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AcrD-Dependent Kanamycin-Induced Antibiotic Resistance of *Escherichia coli* K-12 is not Modulated by the *acrAB* Repressor, AcrR

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Sub-inhibitory levels of kanamycin induce cross-resistance against other classes of antibiotics in *Escherichia coli*. AcrD, an aminoglycoside transporter in *E. coli*, is responsible for providing adaptive resistance to kanamycin, however the regulation of AcrD is unknown. As the system of *acrB* up-regulation must first involve removal of its repressor AcrR, it is possible that AcrR might also have an effect towards AcrD-mediated aminoglycoside resistance. In this study, we explored the possibility that kanamycin-pretreatment of *E. coli* results in induction of *acrD* via removal of AcrR repression. The Δ*acrR E. coli* WOLF-1A was screened alongside a wild-type strain for changes in *acrD* expression and kanamycin minimal inhibitory concentration (MIC) following pretreatment with sub-MIC levels of kanamycin. MICs were determined using the broth-dilution method, and *acrD* expression was monitored by gel electrophoresis and analyzed by integrated spot density values of cDNA transcripts, prepared by RT-PCR of extracted RNA. It was found that kanamycin pretreatment increased the kanamycin MIC for wild-type *E. coli*, but decreased the MIC for *E. coli* WOLF-1A. Electrophoresis band intensities for *acrD* were consistent amongst all treatments, suggesting that *acrR* does not regulate the aminoglycoside transporter *acrD*.

Drug-resistant pathogenic bacteria area prevalent issue in clinical settings; the importance of studying mechanisms of bacterial resistance cannot be understated, and as such, research of antibiotic resistance has been extensive. Generally, in gram-negative bacteria, efflux pumps form complexes of three main components: a cytoplasmic membrane pump linked to an outer membrane channel by a membrane fusion protein (1).AcrD is a component of a cytoplasmic membrane efflux pump in *Escherichia coli* that complexes with the universal AcrA periplasmic membrane fusion protein and the outer membrane channel protein TolC to export aminoglycosides (like kanamycin) and some amphiphilic molecules out of the cell. Similarly, the efflux pump AcrB in *E. coli* works in a complex with AcrA and TolC to export a broader range of antibiotics from the cell, along with some dyes and detergents (2).

Following pre-treatment with sub-inhibitory levels of bactericidal antibiotics (such as kanamycin), antibiotic resistance is induced, including resistance to other classes of antibiotics (1, 3). As this resistance is mediated by the AcrAB efflux pump, and kanamycin resistance is associated with the aminoglycoside transporter AcrD (4), it is possible that the two pathways intersect at some regulatory component. While it is known that *acrAB* is negatively regulated by the AcrR repressor (5), the regulation of *acrD* is unknown – but the fact that AcrR-repression must first be removed from the *acrAB* promoter before upregulation suggests that *acrR* may be involved (either directly or indirectly) in the expression of *acrD*.

In this study, levels of *acrD* expression were determined for *E. coli* WOLF-1A (an Δ*acrR* strain) and for wild-type (WT) *E. coli* BW25113, following pre-treatment with sub-inhibitory kanamycin. Minimal inhibitory concentrations (MICs) of kanamycin and *acrD* transcript expression levels were determined for these respective strains. If AcrR is indeed an important regulatory repressor of *acrD*, then expression levels of *acrD* should be observably higher in the Δ*acrR* strain, which should also correlate with sub-inhibitory kanamycin induced resistance shown in previous studies (1, 4).

MATERIALS AND METHODS

Bacterial strains used. *E. coli* BW25113 (a K-12 derivative) cells were retrieved from the culture collection of the Microbiology and Immunology Department at the University of British Columbia. *E. coli* JW0453-1 (Δ*acrR* strain) was obtained from the Coli Genetic Stock Center (CGSC) (6).

Culture methods. All strains were grown in Luria Bertani (LB) medium and incubated at 30 ºC. Overnight cultures were prepared by inoculating test tubes of LB with a loopful of culture and shaken at 100 rpm at 30 ºC for 18–24 hours. Overnight cultures were diluted in LB and shaken at 30 ºC until an OD_{600} of 1.0 was reached.

