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Treatment with Sub-inhibitory Kanamycin Induces Adaptive Resistance to Aminoglycoside Antibiotics via the AcrD Multidrug Efflux Pump in *Escherichia coli* K-12

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Research has shown that exposing *Escherichia coli* cells to sub-inhibitory concentrations of kanamycin induces adaptive resistance upon subsequent exposure to lethal levels of both structurally-similar and unrelated antibiotics. AcrD is an efflux pump that forms a complex with the outer membrane pore TolC and the periplasmic membrane fusion protein AcrA. Together, this complex works to export a variety of aminoglycosides and amphiphilic compounds from the cell. The involvement of AcrD in the observed adaptive resistance was assessed by comparing the induction of adaptive resistance upon exposure to kanamycin in wild-type and *acrD*-deficient *Escherichia coli* strains. Both strains were pretreated with sub-inhibitory concentrations of kanamycin and subsequently exposed to lethal concentrations of ampicillin, kanamycin, nalidixic acid, streptomycin, and tetracycline. Growth was monitored over 18 hours via optical density readings. Comparing the relative growth of the different treatments revealed that adaptive resistance was only induced in the wild-type strain towards kanamycin and streptomycin. This indicates that AcrD is necessary for the induced adaptive resistance and that it is specific to aminoglycosides. Additionally, molecular techniques were used to assess the level of expression of *acrA*, which was found to be upregulated in both the wild-type and *acrD* deletion strains following pretreatment with kanamycin. RNA was isolated from pretreated and control wild-type and *acrD* deletion cells, converted to cDNA, and analyzed using semi-quantitative PCR to analyze levels of *acrA* expression. These results are consistent with the hypothesis that AcrD is an inducible protein whose up regulation contributes to the previously observed adaptive resistance.

Overuse and misuse of antibiotics has led to an increase in antibiotic-resistant pathogenic bacteria, which is a rapidly growing health concern throughout the world. Nosocomial and community-acquired antibiotic-resistant infections often require treatment with less effective, and perhaps more toxic, antibiotics. This increase in resistance has also led to higher health care costs, as well as a depletion in the number of effective antibiotics that can be used to treat infections (12). Because of this growing problem, it is essential that we gain a better understanding of the causes and mechanisms of resistance (9).

Previous studies have shown that pretreatment of *Escherichia coli* B23 cells with sub-inhibitory concentrations of kanamycin, an aminoglycoside antibiotic, provided protection against subsequent exposure to kanamycin, streptomycin, tetracycline, and ampicillin. While streptomycin is also an aminoglycoside antibiotic, tetracycline and ampicillin are structurally unrelated to kanamycin (3). Interestingly, the resistance to aminoglycoside antibiotics appeared to last longer than resistance to tetracycline and ampicillin (3).

There are multiple mechanisms that can lead to antibiotic resistance, including mutations in the structures targeted by the antibiotic, production of enzymes that specifically degrade antibiotics, and mutations in genes that lead to increased expression of efflux pump systems (12). The cross-resistance observed in the previously described studies appears to be transient (3), which indicates that a gene mutation is not likely to be responsible for the decreased sensitivity to the antibiotics. In this study, we sought to investigate whether upregulation of a specific efflux system plays a role in the transient resistance observed.

AcrD is part of an efflux pump that is known to mediate the export of a variety of aminoglycosides, as well as a few amphiphilic compounds including sodium dodecyl sulfate (SDS), deoxycholate, and novobiocin (7). It forms a complex with the periplasmic membrane fusion protein AcrA, and together with the outer membrane pore TolC, the tripartite complex acts to

export these toxic substances (7). Hocquet *et al.* showed that the genes encoding the MexXY-OprM efflux pump (an AcrD homolog) in *Pseudomonas aeruginosa* exhibited inducible expression following pretreatment with aminoglycosides which led to adaptive resistance to subsequent treatment with inhibitory levels of aminoglycosides (8).

This study investigated the role of AcrD in the observed adaptive resistance by repeating the experiments performed by Chen *et al.* (3) with an *E. coli* K-12 *acrD* deletion strain (STVV11W-1) and the wild-type parent strain (BW25113). Kanamycin-pretreated cells were exposed to five different antibiotics to determine the specificity of adaptive resistance for both aminoglycosides (kanamycin, streptomycin) and antibiotics with mechanistically distinct modes of action. Aminoglycosides target prokaryotic protein synthesis; they bind irreversibly to the 30S subunit of the bacterial ribosome, thereby preventing entry of aminoacyl-tRNA into the acceptor site (A-site) (6). Tetracyline functions to inhibit prokaryotic protein synthesis by a similar mechanism of action but is structurally different than aminoglycosides (4). The unrelated beta-lactam antibiotic ampicillin exerts its antibacterial activity by inhibiting transpeptidation during cell wall synthesis (11). Finally, the bacteriostatic gyrase inhibitor nalidixic acid was also used to test adaptive resistance following kanamycin pretreatment.

