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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

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***Escherichia coli* K-12 contains a number of inducible drug efflux complexes such as the well characterized AcrAB-TolC complex. Drug efflux complexes are important when considering the mechanisms of antibiotic resistance development. The cytoplasmic membrane drug transporter AcrD is known to be involved in the development of aminoglycoside resistance. Since AcrD is a homolog of AcrB, it has been suggested that AcrD also forms a tripartite efflux complex with the periplasmic fusion protein AcrA and outer membrane channel protein TolC. Our study aims to determine the importance of AcrD, AcrA and TolC in the development of adaptive resistance against aminoglycosides and to evaluate their functional interdependence. Minimal inhibitory concentration assays of 3 and 24 h were performed on sub-inhibitory kanamycin pre-treated *acrA*, *acrD* and *tolC* deletion strains, to assess the development of resistance to inhibitory concentrations of kanamycin, streptomycin, ampicillin and tetracycline. A 24 h, long term protection assay indicated that *acrA* and *tolC* mutants were more susceptible and impaired in their ability to develop adaptive resistance against aminoglycosides. Protection against aminoglycosides during the 3 h protection assays was not detected, however transient cross resistance to ampicillin was observed. Results suggested that AcrA and TolC are involved in the gradual development of low level resistance to aminoglycosides following sub-inhibitory kanamycin pre-treatment.**

The continual emergence of multidrug resistant bacteria presents a serious challenge to the effectiveness of currently available antibiotics and stresses the importance of discovering novel targets for new antibiotics. Chen *et al.* and Sidhu *et al.* have demonstrated that pretreatment with sub lethal concentrations of an antibiotic can induce transient and long term resistance to further lethal doses of structurally and functionally related and unrelated antibiotics (1, 2). *Escherichia coli*(*E. coli*)contains several inducible tripartite drug efflux complexes such as AcrAB-TolC, AcrAD-TolC, EmrAB-TolC and MdtEF-TolC (3).Exposure of *E. coli* to sub-inhibitory concentrations of kanamycin and fluoroquinolones has resulted in an increase in expression of the efflux system genes *acrA, acrB, tolC, mdfA* and *norE* (1, 4). The structure and function of the AcrAB-TolC multidrug efflux system has been well characterized. Nikaido *et al.*, Ge *et al.,* and Kim *et al.* have shown that AcrA, AcrB and TolC are essential components, and form an efflux system with a wide substrate specificity range which includes antibiotics, detergents, dyes and toxins (5, 6 & 7).

AcrD, a homolog of AcrB, also belongs to the resistance nodulation division (RND) of membrane proteins and is an H^+ substrate antiporter. However, unlike AcrB which has broad substrate specificity and is able to pump lipophilic and amphiphilic drugs, AcrD is only known to pump aminoglycosides, a hydrophilic class of drugs (8).Despite the difference in functional specificity, AcrD may also require the periplasmic fusion protein AcrA and outer membrane aperture protein TolC for its aminoglycoside efflux function *in vivo* (9).Aires & Nikaido's study have shown the requirement of AcrA for the activation of AcrD efflux activity in proteoliposome vesicles *in vitro* (10). Rosenberg *et al.* however, have demonstrated that while AcrD mutation resulted in *E. coli* being more susceptible to aminoglycosides, absence of AcrA and TolC did not (6).

Our study examined the susceptibility of *acrA, acrD* and *tolC* mutants towards aminoglycosides, and the extent of impairment of adaptive resistance development towards aminoglycosides, β-lactams and tetracycline, following treatment with sub-inhibitory concentration of kanamycin. Differences in aminoglycoside susceptibility of mutants were expected to indicate the functional relationships between AcrA, AcrD and TolC. Kanamycin pre-treated and untreated wild-type and *acrA, acrD* and *tolC* deletion strains were subjected to different concentrations of kanamycin, streptomycin, tetracycline and ampicillin, and growth was observed for 3 h and 24 h. Results indicated that *acrA* and *tolC* deletion strains were more susceptible to aminoglycosides and ampicillin, whereas no difference in susceptibility to tetracycline was observed. The *acrA* and *tolC* deletion strains also showed impairment in the development of adaptive resistance to all antibiotics tested. However *acrD* deletion strain showed unexpected results, and was neither impaired in the development of resistance nor more susceptible to the 4 antibiotics compared with the wild type strain.