Antibiotic stock solutions. Stock solutions of kanamycin (Gibco®) and ampicillin (Sigma-Aldrich®) were prepared by dissolving the antibiotic in distilled water to a final concentration of 100 mg/ml. The solution was sterilized using a 0.22 µm nitrocellulose filter (EMD Millipore®). Dilutions to 10 mg/ml and 1 mg/ml were done using the 100 mg/ml stock and all solutions were stored at –20 ºC.

Preparation of competent *E. coli* JW0453-1 cells for transformation. *E. coli* JW0453-1 were grown to an OD_{600} of 0.6 in of LB broth at 30 ºC for 1-2 hours with mild aeration (~100 rpm). Fifteen hundred µl of this culture was centrifuged for 15 minutes at 5000 rpm at 4ºC. The pellet was washed twice with 1.0 ml of 10% (w/v) glycerol and re-centrifuged. The final cell pellet was resuspended in 50 µl of 10% (w/v) glycerol and kept on ice.

Removal of kanamycin resistance from *E. coli* JW0453-1. Competent *E. coli* JW0453-1 cells were transformed with pCP20 to remove the kanamycin resistance gene. pCP20 contains an ampicillin resistance gene and FLP recombinase. Forty µl of the competent cell suspension was mixed with 1 ul of 458 ng/µl pCP20 and electroporated with the Bio-Rad® MicroPulser™. The cells were recovered in 1 ml of SOC Medium (Invitrogen™) at 30 ºC for 1 hour. The recovered cells were spread-plated onto LB + ampicillin (100 µg/ml) agar plates and incubated at 30 ºC

TABLE 1. NCBI reference sequence, forward and reverse primers sequences used for amplifying *gapA* -1, -2, *acrD* -1. -2, and *acrA* and respective gene and amplified product size.

	Reference sequence		Primer sequence (5' to 3')	Gene size (bp)	Product size (bp)
gapA 1	NC_000913.2	Forward	GTCGCATTGTTTTCCGTGCT	996	701
		Reverse	CAGACGAACGGTCAGGTCAA		
acrD 2	NC_000913.2	Forward	TGCTGGCAATCCTGTTGTGT	3114	320
		Reverse	CTGCGGTAACTTTCGCATGG		
acrA 3	NM_008217.4	Forward	GTCTATCACCCTACGCGCTATCT	1194	1169
		Reverse	CTGCGGTAACTTTCGCATGG		

overnight. Ampicillin-resistant colonies were streaked onto LB agar plates and grown at 42ºC overnight to activate FLP recombinase and inhibit pCP20 plasmid replication. Colonies were grid plated onto LB + ampicillin (100 µg/ml) and LB + kanamycin (100 µg/ml) agar plates. A double-sensitive colony was selected and renamed WOLF-1A for all subsequent experimentation.

Determining minimum inhibitory concentration of kanamycin for *E. coli* BW25113 and WOLF-1A. The MIC was determined by titrating kanamycin in varying concentrations and looking for visible growth. Working solutions of kanamycin in LB broth were made as follows: 0, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 100 µg/ml. *E. coli* strains were mixed to an OD_{595} of 0.005 in working solutions of kanamycin to a total volume of 3 ml in glass test tubes. Following 18 hours of incubation at 37 ºC, the tubes were observed visually for signs of bacterial growth. The minimum concentration of kanamycin in which there was no observable bacterial growth was deemed the MIC.

Kanamycin resistance assay for *E. coli* BW25113 and WOLF-1A. Overnight cultures of *E. coli* BW25113 and WOLF-1A were diluted to 0.2 OD_{595} in LB and grown at 30°C to 0.6 OD_{595}. The culture was then diluted 1:10 into either pretreatment or no pretreatment conditions. The pretreatment cultures were treated with 50% of the determined MIC of kanamycin. All cultures were incubated for 1.0 hour at 30°C. Following incubation, the culture was centrifuged at 9800 x g for 10 minutes and resuspended in LB broth. The cultures were then plated in triplicate into 96-well plates in varying concentrations of kanamycin to a final volume of 300 µl. The plate was incubated at 37°C for 18 hours and observed visually for signs of bacterial growth. The concentration of kanamycin in which there was no observable bacterial growth was deemed the MIC.