We found that deletion of *acrD* impaired the development of induced resistance to aminoglycoside antibiotics following pretreatment with kanamycin. We also investigated the expression levels of efflux pump components and found that pretreatment with kanamycin led to increased expression of *acrA*. Together, these results demonstrate the importance of antibiotic efflux by AcrD in adaptive resistance.

MATERIALS AND METHODS

Bacterial strains. *E. coli* BW25113 cells were obtained from the MICB 421 culture collection of the Microbiology and Immunology Department at the University of British Columbia. *E. coli* JW2454-1 (an *acrD* deletion strain) was obtained from the KEIO collection at the Coli Genetic Stock Centre at Yale University (1).

Culture Methods. Overnight cultures of BW25113 and JW2454-1 strains were prepared by inoculating 5 ml LB with a loopful of bacteria. The cultures were incubated overnight at 30°C in an air shaker. Working cultures of each strain were prepared by diluting the overnight culture 1:20 in LB and growing them to an OD_{600} of 1.0 by incubating them for 2 h at 30°C in the air shaker.

Preparation of competent JW2454-1 cells. JW2454-1 cells were grown to an OD_{600} of 0.6 in 10 ml LB broth at 30°C for 2 h with mild aeration. 1.5 ml of culture was centrifuged for 1 min at 5000 rpm. The pellet was re-suspended in and washed twice in cold H_2O. The pellet was re-suspended in 100 µl cold 10% glycerol and re-centrifuged. After the last centrifugation the pellet was re-suspended in 40 µl 10% glycerol and kept on ice.

Removal of the kanamycin resistance gene in JW2454-1. The kanamycin resistance gene inserted in the JW2454-1 genome was removed by transforming the cells with pCP20, which carries an ampicillin resistance gene and FLP recombinase. 25 µl of competent JW2454-1 cells were mixed with 1 µl of 58 ng/µl pCP20 and electroporated in a BioRad MicroPulser. Transformed cells were recovered in 1 ml Luria-Bertani (LB) Broth (10 g tryptone; 5 g yeast extract; 10 g NaCl; distilled water, 1 L [pH 7.0]) at 30°C for 1 h, plated on LB + ampicillin (100 µg/ml) agar plates and incubated overnight at 30°C. Ampicillin-resistant colonies were streaked on LB agar plates and incubated at 42°C to activate the FLP recombinase and inhibit plasmid replication. After a re-streaking on LB agar, these colonies were grid plated on LB + ampicillin (100 µg/ml) and LB + kanamycin (30 µg/ml) agar plates. A doubly-sensitive colony was selected, renamed STVV11W-1, and used in all subsequent assays.

Antibiotic stock solutions. Stock solutions of kanamycin monosulfate (Sigma #K-4000) and streptomycin sulfate (Sigma #S-6501) were prepared by dissolving each antibiotic in distilled water to a final concentration of 5 mg/ml. Ampicillin sodium (Sigma #A-9518) was dissolved in distilled water to a final concentration of 2.5 mg/ml. Tetracycline hydrochloride (Sigma #T-3383) was dissolved in 70% ethanol to a final concentration of 5 mg/ml. Nalidixic acid (Winthrop #N-614) was dissolved in 0.1 M sodium hydroxide to a final concentration of 5 mg/ml. All solutions were filter sterilized using a Millipore 0.22 µm nitrocellulose membrane (GSWP 02500). The sterile antibiotic solutions were stored at -20°C.

Determination of minimal inhibitory concentrations. The minimum inhibitory concentration (MIC) of ampicillin, kanamycin, nalidixic acid, streptomycin and tetracycline was determined for both BW25113 and STVV11W-1 cells. Working antibiotic solutions were made up in LB as follows: 2, 4, 8, 16, 20, 26, 32 µg/ml for kanamycin and tetracycline; 1, 2, 3, 4.4, 5.8, 7 and 8 µg/ml for ampicillin; and 20, 28, 36, 44, 52, 60 and 68 µg/ml for nalidixic acid and streptomycin. 100 µl of each antibiotic solution was added in duplicate to a Becton-Dickinson Falcon (#30115) 96-well plate. Working cultures of the two strains were diluted to OD_{600} of 0.005, and 100 µl of culture was added to the antibiotics in the 96-well plates. The plates were incubated for 24 h at 30°C. The plates were interpreted visually and the MIC was estimated to be the lowest concentration at which there was no visible growth. Sub-inhibitory concentrations were defined as half the MIC of kanamycin.