MATERIALS AND METHODS

Bacterial strains and culture methods. *E. coli* BW25113 (wild-type) and STVV11W-1 *(*Δ*acrD*) cells were obtained from the MICB 421 culture collection of the Microbiology and Immunology Department at the University of British Columbia. *E. coli* JW0452-3 (Δ*acrA*) and JW5503-1 (Δ*tolC)* cells were obtained from the Coli Genetic Stock Centre at Yale University (12). The cultures were grown in Luria Bertani (LB) medium

TABLE 1 Effect of kanamycin pre-treatment on antibiotic resistance of 4 strains of *E. coli* K-12, represented by the observed MIC in pre-treated and untreated cultures for antibiotics kanamycin (KAN), streptomycin (STR), ampicillin (AMP), and tetracycline (TET).

a) Untreated

	MIC (ug/ml)			
	BW25113	STVV11W-1	ASQ12W-1	ASQ12W-2
KAN	16.0	19.0	13.0	10.0
STR	14.0	22.0	14.0	14.0
AMP	3.5	2.9	2.2	1.5
TET	2.0	2.0	1.0	1.0

b) Pre-treated

	MIC (ug/ml)			
	BW25113	STVV11W-1	ASQ12W-1	ASQ12W-2
KAN	19.0	22.0	13.0	13.0
STR	18.0	26.0	18.0	14.0
AMP	3.5	2.9	2.9	2.2
TET	2.0	2.0	1.0	1.0

(1% w/v Tryptone, 1% w/v yeast extract, 0.5% w/v NaCl, adjusted to pH 7.2) overnight with aeration at 30°C and 170 rpm. Working cultures of each strain for the transient antibiotic resistance assay were prepared by diluting the overnight cultures 1:20 in LB and growing them for 3 h on a shaking platform at 37°C and 180 rpm. Working cultures of each strain for the 24h antibiotic resistance assay were prepared by diluting the overnight cultures 1:20 in LB and growing them to an OD_{595} of 0.600 by incubating them for an estimated 2-3h at 30°C and 170 rpm.

Preparation of antibiotic stock and working solutions. Stock solutions of kanamycin monosulfate (Sigma #K-4000), tetracycline hydrochloride (Sigma #T-3383), and streptomycin sulfate (Sigma #S- 6501) were prepared at a final concentration of 5mg/ml. Stock solution of ampicillin sodium (Sigma #A-9518),was prepared at a final concentration of 2.5mg/ml. All solutions were filter-sterilized using a 0.45µm Millipore filter (#WINNEX-13) and stored at 4°C. Working solutions of antibiotics were prepared by stock solution serial dilutions in 10ml sterile LB to achieve the following final concentrations: kanamycin - 2, 4, 8, 16, 20, 26, 32, 38, 44 and 50µg/ml; tetracycline - 2, 4, 8, 16, 20, 26, and 32µg/ml; ampicillin - 1, 2, 3, 4.4, 5.8, 7, 8, 9.5, 11 and 13µg/ml and streptomycin - 20, 28, 36, 44, 52, 60, 68, 76, 84 and 92µg/ml.

Preparation of electrocompetent mutant cells. Δ*acrA* and Δ*tolC* cells were grown to an OD_{595} of 0.6 in LB broth at 37°C with mild aeration in the air shaker. Cells were chilled on ice for 15min. All centrifuge tubes, solutions and glassware to be used for this procedure were kept on ice. Each culture (1.5 ml) was centrifuged at 4°C for 7.5min at 13200 rpm. The cell pellets were resuspended and washed twice with cold dH_2O. The pellets were resuspended in cold sterile 10% glycerol (Life technologies, Cat# 15514-011) and re-centrifuged at 4°C for 7.5min at 13200 rpm. After the last centrifugation the pellets were resuspended in 10% glycerol and kept on ice.

Removal of the kanamycin resistance gene in JW5503-1 and JW0452-3. The procedure for transformation was adopted from the study by Sidhu *et al.* (1). The kanamycin resistance gene inserted in the supplied Δ*acrA* and Δ*tolC* strains was removed by transforming the cells with pCP20 plasmid, carrying an ampicillin resistance gene and temperature-inducible FLP recombines. Electrocompetent Δ*acrA* and Δ*tolC* cells were mixed with pCP20 (479ng/µl) and electroporated at 2.5 kV with a Bio-Rad MicroPulser (Bio-Rad #165-2100). Transformed cells were recovered in SOC media and incubated at 30°C for 1h. After 1 h, cells were plated on LB + ampicillin (50µg/ml) agar plates and incubated overnight at 30°C. Ampicillin-resistant colonies were identified and streaked on LB agar plates and incubated at 42°C for 24h to activate the FLP recombinase and inhibit plasmid replication. To identify ampicillin and kanamycin sensitive colonies, cells were replica plated on LB + ampicillin (50µg/ml), LB + kanamycin (30µg/ml), and LB agar plates. Double sensitive colonies were picked and used to start fresh cultures. Δ*tolC* strain was renamed ASQ12W-1 and Δ*acrA* was renamed ASQ12W-2.

