure of the Transcriptional Regulator AcrR from *Escherichia coli.* J Mol Biol 374:591–603.
29. **Pan W, Spratt BG.** 1994. Regulation of the permeability of the gonococcal cell envelope by the mtr system. Mol Microbiol **11**:769–775.
30. **Yang K, Han L, Vining LC.** 1995. Regulation of jadomycin B production in *Streptomyces venezuelae* ISP5230: involvement of a repressor gene, *jadR2*. J Bacteriol **177**:6111–6117.
31. **Nishino K, Yamaguchi A.** 2004. Role of Histone-Like Protein H-NS in Multidrug Resistance of *Escherichia coli.* J Bacteriol **186**:1423–1429.
32. **Mcmurry LM, Oethinger M, Levy SB.** 1998. Overexpression of *marA, soxS* , or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. FEMS Microbiol Lett **166**:305–309.
33. **Su C-C, Rutherford DJ, Yu EW**. 2007. Characterization of the multidrug efflux regulator AcrR from *Escherichia coli*. Biochem Biophys Res Commun **361**:85–90.
34. **Ramos JL, Martínez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K, Zhang X, Gallegos MT, Brennan R, Tobes R**. 2005. The TetR Family of Transcriptional Repressors. Microbiol Mol Biol Rev **69**:326–356.
35. **Belmans G, Liu E, Tsui J, Zhou B.** 2016. AcrS Is a Potential Repressor of *acrA* Expression in *Escherichia coli* and Its Deletion Confers Increased Kanamycin Resistance in *E. coli* BW25113. J Exp Microbiol Immunol **20**:12–17.
36. **Ma D, Alberti M, Lynch C, Nikaido H, Hearst JE.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol Microbiol **19**:101–112.
37. **Lau SY, Zgurskaya HI**. 2005. Cell Division Defects in *Escherichia coli* Deficient in the Multidrug Efflux Transporter AcrEF-TolC. J Bacteriol **187**:7815–7825.
38. **Olliver A, Vallé M, Chaslus-Dancla E, Cloeckaert A**. 2005. Overexpression of the Multidrug Efflux Operon *acrEF* by Insertional Activation with IS1 or IS10 Elements in *Salmonella enterica* Serovar Typhimurium DT204 *acrB* Mutants Selected with Fluoroquinolones. Antimicrob Agents Chemother **49**:289–301.
39. **Hay M, Li YM, Ma Y**. 2017. Deletion of AcrS Results in Increased Expression of *acrE* and Confers an Increase in Kanamycin Resistance in *Escherichia coli* BW25113. J Exp Microbiol Immunol **3**:63–69.
40. **Baeva M-E, Golin AP, Mysuria S, Suresh P**. 2018. Plasmid-Mediated Overexpression of AcrS May Decrease Kanamycin Resistance in *Escherichia coli*. J Exp Microbiol Immunol **4**:1–10.
41. **White DG, Goldman JD, Demple B, Levy SB**. 1997. Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA, soxS,* or *robA* in *Escherichia coli*. J Bacteriol **179**:6122–6126.
42. **Okusu H, Ma D, Nikaido H**. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J Bacteriol **178**:306–308.
43. **Weston N, Sharma P, Ricci V, Piddock LJV**. 2018. Regulation of the AcrAB-TolC efflux pump in *Enterobacteriaceae*. Res Microbiol **169**:425–431.
44. **Chubiz LM, Rao CV**. 2011. Role of the *mar-sox-rob* Regulon in Regulating Outer Membrane Porin Expression. J Bacteriol **193**:2252–2260.

45. **Rosenberg EY, Bertenthal D, Nilles ML, Bertrand KP, Nikaido H**. 2003. Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein: Binding of bile salts to Rob regulates AcrAB expression. Mol Microbiol **48**:1609–1619.
46. **Rosner JL, Dangi B, Gronenborn AM, Martin RG**. 2002. Posttranscriptional Activation of the Transcriptional Activator Rob by Dipyridyl in *Escherichia coli.* J Bacteriol **184**:1407–1416.