Transient antibiotic resistance assay. Working dilutions of the antibiotics were prepared in LB to perform the transient antibiotic resistance assay as follows: 3, 4, 5, 6, 7, 8, 9 µg/ml for ampicillin; 20, 24, 27, 30, 33, 36, 40 µg/ml for kanamycin; 30, 33, 36, 38, 40, 42, 44 µg/ml nalidixic acid; 22, 25, 28, 30, 32, 34, 36 µg/ml for streptomycin; and 2, 4, 6, 8, 10, 12, 14 µg/ml for tetracycline. The transient antibiotic resistance assay was adapted from the method described by Chen *et al.* (3). Working cultures of BW25113 and STVV11W-1 were grown to an OD_{595} of 0.6, and diluted 1:20 in fresh LB. Kanamycin was added to the pretreated cultures to a final concentration of 8 µg/ml for STVV11W-1 and 6.5 µg/ml for BW25113. All four cultures were incubated at 30°C for 1 h. Five Sarstedt (#82.1581.011) flat-bottomed 96-well plates were set up to test the four different cultures at each of the working concentrations of the five antibiotics. 100 µl of each antibiotic solution was added to the plates in triplicate for each culture type. The kanamycin was washed from the pretreated cultures by centrifuging at 8600 rpm for 10 min, re-suspending in 15 ml LB, re-centrifuging, and re-suspending in 17 ml LB. The final OD_{595} of each culture was between 0.18 and 0.25. 100µl of culture was added to the appropriate wells and the plates were incubated at 30°C. Growth was monitored by measuring turbidity at 595 nm in a Bio-Rad (Model 3550) microplate reader. Readings were taken every 30 min for 3 h, and every 60 min

TABLE 1. Minimal inhibitory concentrations (MICs) of two *E. coli* K-12 strains, interpreted qualitatively.

Antibiotic	MIC (µg/ml)	
	BW25113	STVV11W-1 (Δ*acrD*)
Ampicillin	2.9	2.9
Kanamycin	13	16
Nalidixic acid	18	18
Streptomycin	14	14
Tetracycline	4	2

for the following 2 h. After 6 h the plates were incubated at 30°C overnight and the turbidity was read at 18 h.

Analysis of the expression of *acrA*. Working (1:20) control and kanamycin-treated cultures of BW25113 and STVV11W-1 were prepared as described above. All four cultures were incubated at 30°C for 1 h. 10^6 cells was taken from each of the four cultures and RNA was isolated using the PureLink™ RNA Mini Kit (Life Technologies) following the manufacturer's instructions. RNA concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific) and integrity was assessed by running 10 µl of each sample on a 2% agarose gel. 0.5 µg of RNA from each sample was used for first strand cDNA synthesis using the SuperScript® III one-step RT-PCR system (Life Technologies) following the manufacturer's instructions. cDNA concentrations were measured as described for RNA concentrations and 1.6 µg of cDNA from each sample was used in a PCR reaction using primers for *acrA* (forward: 5'-GTCTATCACCCTACGCGCTATCT-3', reverse: 5'-GCGCGCACGAACATACC-3') with *Taq* DNA polymerase (Life Technologies) following the manufacturer's instruction. The reaction was held at 94°C for 2 minutes followed by 35 cycles of 94°C for 45 seconds, 50°C for 30 seconds, and 72°C for 1 minute and 30 seconds. 30 µl of each PCR reaction was run on a 4% agarose gel at 100V for 45 minutes, and stained overnight in 0.5 µg/ml ethidium bromide.

Mathematical analysis of results. The triplicate values for all samples at each time point, excluding outliers, were averaged. The amount of growth at the final time point (18 h) was compared to the amount of growth at 6 h by calculating the ratio of the OD_{595} at 18 h to the OD_{595} at 6 h. Ratios were plotted in a bar graph. Statistical significance ($P \leq 0.05$) was determined by performing a 2-factor ANOVA without replication (treatment x antibiotic concentration) followed by Bonferroni post-hoc testing. Pretreated and control cultures of each strain were compared, and significance was reported as a cumulative (grouped) significance over the designated range of antibiotic concentrations.