Determination of Minimal inhibitory concentrations (MIC). This method was adopted from the study by Sidhu *et al.* (1). Working cultures of each strain were prepared by the procedure described above for the long term antibiotic resistance assay and diluted to an initial OD_{595} of 0.005 using a Spectronic 20D + spectrophotometer. One hundred µl of each antibiotic concentration was added to a column in a separate 96-well plate (BD Falcon #30115) for a total of four different antibiotic plates. One hundred µl of culture from each strain was added in duplicate to each 96-well plate. The plates were incubated for 24h at 30°C and interpreted visually. MIC was determined to be the lowest concentration of an antibiotic at which no visible growth was observed. Sub-inhibitory kanamycin concentrations were defined as half of the MIC of kanamycin.

Transient antibiotic resistance assay. The transient antibiotic resistance assay was adapted from the method described by Chen *et al.* (2). Working cultures of each strain were divided into control and pre-treatment cultures. Pre-treatment cultures were treated with sub inhibitory kanamycin concentrations. Both sets of cultures were incubated on a shaking platform for 1h at 37°C and 180 rpm. kanamycin was washed from the pre-treated cultures by centrifugation (IEC Centra MP4 Centrifuge) at 8600 rpm for 10min. Cell pellets were resuspended in LB, re-centrifuged, and resuspended in LB. The resuspended cultures were diluted to an OD_{595} of 0.02. Each antibiotic concentration was added to the columns in two 96 well plates (Sarstedt #82.1581.011) plates for each different antibiotic. Bacterial culture from each strain was added in duplicate to each 96-well plate, for an aggregate of 4 plates for the untreated condition and 4 plates for the pretreated condition. All the plates were incubated at 37°C and growth was monitored by measuring turbidity in a Biotek Epoch Microplate Spectrophotometer**.** Readings were taken at 30min intervals for 150min. Results were displayed as a plot of the relative growth (OD_{595} 150 min: OD_{595} 30 min) of each strain under both incubation conditions vs. the antibiotic concentration tested. A ratio of 1 indicated no relative growth between 30min and 150min.

Long term antibiotic resistance assay. The method adopted to perform the transient antibiotic resistance assay was replicated to set up the 24 h long term MIC determination assay. However, following sub inhibitory kanamycin treatment, both control and pretreated cultures of all four strains were diluted to OD_{595} of 0.005. Plates were incubated for 24h at 30°C and growth was interpreted visually.

Bacterial genomic DNA extraction. Overnight LB broth cultures of wild-type and deletion strains were prepared. DNA extraction was performed using the QIAamp DNA Mini Kit, Cat. No.51304. Final DNA was suspended in 200µl of distilled water. DNA concentration was measured using Thermoscientific NanoDrop 2000c Spectrophotometer.

PCR validation of ASQ12W-1, ASQ12W-2, STVV11W-1 & BW25113. PCR primer sets for *acrA, acrD* and *tolC* were designed using the Primer3Plus online software and obtained from Integrated DNA technologies. Primer sequences were as follows: *acrA* (Forward: 5'-agccctaacaggatgtgacg-3'), (Reverse 5'-gcttcgatgtcgctaccttc-3'); *acrD* (Forward: 5'-caggacggctcagaggtaag-3'), (Reverse: 5'-aacgaggtggtttcatacgc-3'); *tolC* (Forward: 5'-ttttaacgtgggcctggtag-3', (Reverse: 5'-cgtttttcggcttctttcag-3'). The PCR cycle consisted of initial denaturation at 95°C for 2 min, followed by 35 cycles of

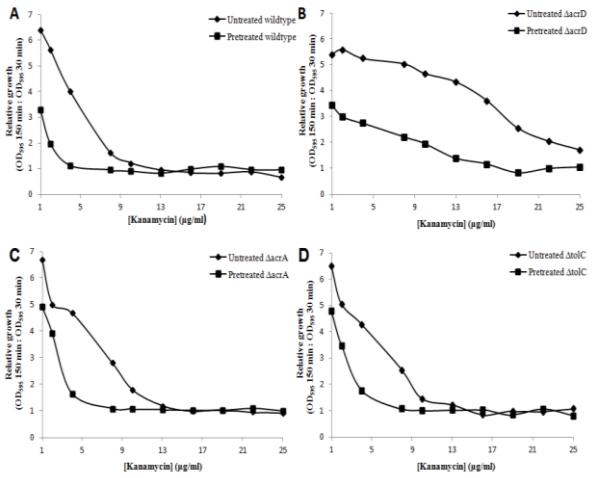

FIG 1 Effect of sub-inhibitory kanamycin pre-treatment on the development of adaptive resistance in the wild type and mutant strains towards increasing concentrations of kanamycin. *E. coli* K-12 (A) Wild type BW25113, (B) Δ*acrD* STVV11W-1, (C) Δ *acrA* ASQ12W-2 and (D) Δ*tolC* ASQ12W-1 were pre-treated or as a control left untreated with sub inhibitory concentrations of kanamycin, and then subsequently treated with varying kanamycin concentrations. Points represent relative growth during the 150 minutes assay duration.

denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min and a final extension step consisting of 72°C for 5 min (Biometra – T Gradient Thermocycler). A 1.5% agarose gel electrophoresis was conducted to visualize PCR products. The gel was run for 45 min at 120 V and stained with ethidium bromide for 20 min before visualization (Alpha Imager).

