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Short-term Adaptive Resistance in *E. coli* K-12 is not dependent on *acrD, acrA* and *tolC*

Kevin Lee, Qiaochu Lin, Andrew Low, and Lisa Luo
Department Microbiology & Immunology, University of British Columbia

Previous studies have shown that pretreatment of *Escherichia coli* with a sub-inhibitory level of kanamycin can induce both short-term and long-term adaptive resistance based on the class of antibiotics, where short term adaptive resistance was observed for both non-aminoglycosides and aminoglycosides while long term adaptive resistance was specific for aminoglycosides. While *acrD* has been shown to be necessary for adaptive resistance to aminoglycosides in long term adaptive resistance, its involvement in short term adaptive resistance was not assessed. The current study examined the necessity for *acrD, acrA* and *tolC*–genes that encode for the three components of the AcrAD-TolC efflux pump that exports aminoglycosides out of the cell –in the physiological phenomenon of adaptive resistance. Using *E. coli* K-12 wild type, Δ*acrD*, Δ*acrA* and Δ*tolC* strains, either pretreated or non-pretreated with a sub-inhibitory level of kanamycin, growth was monitored over 2 hours using turbidity readings to assess the short-term protection to ampicillin, tetracycline, kanamycin and streptomycin, while changes in the minimum inhibitory concentrations of kanamycin and streptomycin were observed to assess long-term protection. When pretreated with kanamycin, all four *E. coli* K-12 strains had increased transient resistance to subsequent lethal exposures of ampicillin and tetracycline but showed increased transient susceptibility to subsequent lethal exposures of the aminoglycosides. In addition, the kanamycin pretreatment did not induce long-term adaptive resistance to aminoglycosides in any of the four *E. coli* K-12 strains. Our data suggest that *acrD, acrA* and *tolC* are not necessary for short-term adaptive resistance. The necessity of *acrA* and *tolC* for long-term adaptive resistance could not be conclusively determined because there was no long-term adaptive resistance observed in the *E. coli* parental strain. As such, it is inconclusive as to whether *acr*D, *acr*A, and *tol*C are involved in the long-term physiological phenomenon of adaptive resistance.

Adaptive resistance is defined as a reduction in the susceptibility of the bacteria to an antibiotic as a result of exposure to a sub-inhibitory concentration of the antibiotic (1). Previous studies have shown that pretreatment of *E. coli* B23 cells with a sub-inhibitory level of kanamycin induced short-term resistance (less than 2 hours) to a variety of antibiotics groups that are structurally different: ampicillin, tetracyline, and aminoglycosides (2). However, the same results were not observed for long-term resistance (24 hours), where the cells were only protected against aminoglycosides (2). It has also been shown that upon pretreatment with kanamycin, *acrD* is necessary for inducing long-term adaptive resistance against aminoglycosides in *E. coli* K-12 (3). However, its role in short-term adaptive resistance had not been studied. Furthermore, the necessity of the *acrA* and *tolC* genes that encode for the other components of the efflux pump, of which AcrD is a part, had not been studied in adaptive resistance.

Efflux pumps are protein complexes that prevent antibiotics from reaching their target sites by removing the antibiotic from the periplasm or cytoplasm. One such efflux pump is the three component system in *E. coli* made up of AcrA, AcrD and TolC, also known as the AcrAD-TolC multidrug efflux transport system (4).AcrA is a periplasmic membrane fusion protein, and TolC is an outer membrane pore (4). AcrD is a transmembrane transporter protein belonging to the resistance nodulation division family of proteins (5). Together, these three proteins work to transport a variety of molecules from both the cytoplasm and periplasm to the extracellular space (6), with the substrate specificity of this efflux pump determined bard (4). In particular, AcrD is known to be responsible for conferring resistance to aminoglycosides (a very hydrophilic class of molecules) as well as some amphiphilic compounds (4).