47. **Rosner JL, Martin RG. 2009**. An Excretory Function for the *Escherichia coli* Outer Membrane Pore TolC: Upregulation of *marA* and *soxS* Transcription and Rob Activity Due to Metabolites Accumulated in tolC Mutants. J Bacteriol **191**:5283–5292.
48. **Han TH, Lee J-H, Cho MH, Wood TK, Lee J**. 2011. Environmental factors affecting indole production in *Escherichia coli*. Res Microbiol **162**:108–116.
49. **Hirakawa H, Inazumi Y, Masaki T, Hirata T, Yamaguchi** A. 2004. Indole induces the expression of multidrug exporter genes in *Escherichia coli*: Indole signal induces some drug exporters. Mol Microbiol **55**:1113–1126.
50. **Bateni F, Dickey M, Hanafi H, Vickers B**. 2016. BaeR Is Required for Upregulation of Expression of *acrD* in *Escherichia coli* Following Treatment with Subinhibitory Concentrations of Kanamycin. J Exp Microbiol Immunol **20**:56–60.
51. **Besse S, Raff D, Thejomayen M, Paxton T**. 2014. Sub-Inhibitory Concentrations of Kanamycin May Induce Expression of the Aminoglycoside Efflux Pump *acrD* Through the Two-Component Systems CpxAR and BaeSR in *Escherichia coli* K-12. J Exp Microbiol Immunol **18**:1–6.
52. **Sidhu K, Talbot M, Van Mil K, Verstraete M**. 2012. Treatment with Sub-inhibitory Kanamycin Induces Adaptive Resistance to Aminoglycoside Antibiotics via the AcrD Multidrug Efflux Pump in *Escherichia coli* K-12. J Exp Microbiol Immunol **16**:11–16.
53. **Emami M, Xu S, Chan T**. 2014. AcrS is an Activator of *acrD* Expression in *Escherichia coli* K-12 Following Exposure to Sub-inhibitory Concentration of Kanamycin Pretreatment. J Exp Microbiol Immunol **18:**7–11.
54. **Dick J, Jayme T, McGarry J, Meyer S**. 2013. AcrD-Dependent Kanamycin-Induced Antibiotic Resistance of *Escherichia coli* K-12 is not Modulated by the *acrAB* Repressor, AcrR. J Exp Microbiol Immunol **17**:14–18.
55. **Wei Y, Lee J-M, Smulski DR, LaRossa RA**. 2001. Global Impact of *sdiA* Amplification Revealed by Comprehensive Gene Expression Profiling of *Escherichia coli*. J Bacteriol **183**:2265–2272.
56. **Kawamura-Sato K, Shibayama K, Horii T, Iimuma Y, Arakawa Y, Ohta M**. 1999. Role of multiple efflux pumps in *Escherichia coli* in indole expulsion. FEMS Microbiol Lett **179**:345–352.
57. **Lee J, Jayaraman A, Wood TK**. 2007. Indole is an inter-species biofilm signal mediated by SdiA. BMC Microbiol **7**:42.
58. **Kobayashi K, Tsukagoshi N, Aono R**. 2001. Suppression of Hypersensitivity of *Escherichia coli acrB* Mutant to Organic Solvents by Integrational Activation of the *acrEF* Operon with the IS1 or IS2 Element. J Bacteriol **183**:2646–2653.
59. **Alon Cudkowicz N, Schuldiner S**. 2019. Deletion of the major *Escherichia coli* multidrug transporter AcrB reveals transporter plasticity and redundancy in bacterial cells. PLOS ONE **14**:e0218828.
60. **Shuster Y, Steiner-Mordoch S, Alon Cudkowicz N, Schuldiner S**. 2016. A Transporter Interactome Is Essential for the Acquisition of Antimicrobial Resistance to Antibiotics. PLOS ONE **11**:e0152917.