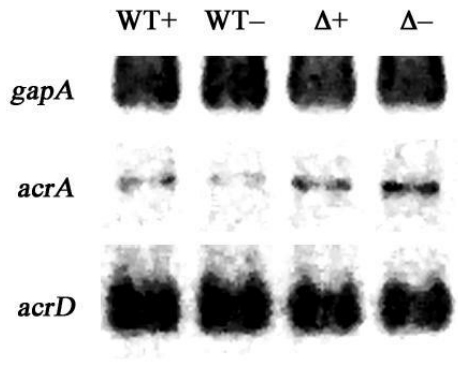

FIG 1 Effect of kanamycin pretreatment on the expression of *gapA*, *acrA* and *acrD*, in *E. coli* BW25113 and *E. coli* WOLF-1A. "WT" refers to *E. coli* BW25113, "Δ" refers to *E. coli* WOLF-1A. "+" refers to kanamycin pretreatment and "–" refers to no kanamycin pretreatment.

Total RNA extraction and Superscript III cDNA conversion. As described, working (1:20) cultures of kanamycin treated and untreated *E. coli* BW25113 and WOLF-1A were prepared. Following 2 hours of incubation at 37°C, 10^8 cells were taken from each respective culture and total RNA was isolated using the PureLink™ RNA Mini Kit (Life Technologies™) according to the manufacturers protocol. Absorbance at 260/280, RNA concentration, and purity was measured using a NanoDrop spectrophotometer (Thermo Scientific™). Five hundred ng of total RNA from respective cells was then reverse transcribed using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen™) according to the manufacturer's protocol. Isolated RNA and cDNA were stored at -80°C.

PCR amplifcation, agarose gel electrophoresis, and semi-quantification via spot density readings. Primer sequences of respective *gapA, acrA, and acrD* genes, PCR temperature conditions, and size of expected amplicons are shown in Table 1. The PCR reaction was carried out with: 1x PCR running buffer without $MgCl_2$ (Qiagen®), 25 mM $MgCl_2$ (Sigma-Aldrich), 10 mM dNTP's, 10 µM forward and reverse primers respectively, Taq (Qiagen®), RNase free water, and cDNA. Initial denaturation was set at 94°C for 5 minutes. All other PCR conditions are given in Table 1. All PCR products were electrophoresed in 2.0% (w/v) agarose gels, run for 1 hr at 100 volts, and stained for 15 minutes in 0.5 µg/ml of ethidium bromide. The PCR amplicons were visualized under ultraviolet light using the MultiImage light cabinet (Alpha Innotech®). Integrated spot density values were taken of each respective band.

Statistical analysis of results. Excluding outliers, the integrated density values for the three replicates of respective WT BW 25113 and KO WOLF-1A treated and untreated cells were averaged. Density values for *acrA* and *acrD* were divided by *gap* density values to standardize. Ratios were plotted in a bar graph. Statistical significance (* $p<0.05$, ** $p<0.01$) was determined by performing unpaired T test for respective samples comparing WT and KO cells (Figure 2).

RESULTS

Pretreatment with sub-inhibitory kanamycin affected kanamycin MICs differently for *E. coli* BW25113 and *E. coli*WOLF-1A. The baseline kanamycin MICs for untreated *E. coli* BW25113 and *E. coli*WOLF-1A were found to vary from 18–20 µg/ml and 20–22 µg/ml, respectively (data not shown); implying that the Δ*acrR*WOLF-1A was slightly more resistant to kanamycin than the WT BW25113. Following kanamycin pretreatment, relative to the untreated conditions the MIC for BW25113 increased 1.2 fold, while that for WOLF-1A decreased 1.1 fold. This suggested that sub-inhibitory kanamycin exposure increased the resistance of BW25113 to kanamycin, but sensitized the WOLF-1A strain slightly.