The mode of action for aminoglycosides is not fully understood. However, they have been shown to have several potential antibiotic mechanisms, including inhibition of protein synthesis. They disrupt the proofreading process and causes increased rate of error in synthesis with premature termination and they inhibit ribosomal translocation where the peptidyl-tRNA moves from the A-site to the P-site (7). Tetracycline antibiotics, like aminoglycosides, inhibit protein synthesis by interfering with the binding of aminoacyl-tRNA to the mRNA-ribosome complex. They act by binding to the 30S ribosomal subunit at the A-site and blocking the sequential attachment of the tRNA so protein biosynthesis cannot occur (8). However, they are structurally different from aminoglycosides (3). Ampicillin belongs to the beta-lactam group of antibiotics: it works as a competitive inhibitor of the enzyme transpeptidase, an essential enzyme for bacterial cell wall synthesis, thereby inhibiting peptidoglycan production, which ultimately leads to cell lysis (9).

In order to further our understanding of adaptive resistance, in this study, we set out to assess if the genes for the AcrAD-TolC efflux pump are involved in the physiological phenomenon of adaptive resistance. Since *acrD* has been shown to be involved in long-term adaptive resistance (3), and AcrD has been shown to work in

complex with both AcrA and TolC (4), we hypothesized that *acrD*, *acrA* and *tolC* are necessary for both short-term and long-term adaptive resistance to aminoglycosides. Given that Sidhu *et al.* (3) showed AcrD was only involved in the long-term adaptive resistance specific to aminoglycosides, it was unclear why short-term adaptive resistance to other classes of antibiotics was observed. In our study, we found that *acrD*, *acrA* and *tolC* are not involved in short-term adaptive resistance and their role in long-term adaptive resistance is inconclusive. Therefore, further work is needed to elucidate the other mechanisms, independent of this particular efflux pump, behind the previously observed short-term adaptive resistance.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* BW25113 (wild type, parental strain) and *E. coli* STVV11W-1 (Δ*acrD* strain) were obtained from the MICB 421 culture collection of the Microbiology and Immunology Department at the University of British Columbia (3). *E. coli* JW0452-3 (Δ*acrA* strain) and JW5503-1 (Δ*tolC* strain) were obtained from the Keio collection (10) available at the Coli Genetic Stock Centre at Yale University.

Culture Methods. All 4 strains were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, all dissolved in distilled water); spread plate cultures were plated on LB agar plates (LB broth with 1.5% agar) with or without antibiotics. Overnight cultures were prepared by inoculating 5 ml of LB with a loop full of bacteria and incubated overnight at 30°C in an air shaker (New Brunswick Scientific Excella E24 Incubator Shaker Series).

Preparation of antibiotic stock solutions. Stock solutions of kanamycin monosulfate, streptomycin sulfate and ampicillin sodium were prepared by dissolving each antibiotic in dH_2O to a final concentration of 1 mg/ml. Tetracycline hydrochloride was dissolved in 70% ethanol to a final concentration of 1mg/ml. All antibiotic solutions were sterilized by filtration through 0.22 µm syringe filter. The sterile antibiotic solutions were stored at -20°C and protected from direct sunlight with aluminum foil.

Preparation of competent *E. coli* JW0452-3 and *E. coli* JW5503-1 cells. Overnight cultures were diluted in LB to an OD_{600} of 0.4 and grown to an OD_{600} of 0.6 in order to generate working cultures of each strain in 30 ml LB. The working cultures were chilled on ice for 10 minutes. 20 ml of each culture was pelleted at 1000 x g for 15 min at 4 °C with the IEC Centra MP4R centrifuge; the resulting pellets were resuspended in and washed twice with 20 ml in ice cold 10% glycerol. After the third centrifugation step, each pellet was resuspended in 1 ml of cold 10% glycerol and centrifuged. After this last centrifugation step, each pellet was resuspended in 200 µl of 10% glycerol and kept on ice or stored at -80°C.