Restriction digestion confirmation of deletion strains. The presence of *acrA* or *acrD* deletions in mutant strains was further verified by performing single digests of PCR amplified genomic DNAsequences at *PstI* or *HaeIII* restriction sites. A total of four restriction digests were set up. *PstI* digest: 1 µg DNA Δ*acrA* (1.64µl) and wild-type (1.15µl), 1 µl PstI enzyme (Invitrogen C# 15215-015) and 2µl 10X React 2 Buffer (Invitrogen C# 16302-010). *HaeIII* digest: 1 µg DNA Δ*acrD* (1.80µl) and wild-type (1.65µl), 1µl *HaeIII* enzyme (Invitrogen C#15205-016) and 2µl 10X React 2 Buffer (Invitrogen C# 16302-010). Both *PstI* and *HaeIII* reactions were incubated at 37°C for 1h, and then visualized with 1.5% agarose gel electrophoresis as described above.

RESULTS

AcrA and TolC are more sensitive than AcrD to multiple antibiotics. BW25113 and Δ*acrD*STVV11W-1 had higher MICs for aminoglycosides and ampicillin as compared to the Δ*tolC* ASQ12W-1, and Δ*acrA* ASQ12W-2 cells (Table 1a). Since the results of MIC determination for all strains were identical to those of the kanamycin untreated cultures in the long-term antibiotic resistance assay, they are represented by a common table (Table 1a). The kanamycin and ampicillin MICs for the four strains did not follow a common trend, MICs varied between 10-19 µg/ml for kanamycin and 1.5-3.5 µg/ml (Table 1a). The MICs for tetracycline in BW25113 and STVV11W-1(2 µg/ml) was higher by two-fold than the mutants ASQ12W-1 and ASQ12W-2 (1 µg/ml). The MICs for streptomycin in STVV11W-1 was 1.6 times higher than that of wild-type, ASQ12W-1 and ASQ12W-2 cells.

Pre-treatment with sub-inhibitory levels of kanamycin resulted in minimal increased resistance to aminoglycosides for selective mutant strains after long-term incubation. Results of the MIC assay to determine development of adaptive resistance in all four *E. coli* strains to inhibitory concentrations of kanamycin, streptomycin, ampicillin and tetracycline are presented in Table 1b. For kanamycin, BW25113 and STVV11W-1showed a 1.2-fold increase in concentration while the ASQ12W-2strain showed a 1.3-fold increase; ASQ12W-1 cells remained the same at 13 µg/ml. Similarly, streptomycin treated BW25113 and ASQ12W-1 cells increased by a factor of 1.3 while STVV11W-1 increased by 1.2-fold. Ampicillin-treated pre-treated cells of ASQ12W-1 and ASQ12W-2 strains increased by 1.5 and 1.3-times respectively while the two remaining strains did not show a difference in MICs. Tetracycline treated cells did not show any differences in MICs between non-treatment and pre-treatment with kanamycin. On average, MICs for kanamycin and streptomycin pre-treated cells of all strains were 9-fold higher than ampicillin and tetracycline pre-treated cells.

Sub-inhibitory kanamycin pre-treatment did not result in development of adaptive resistance immediately after pre-treatment. All non-treated cells in the presence of kanamycin showed a higher relative growth at a concentration of 1 µg/ml as compared to pre-treated cells. The relative growth for untreated cells compared to pre-treated cells was greater in all strains; a 2-fold increase in wild-typeBW25113, 1.5-time increase in Δ*acrD*STVV11W-1and 1.4-fold increase in Δ*tolC*ASQ12W-1 and Δ*acrA* ASQ12W-2 cells (Fig. 1A, 1C, and 1D). The relative growth curves of pre-treated ASQ12W-1 and ASQ12W-2 cells showed a similar trend in that the point of inhibition, relative growth of 1, occurred at a concentration of 8 µg/ml; a concentration 2 times higher than the inhibition point of BW25113 cells at 4 µg/ml. The point of convergence of inhibition, where pre- and non-treated cells have a relative growth of 1, occurred at a higher concentration of 13µg/ml for Δ*acrA* ASQ12W-2 cells while convergence occurred between 10 and 13 µg/ml for wild-type BW25113and Δ*tolC*ASQ12W-1cells. A 1.5-fold difference in relative growth between non- and pre-treated Δ*acrD* STVV11W-1cells was held constant over lower concentrations, before converging slightly after 16 µg/ml of kanamycin; non-treated cells did not reach inhibition within the range of antibiotic concentrations tested (Fig. 1B). Results for streptomycin treated cells followed similar trends but at lower initial relative growths for the two treatments, and growth inhibition at lower overall concentrations (results not shown). Relative growth trends for ampicillin treated deletion strains were similar in that pre-treated cells resulted in slightly higher relative growth than untreated cells, 1.5-fold for ASQ12W-1 and ASQ12W-2, and 1.3-fold for STV11W-1 (Fig. 2B-D). All deletion strains