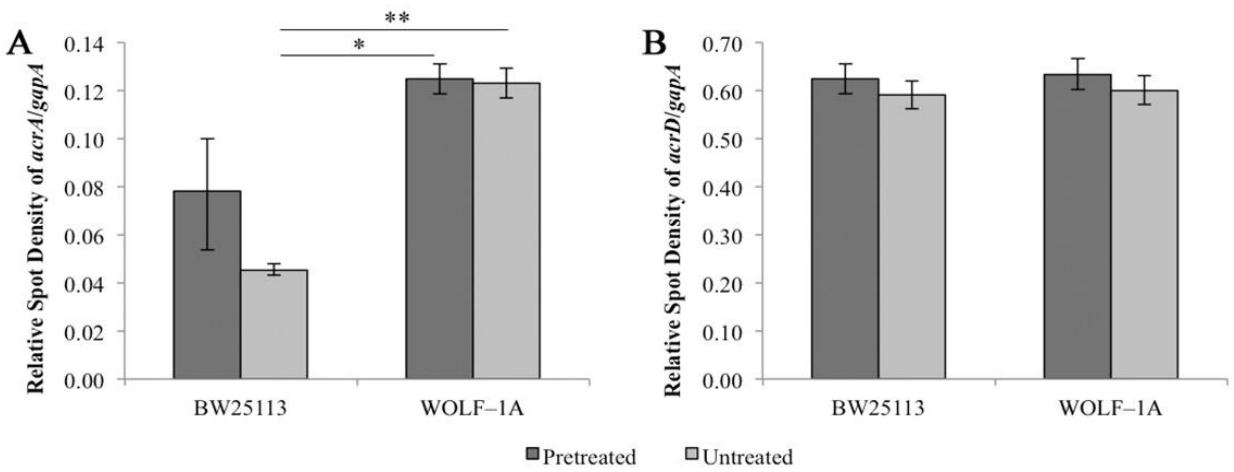

FIG 2 Effect of kanamycin pretreatment on the expression of *acrA* and *acrD*. Integrated spot density results for *E. coli* BW25113 and WOLF-1A (Δ*acrR*) are shown. (A) *acrA* expression standardized to *gapA*. (B) *acrD* expression standardized to *gapA*. All data is shown as the mean with +/- SEM of 3 replicates for WT and *acrR* KO with statistical significance denoted as *P < 0.05, ** P < 0.01.

Kanamycin pretreatment of *E. coli* BW25113 and *E. coli* WOLF-1A affected *acrA* expression. Electrophoresis band intensities of *acrA* fluctuated amongst treatments relative to those of *gapA* (Fig. 2). For *E. coli*WOLF-1A, the *acrA* band intensity was unchanged following kanamycin pretreatment (Fig. 3A). This unchanged band intensity did not correlate with the slight decrease in kanamycin MIC following pretreatment (Fig. 1). For *E. coli* BW25113, the *acrA* band increased in intensity 1.7 fold following kanamycin pretreatment (Fig. 3A). This trend of increased *acrA* expression correlated with the observed increase in kanamycin MIC following pretreatment (Fig. 1). For the untreated conditions, there was a 2.7 fold increase in *acrA* band intensity in the WOLF-1A strain compared to the BW25113 strain (Fig. 3A), indicating pronounced upregulation of *acrA* in the Δ*acrR* strain independent of pretreatment.

Kanamycin pretreatment of *E. coli* BW25113 and *E. coli* WOLF-1A did not influence *acrD* expression. Electrophoresis band intensities of *acrD* did not fluctuate amongst treatments (Fig. 2); rather, they stayed consistent in both strains in each condition (Fig. 3B). This suggests that neither AcrR nor kanamycin pretreatment affects the expression of *acrD* in BW25113.