Removal of the kanamycin resistance gene in *E. coli* JW0452-3 and *E. coli* JW5503-1 cells. The kanamycin resistance gene was removed from each strain following an adapted version of a protocol by Narita and Peng (11). Briefly, 479 ng of pCP20 plasmid was mixed with 60 µl of each of the competent strains in pre-chilled 0.2 cm sterile BioRad MicroPulser cuvettes, followed by electroporation using a BioRad MicroPulser. Immediately after electroporation, 1 ml of pre-warmed SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20mM glucose) was added to each cuvette to rinse and transfer the contents of the cuvettes into sterile test tubes to let the transformed cells recover at 30°C for 2 hours. Transformed cells were then spread plated on LB + ampicillin (50 µg/ml) agar plates, which were incubated overnight at 30°C in order to select for ampicillin resistant colonies. Ampicillin-resistant colonies were then streaked on a LB agar plate and incubated at 42°C. To ensure the loss of the kanamycin resistance gene and the pCP20 plasmid, colonies from the first LB agar plate were re-streaked on a second LB agar plate and incubated at 42°C overnight. Colonies from the second LB agar plate were then grid plated on LB, LB + ampicillin (50 µg/ml) and LB + kanamycin (30 µg/ml) agar plates and incubated at 37°C overnight. A colony of each strain that was both kanamycin and ampicillin sensitive was selected and used for all subsequent assays: the doubly sensitive colony of the *acrA* deletion strain was renamed as LLLL12W-1 and the doubly sensitive colony of the *tolC* deletion strain was renamed as LLLL12W-2.

Minimum inhibitory concentration (MIC) assay. The MICs of kanamycin and streptomycin were determined for all 4 strains using 96 well flat bottom plates in the first set of MIC assays. Two-fold serial dilutions of the antibiotics were made in triplicates. Final concentrations of the antibiotics in the wells were two-fold serial dilutions from 0 to 32 µg/ml for kanamycin and streptomycin, with an additional concentration of 24 µg/ml for streptomycin, and from 0 to 16 µg/ml for tetracycline and ampicillin. The wells with 0 µg/ml of the antibiotics (the control concentration) contained only LB. Working cultures of the 4 strains were generated by diluting the culture to an OD_{600} of 0.005. The plates were incubated for 24 hours at 37°C. The plates were then inspected visually to determine the MIC. Sub-inhibitory concentrations were defined as 0.5X MIC. The second set of MIC assays used the same set of final concentrations for kanamycin and streptomycin. Two working cultures of each of the four strains from each of the overnight cultures were generated as described above. For each of the four strains, one of the working cultures was pretreated by adding a sub-inhibitory concentration of kanamycin (specific to each strain). All 8 cultures were incubated at 37°C for 1 hour in a shaking water bath. After the 1 hour pretreatment, kanamycin was removed from the pretreated cultures by centrifuging the cultures at 8600 rpm (IEC Centra MP4R centrifuge; rotor IEC #836) for 10 minutes and then resuspending in 15 ml LB twice. At the same time, the non-pretreated cultures were also spun down and resuspended as described above. The final OD_{600} of each culture were diluted to 0.005 prior to addition into the wells. The plates were incubated for 24 hours at 37°C.

Transient antibiotic resistance assay. The final concentrations for the transient antibiotic resistance assay were two-fold serial dilutions from 0 to 32 µg/ml for kanamycin, from 0 to 64 µg/ml for streptomycin, from 0 to 16 µg/ml for ampicillin, and from 0 to 8 µg/ml for tetracycline. Overnight cultures of all 4 strains were diluted to an OD_{600} of 0.2 in fresh LB to generate working cultures. Pretreatment and subsequent removal of kanamycin was performed as described above. Non-pretreated cultures were also spun down and resuspended as described above. The final OD_{600} of 0.15-0.35 for each culture were used for the assay and the plates were incubated at 37°C. A Biotek Epoch Microplate Spectrophotometer was used to read turbidity at 600 nm in order to monitor growth. Readings were taken every 20 min until the 2 hour time point.