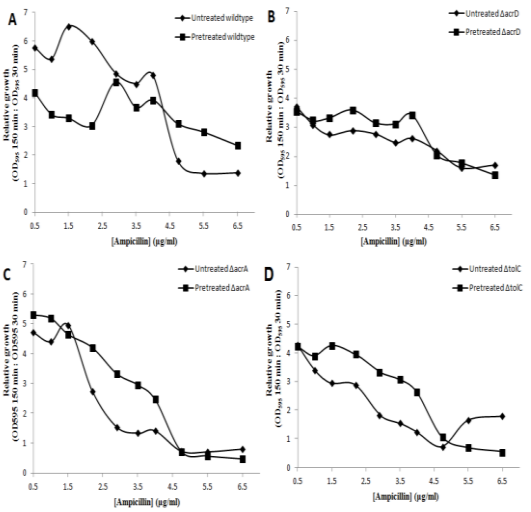

FIG 2 Effect of sub-inhibitory kanamycin pre-treatment on the development of adaptive resistance in the wild type and mutant strains towards increasing concentrations of ampicillin. *E. coli* K-12 (A) Wild type BW25113, (B) Δ*acrD* STVV11W-1, (C) Δ *acrA* ASQ12W-2 and (D) Δ*tolC* ASQ12W-1 were pre-treated or as a control left untreated with sub inhibitory concentrations of kanamycin, and then subsequently treated with varying ampicillin concentrations. Points represent relative growth during the 150 minutes assay duration.

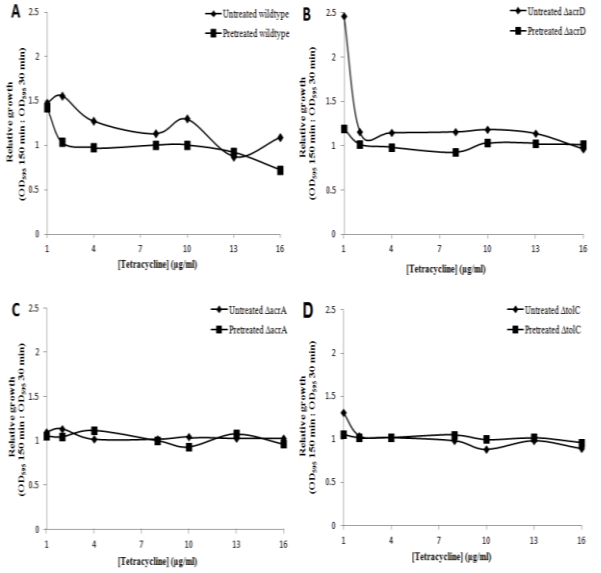

FIG 3 Effect of sub-inhibitory kanamycin pre-treatment on the development of adaptive resistance in the wild type and mutant strains towards increasing concentrations of tetracycline. *E. coli* K-12 (A) Wild type BW25113, (B) Δ*acrD* STVV11W-1, (C) Δ *acrA* ASQ12W-2 and (D) Δ*tolC* ASQ12W-1 were pre-treated or as a control left untreated with sub inhibitory concentrations of kanamycin, and then subsequently treated with varying tetracycline concentrations. Points represent relative growth during the 150 minutes assay duration.

whether pre-treated or untreated displayed a similar relative growth at 0.5 µg/ml before the non-treated relative growth curves diverged from pre-treated curves. Both curves of STVV11W-1 and ASQ12W-2 converged at 4.8 µg/ml and followed the same trend thereafter. In contrast, untreated ASQ12W-1 cells showed increased relative growth after the point of convergence (Fig. 2D). While the relative growth curve of pre-treated wild-type cells of ASQ12W-1, ASQ12W-2 and BW25113 followed a similar trend to that of STVV11W-1, the non-treated curve displays a relative growth of 1.4-times higher at 0.5 µg/ml before falling suddenly to 1 at 4.8 µg/ml (Fig. 2A). Tetracycline-treated cells displayed inhibition of growth, relative growth of 1, for both non- and pre-treated cells regardless of the concentration (Fig. 3A-D). The major difference occurred with untreated STVV11W-1 cells where relative growth was 2-times higher than pre-treated cells before falling to 1 at 2 µg/ml (Fig.3B).