DISCUSSION

Analysis of *acrD* expression in the *E. coli* BW25113 and Δ*acrR* WOLF-1A strains indicated no clear relationship between the two genes. A goal of this study was to explore the potentiality that AcrR was one of the key regulatory elements of cross-resistance to different classes of antibiotics via *acrB* and *acrD* induction. This relationship was thought to be possible as substrates of the AcrAD aminoglycoside efflux pump are known to induce the upregulation of the AcrAB multi-drug efflux pump (7), which is in turn regulated by the repressor AcrR (6,8). The deletion of *acrR* therefore, was hypothesized to result in increased or constitutive expression of *acrD*, however this was not observed. The intensity of *acrD* on the gel was uniform across all conditions and strains (Figs. 2 and 3B), suggesting that the AcrR repressor protein does not affect the transcription of *acrD*. Integrated density values of cDNA banding patterns shows virtually no change in *acrD* expression regardless of the *acrR* genotype or kanamycin pretreatment (Fig. 3B). This indicated that there is no relationship between AcrR or kanamycin-pretreatment and *acrD* gene expression.

Following kanamycin pretreatment, the *E.coli* BW25113 showed a 1.7 fold increase in *acrA* expression (standardized to *gapA*) compared to the untreated strain (Figs 2 and 3A), consistent with the literature (5,7). Further, paralleling expectations that an *acrR* gene deletion would increase the expression of *acrA*, this was observed in WOLF-1A for both pretreated and untreated conditions (Figs 2 and 3A). Interestingly, while pretreatment resulted in a 1.7 fold increase in *acrA* expression for *E.coli* BW25113, untreated WOLF-1A showed a more pronounced 2.7 fold increase in expression relative to untreated *E.coli* BW25113 (Fig 3A). This showed that while pretreatment induced increased expression of *acrA*, complete removal of the *acrAB* repressor AcrR yielded a more prominent increase, as expected by the known role of AcrR as a repressor for *acrAB* (9). Additionally, it is important to note that the level of *acrA* expression remained unchanged in WOLF-1A following kanamycin pretreatment relative to untreated conditions, suggesting that with complete removal of AcrR repression, maximum expression of *acrA* is achieved and thereby uninfluenced by sub-inhibitory kanamycin pretreatment.

Kanamycin MIC assays demonstrated differing trends in kanamycin resistance between WOLF-1A and *E. coli* BW25113 following pretreatment. *E. coli* BW25113

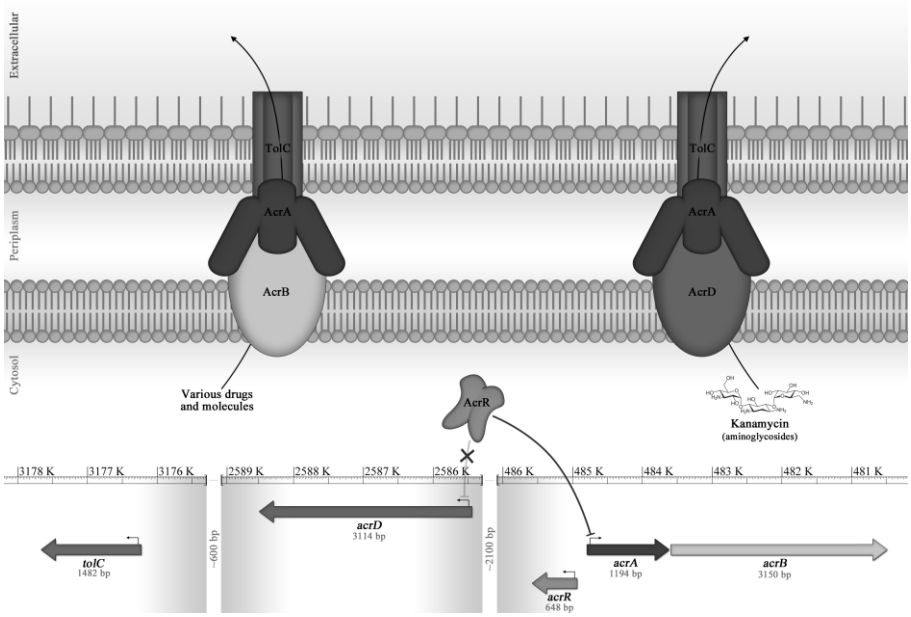

FIG 3 Schematic of the AcrAB:TolC and AcrAD:TolC efflux pumps in the *E. coli* inner and outer membranes, along with the known regulatory aspects of AcrR. AcrA acts as a periplasmic membrane fusion protein (MFP), bridging the efflux pumps AcrB and AcrD with the TolC outer membrane channel. AcrB is a multi-drug efflux pump whereas AcrD is known to be an aminoglycoside transporter. AcrR represses the transcription of *acrA*, while repression of *acrD* is not evident. The red 'X' interrupting the hypothesized inhibition of *acrD* by AcrR implies the results of this study.