61. **Ruiz C, Levy SB**. 2014. Regulation of *acrAB* expression by cellular metabolites in *Escherichia coli*. J Antimicrob Chemother **69**:390–399.
62. **Helling RB, Janes BK, Kimball H, Tran T, Bundesmann M, Check P, Phelan D, Miller C**. 2002. Toxic Waste Disposal in *Escherichia coli*. J Bacteriol **184**:3699–3703.
63. **Chubiz LM, Rao CV**. 2010. Aromatic Acid Metabolites of *Escherichia coli* K-12 Can Induce the *marRAB* Operon. J Bacteriol **192**:4786–4789.
64. **Alian S, Qazi U, Sou J**. 2013. AcrA and TolC are important Efflux Components in the development of low level Adaptive Aminoglycoside Resistance in *Escherichia coli* K-12 Following Sub-inhibitory Kanamycin Pre-Treatment. J Exp Microbiol Immunol **17**:1–7.
65. **Dersch P, Khan MA, Mühlen S, Görke B**. 2017. Roles of Regulatory RNAs for Antibiotic Resistance in Bacteria and Their Potential Value as Novel Drug Targets. Front Microbiol **8**:803.
66. **Parker A, Gottesman S**. 2016. Small RNA Regulation of TolC, the Outer Membrane Component of Bacterial Multidrug Transporters. J Bacteriol **198**:1101–1113.
67. **Hobbs EC, Yin X, Paul BJ, Astarita JL, Storz G**. 2012. Conserved small protein associates with the multidrug efflux pump AcrB and differentially affects antibiotic resistance. Proc Natl Acad Sci **109**:16696–16701.
68. **Kobylka J, Kuth MS, Müller RT, Geertsma ER, Pos KM**. 2020. AcrB: a mean, keen, drug efflux machine. Ann N Y Acad Sci **1459**:38–68.

69. **Weatherspoon-Griffin N, Yang D, Kong W, Hua Z, Shi Y**. 2014. The CpxR/CpxA Two-component Regulatory System Up-regulates the Multidrug Resistance Cascade to Facilitate *Escherichia coli* Resistance to a Model Antimicrobial Peptide. J Biol Chem **289**:32571–32582.
70. **O'Neill Z, Bulka O, Marziali M, Nah R**. 2018. Construction of *luxCDABE* reporter plasmids to investigate regulation of the *acrAB* and *acrEF* operons by the AcrS repressor in *Escherichia coli* BW25113. J Exp Microbiol Immunol **4**:1–9.
71. **Poole K**. 2005. Efflux-mediated antimicrobial resistance. J Antimicrob Chemother **56**:20–51.
72. **Nikaido H**. 1998. Multiple antibiotic resistance and efflux. Curr Opin Microbiol **1**:516–523.
73. **Kinana AD, Vargiu AV, May T, Nikaido H**. 2016. Aminoacyl β-naphthylamides as substrates and modulators of AcrB multidrug efflux pump. Proc Natl Acad Sci **113**:1405–1410.
74. **Horiyama T, Nishino K**. 2014. AcrB, AcrD, and MdtABC Multidrug Efflux Systems Are Involved in Enterobactin Export in *Escherichia coli*. PLoS ONE **9**:e108642.
75. **Hirata T, Saito A, Nishino K, Tamura N, Yamaguchi A**. 2004. Effects of Efflux Transporter Genes on Susceptibility of *Escherichia coli* to Tigecycline (GAR-936). Antimicrob Agents Chemother **48**:2179–2184.
76. **Yang S, Lopez CR, Zechiedrich EL**. 2006. Quorum sensing and multidrug transporters in *Escherichia coli*. Proc Natl Acad Sci **103**:2386–2391.
77. **Elkins CA, Mullis LB**. 2006. Mammalian Steroid Hormones Are Substrates for the Major RND- and MFS-Type Tripartite Multidrug Efflux Pumps of *Escherichia coli*. J Bacteriol **188**:1191–1195.