RESULTS

AcrD affects MICs to multiple antibiotics. The MICs of ampicillin (2.9 µg/ml), nalidixic acid (18 µg/ml) and streptomycin (14 µg/ml) were found to be the same for both the BW25113 cells and the *acrD* deficient STVV11W-1 cells (Table 1). The MIC of tetracycline was two times higher for the BW25113 cells (4 µg/ml) compared to the STVV11W-1 cells (2 µg/ml) (Table 1). The MIC for kanamycin was marginally (1.2 times) higher for the STVV11W-1 cells (16 µg/ml) compared to the BW25113 cells (13 µg/ml) (Table 1); as a result, the STVV11W-1 cells were pretreated with a higher concentration of kanamycin than the BW25113 cells.

Pretreatment with sub-inhibitory levels of kanamycin resulted in resistance to subsequent treatment with aminoglycosides in BW25113 cells. Wild-type (BW25113) and *acrD* deficient (STVV11W-1) cells were pretreated with kanamycin and then treated with ampicillin, kanamycin, nalidixic acid, streptomycin or tetracycline to compare their adaptive resistance (Fig. 1). Wild-type cells showed significantly greater growth ratios ($P \leq 0.05$) across all tested concentrations of kanamycin and streptomycin following kanamycin pretreatment (Fig. 1A, 1B). Specifically, pretreated BW25113 cells showed between 2.5- and 4.1-fold greater relative growth upon treatment with inhibitory concentrations of kanamycin compared to non-pretreated cells (Fig. 1A). Similarly, this same strain showed between 2.5- and 3.5-fold greater relative growth when treated with streptomycin following pretreatment (Fig. 1B). No significant difference in growth was observed for these cells for the other antibiotics tested (Fig. 1C, 1D, 1E). In the absence of kanamycin pretreatment, *acrD* deficient and wild-type cells were equally susceptible to the various antibiotics (Fig. 1). Pretreated STVV11W-1 cells showed no significant increase in resistance after pretreatment for any of the antibiotics tested, suggesting a role for AcrD in adaptive resistance. The non-pretreated STVV11W-1 cells showed no significant increase in resistance compared to non-pretreated BW25113 cells (Fig. 1).

Pretreatment with sub-inhibitory levels of kanamycin results in increased expression of *acrA* in BW25113 and STVV11W-1. Increased *acrA* expression was observed in both pretreated BW25113 and pretreated STVV11W-1 compared to the non-pretreated cells, indicating an upregulation of *acrA* expression following sub-inhibitory treatment with kanamycin independent of *acrD* (Fig. 2). The wild-type BW25113 cells consistently showed higher levels of *acrA* expression compared to the *acrD* deficient cells. This indicates that *acrA* expression may be affected by the presence of AcrD.

DISCUSSION

The major finding of this study is that kanamycin-induced adaptive resistance to subsequent treatment with kanamycin and streptomycin is mediated by AcrD. Consistent with the observations made by Chen *et al.* (3), this adaptive resistance was seen upon subsequent