Molecular verification of deletion mutants through PCR. Primers specific for internal sequences within *acrA*, *acrD* and *tolC* were designed to confirm gene deletion in the strains. BW25113 wild-type strain displayed products for acrA primers, acrD primers and tolC primers at 233 bp, 207 bp and 228 bp product sizes respectively. The absence of product in lane 5 for ASQ12W-1, confirmed the deletion of *tolC* but bands observed for Δ*acrA* and Δ*acrD* mutants suggested presence of the genes (Fig. 4). Restriction endonuclease digests of *acrA* and *acrD* PCR reactions, did not display any band for both the undigested and digested *acrA* amplification reactions, however a band indicating fragment at 100 bp in lane 9 for STVV11W-1 was observed (Fig. 5). The restriction digest demonstrated that while ASQ12W-2 was in fact a deletion strain due to absence of product in lane 7, STVV11W-1 may still contain an intact *acrD* as a band was observed in lane 9.

DISCUSSION

Since AcrD has been shown to be involved in aminoglycoside efflux, *acrD* mutant or deletion strains were expected to be more susceptible to kanamycin and streptomycin, than the wild type strains and should have been inhibited at a lower MIC. However, *acrD* deletion strain required a higher MIC than the wild type (Table 1a). The MIC results are consistent with the results obtained by Sidhu *et al.*, but contrary to the findings of Rosenberg *et al.*, and raises suspicion about the *acrD* mutational status of STVV11W-1 (1, 13). Δ*acrA* and Δ*tolC* deletion strains were also observed to be more sensitive to kanamycin, but only marginally more to ampicillin, as compared to the isogenic wild type. The observation suggested that absence of AcrA and TolC impaired the tolerance of *E.coli* K -12 to kanamycin. Since all four strains show similar susceptibility to tetracycline, AcrD, AcrA and TolC can be considered equally unimportant in contributing to tetracycline tolerance.

Kanamycin pre-treatment generates a protective effect to subsequent higher concentration of aminoglycosides in wild-type and Δ*acrD* during a 24 h incubation period. However since the MIC upshift is small (15% and 13%), pre-treatment did not result in a

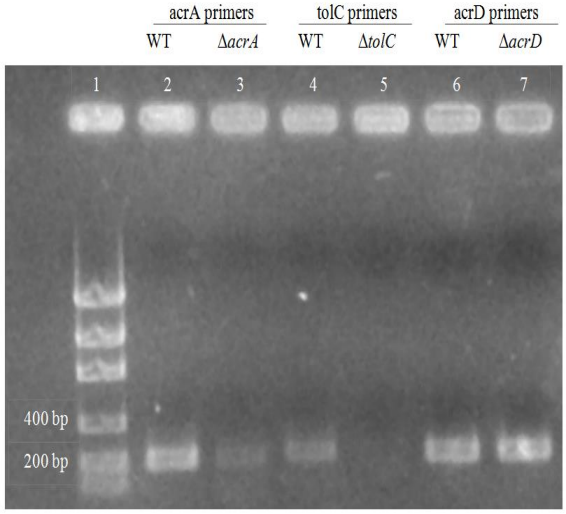

FIG 4 PCR assessment of *acrA*, *acrD* and *tolC* genes in wild-type and deletion strains. Lane 1: Invitrogen Low DNA Mass Ladder. Product amplification of gene products detected in BW25113 (wild-type) strain as well as Δ*acrA* (lane 3) and Δ*acrD* (lane 7).

high level of resistance. A similar small up-regulation in resistance of Δ*acrA* (22%) to kanamycin and Δ*tolC* (33%) to streptomycin indicated that the missing components were possibly not necessary for tolerating the corresponding antibiotics. Kanamycin pre-treatment did not induce cross resistance to ampicillin and tetracycline in any strain (Table 1b). The absence of cross resistance was also observed by Chen *et al.* and emphasized the involvement of different mechanisms and components for developing resistance to non aminoglycoside antibiotics (2).

Relative growth measurements 3 h following kanamycin pre-treatment were done to examine the kinetics of induced resistance development to aminoglycosides, and the induction of transient protection against structurally and functionally unrelated antibiotics i.e. tetracycline and ampicillin (Fig. 1, 2 & 3). Results indicated a common trend of growth suppression under the kanamycin pre-treatment condition as compared to the untreated controls. The growth suppression effect was particularly profound for pre-treated wild-type, Δ*acrA* and Δ*tolC* being exposed to inhibitory aminoglycoside concentrations (Fig. 1A, B, C, streptomycin graph not shown), and can be related to the mechanism of aminoglycoside action. Aminoglycosides inhibit protein synthesis by binding irreversibly to the 30S ribosomal subunit and 16S rRNA, and preventing the specific binding of aminoacyl-tRNA to the mRNA-ribosome complex resulting in non-functional proteins (14). Consequently, kanamycin pre-treatment will lead to an abundance of free but functionally impaired ribosomes in the cell. Insufficient protein synthesis results in delayed growth, but does not kill the bacteria because the concentrations used are sub-inhibitory. Bacteria are known to exhibit kanamycin resistance by O-Adenyltransferase mediated inactivation of kanamycin molecules, down regulation of outer membrane porin proteins e.g. OmpF, and active extrusion through drug efflux pumps (15). Therefore the observed growth suppression with increasing treatment concentrations was a consequence of kanamycin pretreatment (Fig 1A, B & C).