MICs were as expected following pretreatment – that is, the kanamycin MIC increased (Fig. 1) as has been shown in the literature (4). There was a slight increase in MIC's for the *E. coli* BW25113 following pretreatment (Fig. 1). The *acrA* expression levels also increased slightly (Fig 2 and Fig. 3A), while *acrD* levels showed no observable change in expression (Fig.2 and Fig. 3B), as described above. Assuming the AcrAB efflux pump is not responsible for the export of kanamycin (9), and considering that net *acrD* expression was unchanged, it is possible that the increase in *acrA* facilitated accelerated association of the AcrAD complex (Fig. 4). AcrA concentration may be the limiting factor in assembly of the aminoglycoside efflux complex AcrAD. Contrary to *E. coli* BW25113, WOLF-1A became slightly more susceptible to kanamycin following pretreatment (Table 2), despite untreated WOLF-1A having a relatively higher initial baseline MIC. However, this decrease in resistance was only 1.1 fold (Table 2), which is relatively small and within the range of statistical error. If *acrD* is unaffected by AcrR, then knocking out *acrR* and subjecting the cells to stressful conditions (in the form of sub-inhibitory kanamycin) could slow the growth of cells, and consequently reduce the culture's kanamycin MIC. It should also be considered that AcrR may have an unknown function that is affected by its removal, and this loss of function may be a confounding factor affecting this result. A further complication is the arguable possibility that the AcrAB complex may be able to extrude kanamycin (7), in which case we would expect the increased *acrA* expression to promote greater occurrences of AcrAB interaction, and thereby increased resistance to kanamycin in WOLF-1A. It is also important to note that some research suggests that AcrD may be able to function in the absence of AcrA (2), however this is disputed (10), and was not confirmed in this study.

No relationship was found between AcrR or kanamycin-pretreatment and induction of *acrD* gene expression. Pretreatment of *E. coli* BW25113 with sub-inhibitory kanamycin increased *acrA* expression and kanamycin resistance, however complete removal of *acrR* (*E. coli* WOLF-1A) yielded the most prominent levels of *acrA* expression – which were independent of kanamycin pretreatment. Despite the increase in kanamycin MIC for *E. coli* BW25113, the trend observed for WOLF-1A was opposite, but minimal. Thus, the repressor of the *acrAB* operon, AcrR, is not involved in AcrD-dependent kanamycin resistance in *E. coli.*

TABLE 2 Minimal inhibitory concentrations of kanamycin for untreated (–) and pretreated (+) *E. coli* BW25113 and WOLF-1A.

	Kanamycin MIC (µg/ml)			
	BW25113		WOLF-1A (Δ*acrR*)	
	–	+	–	+
Assay 1	20	24	22	20
Assay 2	18	22	22	20

FUTURE DIRECTIONS

Similar experiments should be done with an *acrB* knockout strain to provide further evidence of any involvement of the AcrAB complex in kanamycin-induced antibiotic resistance. *acrB* upregulation may be partially responsible for the observed MIC increase in BW25113.Membrane fusion proteins are an integral component of drug efflux pumps (1). An *acrA* knockout strain could be used to investigate any involvement of alternative membrane fusion proteins in kanamycin-induced antibiotic resistance. If other proteins are involved in the AcrD efflux system, then the involvement of *acrA* may be minimal, and kanamycin resistance should still be observed. Furthermore, experimentation on the length of sub-inhibitory kanamycin treatment and the concentration of kanamycin during treatment could optimize the observed antibiotic resistance for future studies involving kanamycin-mediated antibiotic resistance. Finally, as a control to confirm the genotype of the Δ*acrR*WOLF-1A, *acrR* should be amplified by PCR followed by gel electrophoresis.

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