exposure to aminoglycoside antibiotics in wild-type

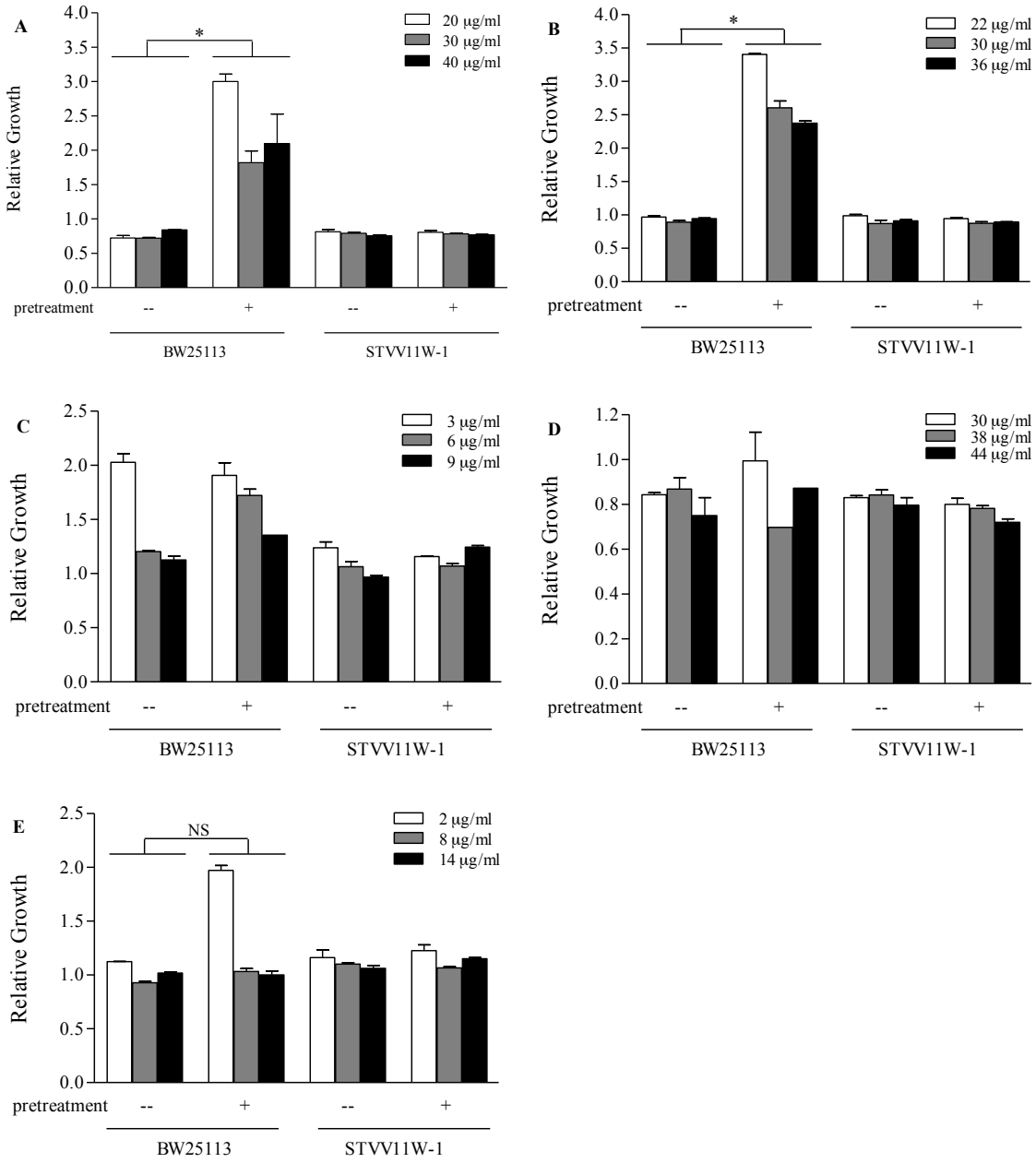

FIG. 1. Effect of AcrD on adaptive resistance induced by kanamycin pretreatment. *E. coli* K-12 wild-type (BW25113) or *acrD* deficient cells (STVV11W-1) were pretreated with kanamycin, and then subsequently treated with (A) kanamycin, (B) streptomycin, (C) ampicillin, (D) nalidixic acid, or (E) tetracycline. Relative growth was calculated as the ratio between final and initial OD_{595}. Statistical significance denoted as *$P \leq 0.05$; NS, $P > 0.05$.

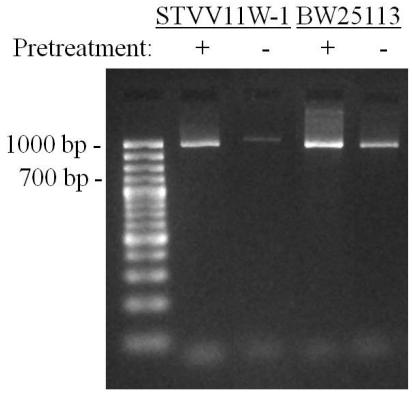

FIG 2. Effect of AcrD on the expression of *acrA* after pretreatment with kanamycin in two strains of *E. coli* K-12.

study, no increase in resistance to the other three antibiotics tested was observed (Fig. 1C, 1D, 1E) (3). Adaptive resistance to any of the five antibiotics tested was not observed in the *acrD* deletion strain (Fig. 1). This lack of adaptive resistance to non-aminoglycoside antibiotics demonstrates the specific nature of resistance induced by pretreatment with kanamycin. Sub-inhibitory levels of this antibiotic presumably work to induce expression of AcrD which subsequently exports a specific subset of compounds, in this case aminoglycosides. This is consistent with findings by Barclay *et al*. who observed a limited effect of aminoglycoside-induced adaptive resistance on the antibacterial activity of other non-aminoglycoside antibiotics (2). They found that five of the six tested antibiotics had no altered bactericidal effects in the event of adaptive resistance (2).