The 3 h duration of the short term protection assays did not allow the bacteria adequate time to overcome the effects of kanamycin and resume normal physiological growth. Since up-regulation of resistance generating elements is expected to occur following kanamycin pretreatment, no adaptive resistance was observed. As indicated by the results of the 24 h antibiotic resistance assay (Table 1b), wild-type and Δ*acrD* cells were able to grow at higher kanamycin concentrations after pre-treatment with sub-inhibitory concentrations. If the duration of the short term assay was extended beyond 150 minutes, a similar pattern of improved survival and growth at inhibitory aminoglycoside concentrations would be expected for wild-type cells, and the relative growth patterns of wild-type, Δ*acrA* and Δ*tolC* deletion strains would provide information about the requirement of AcrA, AcrD and TolC for the development of adaptive resistance. A second possible explanation for the improved relative growth of control cultures in comparison to the pretreated cultures is the absence of a mock LB washing step for the former, which the latter were subjected to in order to remove intracellular kanamycin molecules. This may have resulted in stress of pre-treated cells due to osmotic shock and ion imbalance, while cells in the control culture were not subject to any comparable stress and possessed a normal homeostatic environment. Therefore a mock washing step for the untreated cultures may have been an important experimental perimeter, and should be introduced as a control.

In addition to the growth suppression effect, the observed transient cross resistance to inhibitory concentrations of ampicillin was also due to the requirement for ampicillin efficacy (Fig. 2 A-D). Ampicillin is a β-lactam antibiotic, and following entry into the periplasm through outer membrane porins in the cell wall, inhibits the transpeptidation step between amino acid side chains in adjacent peptidoglycan polymers (16). Since transpeptidation imparts rigidity to the cell wall and prevents osmolysis, cells without these linkages will burst due to loss of peptidoglycan structural integrity. Therefore, the effects of ampicillin are observable only when cells are growing. Relative growth of bacteria upon exposure to ampicillin was higher for the pretreated cells, because cells grew slower as compared to cells in the untreated cultures, and thus they were less affected by the action of ampicillin. Δ*acrA* and Δ*tolC* grew better at lower

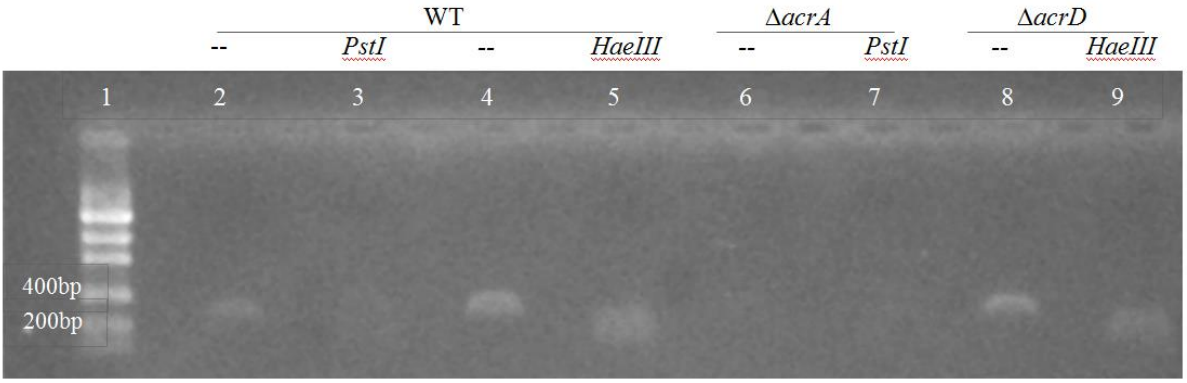

FIG 5 Restriction endonuclease digest confirmation of *acrA* and *acrD*-deficient strains. Lane 1: Invitrogen Low DNA Mass Ladder. Lanes 2 to 5 depict positive control with BW25113 PCR products of *acrA* (lanes 2 and3) and *acrD* (lanes 4 and 5). *PstI* digest of *acrA* PCR product confirms absence of initial PCR product while *HaeIII* digest of *acrD* PCR product shows the presence of initial PCR product.

ampicillin concentrations, because the absence of TolC and AcrA resulted in comparatively lesser porins in the outer membrane for ampicillin molecules to enter into the periplasm (17). At greater ampicillin concentrations, decreased porin availability was insufficient to prevent bacterial lysis. Tsukagoshi *et al.* have reported that the AcrAB-TolC pump is also involved in the expulsion of β-lactams (18). The decreased relative growth of Δ*acrA* and Δ*tolC* strains compared to the wild-type and Δ*acrD*, can therefore, also be attributed to the deficiency of AcrA and TolC.