Despite the similarity in the initial MICs of each antibiotic between the mutant and wild-type strains (Table 1), the increase in resistance to aminoglycosides following pretreatment was only observed in the wild-type strain (Fig. 1A, 1B). This indicates that the aminoglycoside efflux pump AcrD is necessary for adaptive resistance. Interestingly, the MIC of kanamycin was 1.2 times higher in the *acrD* deletion strain than in the wild-type strain (Table 1). Given that the AcrD efflux pump system appears to be involved in removing kanamycin from the cells, it was expected that the *acrD* deletion strain would be more susceptible to kanamycin rather than less susceptible. Nevertheless, the *acrD* deletion strain was more susceptible to aminoglycosides in general upon subsequent treatment (Fig. 1A, 1B), supporting the role of AcrD in resistance to this class of antibiotics.

To further investigate the involvement of the AcrD efflux pump in adaptive resistance, semi-quantitative RT-PCR was used to determine if pretreatment with kanamycin leads to increased expression of efflux pump components. AcrA is a periplasmic fusion protein that acts in concert with the cytoplasmic membrane protein AcrD to export aminoglycosides (7). Consistent with our hypothesis, pretreatment with kanamycin led to an increase in expression of *acrA*. Interestingly, this increased expression was observed in both the wild-type and the mutant strains (Fig. 2). However, the deletion of *acrD* in the mutant impaired its ability to develop adaptive resistance, likely due to its inability to form the AcrAD-TolC efflux complex.

AcrA also forms a tripartite complex with AcrB and TolC, which act together to export a range of toxins from *E. coli* (10). Despite the increased expression of *acrA,* there was no increase in resistance in the mutant strain, indicating that the deletion of *acrD* contributes to adaptive resistance directly, as opposed to indirectly via mechanisms such as regulation of other efflux components. Consequently, the adaptive cross-resistance to non-aminoglycoside antibiotics observed previously cannot be attributed to the formation of the AcrAB-TolC complex (3).

Incorrect use of antibiotics may lead to incomplete clearance of bacterial infections and induction of adaptive resistance, which stresses the importance of completing the full course of prescribed antibiotics. As demonstrated in this study, exposing bacteria to sub-lethal concentrations of antibiotics can lead to increased efflux pump expression, providing adaptive antibiotic resistance upon exposure to inhibitory concentrations of similar antibiotics. This effect has also been previously demonstrated *in vivo* during antibiotic treatment of infected patients (5). Increased efflux pump expression can act synergistically with decreased expression of outer membrane porins to decrease susceptibility to antibiotics. Both of these responses are under the control of the global regulator MarA, which suggests pretreatment with kanamycin may also lead to decreased expression of outer membrane porins (5).

In conclusion, our study indicates that the presence of AcrD is required for adaptive resistance to kanamycin and streptomycin following pretreatment with sub-inhibitory levels of kanamycin. This pretreatment also leads to increased expression of *acrA*. Thus, the two proteins may work in conjunction for the effective efflux of certain aminoglycoside antibiotics.

FUTURE DIRECTIONS

While the *acrD* deficient mutant lacked any adaptive resistance, it still showed upregulation of *acrA* following pretreatment with kanamycin. Given the known interaction between AcrA and AcrD and the global control by the regulator MarA (5, 8), *acrD* expression is potentially altered by pretreatment with kanamycin. In this study, we attempted to assess *acrD* expression following pretreatment; however, the primers chosen gave a very small product that was undetectable on the gel. Further studies should be done to examine the expression of *acrD* with improved primer design. Additionally, the expression experiments should be repeated with the incorporation of a house-keeping gene as a control. Like the *acrD* expression assay, the PCR product using the control gene *rpoN* was too small to be observed on an agarose gel. It is also important to confirm that increased levels of gene expression correlate to increased amounts of the gene product. Western blot analysis for AcrA and/or AcrD may be useful to this effect. Expected results for *acrD* expression would be similar to the observed results for *acrA* expression for the wild-type cells.

To verify the involvement of AcrA in adaptive resistance following pretreatment with sub-inhibitory concentrations of kanamycin, this experiment could be repeated using an *acrA* knockout strain. It would be expected that the mutant strain would not show adaptive resistance.

Moreover, to date, investigations into the induction of adaptive resistance have used kanamycin to pretreat the cells. Pretreating with other classes of antibiotics would reveal whether or not this phenomenon is restricted to pretreatment with aminoglycoside antibiotics.

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