The lack of adaptive resistance to tetracycline is also related to the kinetics and mechanism of tetracycline action (Fig 3 A-D). Like ampicillin, tetracycline also penetrates the outer membrane through pores such as OmpC and OmpF (19). However, its greater lipophilicity compared to aminoglycosides facilitates faster entry into the cell where it inhibits protein synthesis by preventing aminoacyl-tRNA binding to ribosomes in a reversible fashion (20). Rapid protein synthesis inhibition by tetracycline compared to aminoglycosides is also due to its capability of binding to more sites on the ribosome to exert its effect (20). Additionally, compared to the action of ampicillin, cells are not required to reach a particular size before the action of tetracycline becomes effective. The marginally improved relative growth patterns for Δ*acrD* and wild-type indicated the possible involvement of AcrA and TolC in conferring low level resistance at low tetracycline concentrations (Fig 3 A, B). A similar minor improvement in resistance was also seen for Δ*acrD* and wild-type to ampicillin over that displayed by Δ*acrD* and wild-type during the 24 h protection assays (Table. 1b).

The lower susceptibility of Δ*acrD* to aminoglycosides in both the 3 h and 24 h protection assays compared with Δ*acrA* and Δ*tolC* was unexpected, because AcrD has been determined to be the aminoglycoside specific transporter, whereas AcrA and TolC are accessory components required for periplasmic extrusion (13, 21). Consequently, the absence of AcrD should have rendered the bacteria more susceptible to aminoglycosides, instead of the observed wild type phenotype. PCR amplification and subsequent restriction digest with *HaeIII* provided presumptive evidence that Δ*acrD* possessed *acrD* sequences in its genome. Since the primers were designed in such a way that amplicons would only be generated if the central *acrD* sequences were present, the bands on the gel indicated that either the generation of *acrD* mutant by Baba *et al.* was unsuccessful (12), or that the Δ*acrD* strain possessed *acrD* duplications in its genome. As a consequence of these observations we argue that the lack of development of adaptive resistance in STVV11W-1 observed by Sidhu *et al.* in their study may not be due to the absence of *acrD* (1). Therefore, explanation of the observed results for Δ*acrD* requires further investigation of its genome and proteome.

Pre-treatment of *E.coli* K-12 with 50% of inhibitory concentration of kanamycin, resulted in gradual development of low level resistance to subsequent treatment of inhibitory concentrations of aminoglycosides. Pretreatment also generated a transient moderate level of cross resistance to inhibitory concentrations of ampicillin. However, kanamycin does not induce any cross resistance to tetracycline. Our study also indicated that Δ*acrA* and Δ*tolC* strains were generally more sensitive to aminoglycosides and hydrophilic β lactams, and pretreatment of the mutants with sub inhibitory kanamycin concentrations resulted in occasional development of very low level resistance to inhibitory aminoglycoside concentrations over 24 h. Immediately following kanamycin pretreatment, adaptive resistance in Δ*acrA* and Δ*tolC* developed only to β lactams, however, it is transient and at lower levels than the isogenic wild type strain. Therefore, AcrA and TolC are involved in the natural resistance and development of induced resistance in *E.coli* K-12 to the action of aminoglycosides.

FUTURE DIRECTIONS

The results of the MIC assays can provide support to the existence of a functional dependence between AcrD, AcrA and TolC .Therefore, it is vital to first determine whether STVV11W-1 is in fact an *acrD* mutant, or whether the detection of the *acrD* sequence was a result of non-specific amplification by our primer of a different site in the genomic DNA. It can be useful to perform PCR using several different primer designs which can amplify highly specific sequences at different sites along the *acrD* gene. If certain primers do detect the presence of *acrD* sequences in STVV11W-1, it should also be investigated whether these sequences are being transcribed by using mRNA detection methods such as RT-PCR. Technical errors such as, the omission of a mock-washing step for the control cultures should be avoided.

The role of AcrD in aminoglycoside efflux can alternatively be tested by creating an *acrD* deletion using a homologous recombination based system. For this purpose the lambda red recombinase system can be conveniently used. It has also not yet been discovered whether AcrD physically interacts and forms a complex with AcrA and TolC in the presence or absence of a substrate. The existence of a physical link could be determined by creating and transforming wild type or mutant *E. coli* K-12 cells with plasmids expressing fluorescently tagged AcrD, AcrA and TolC proteins, and studying their distribution in the bacterial cell envelope before and after antibiotic treatment. Physical linkage can also be tested by creating His tagged proteins; using plasmid based cloning methodologies, and performing immunoprecipitation with anti-His antibodies following aminoglycoside treatment and membrane protein extraction. The isolation of a multi-subunit protein complex would verify the presence of an AcrAD-TolC complex.

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