Mathematical Analysis of Results. For the MIC assay, the MIC value of each antibiotic for each of the four *E. coli* K-12 strains was estimated and defined as the concentration following the last concentration with visible growth. For the transient antibiotic resistance assay, the triplicate OD_{600} values for all samples at each time point were averaged. Using these OD_{600} values, the growth rate was calculated between the t=0 and t=100 min for each *E. coli* K-12 strain at each concentration of each antibiotic, using the growth rate formula for bacteria growing in

TABLE 1. The MICs of kanamcyin and streptomycin for the four *E. coli* K-12 strains, with (+) or without (-) pretreatment with their corresponding sub-inhibitory levels of kanamycin, interpreted visually.

	MIC (µg/ml)							
	WT		Δ*acrD*		Δ*acrA*		Δ*tolC*	
	+	−	+	−	+	−	+	−
Kanamycin	8	8	16	16	8	8	8	8
Streptomycin	24	24	32	32	16	16	8	8

exponential phase (12). t=0 was defined as the time point at which the pretreated or control cells were treated with the different antibiotic solutions. Each growth rate of each culture (pretreated or non-pretreated) at each antibiotic concentration was then normalized to the growth rate of the corresponding pretreated or non-pretreated culture of the same strain that was treated with LB (no antibiotics). This normalization was done to account for differences in the growth rates of each culture within each strain solely due to the pretreatment, or lack thereof. The 95% confidence limits were calculated by combining error in the processed results (12). Overlapping error bars were interpreted as indicating a non-significant difference between measurements.

RESULTS

Loss of *acrD*, *acrA* and *tolC* influences the inherent MICs of kanamycin and streptomycin for non-pretreated strains. As seen in Table 1, the MIC of kanamycin for *E. coli* LLLL12W-1 and *E. coli* LLLL12W-2 was the same as that for *E. coli* BW25113, at 8 µg/ml, indicating the deletions of *acrA* and *tolC*, respectively, did not have an effect on the kanamycin MIC. The loss of *acrA* and *tolC* did, however, make the deletion strains intrinsically more sensitive to streptomycin as the MIC for *E. coli* BW25113 (24 µg/ml) was 1.5X and 3X higher than those for *E. coli* LLLL12W-1 and *E. coli* LLLL12W-2 (16 and 8 µg/ml, respectively). Also surprising was the fact that the MIC of kanamycin for *E. coli* STVV11W-1 (16 µg/ml) was 2X higher than that for *E. coli* BW25113, indicating that loss of *acrD* makes the deletion strain intrinsically more resistant to kanamycin. This increase in intrinsic resistance due to the loss of *acrD* was also observed for streptomycin as the MIC of streptomycin for *E. coli* STVV11W-1 (32 µg/ml) was 1.3X greater than that for *E. coli* BW25113. These MIC results for *E. coli* STVV11W-1 were unexpected because it was expected that the loss of *acrD* would make the deletion strain more susceptible to aminoglycosides, given the previously shown role of *acrD* in aminoglycoside resistance (4). Because the *E. coli* STVV11W-1 was intrinsically more resistant to kanamycin, this strain was subsequently pretreated with a higher concentration of kanamycin than the other three strains.

Pretreatment with sub-inhibitory levels of kanamycin resulted in short-term cross adaptive resistance to non-aminoglycosides, but increased susceptibility to aminoglycosides in the short-term, in all four *E. coli* K-12 strains used in this study. As seen in Figs. 1A and 1B, for each of the four *E. coli* K-12 strains, the pretreated strains had significantly higher normalized growth rates than the respective non-pretreated strains upon treatment with a lethal concentration (MIC data not shown for ampicillin or tetracycline) of ampicillin and tetracycline, indicating that the kanamycin pretreatment induced short-term cross-adaptive resistance to aminoglycosides. However, in Figs. 1C and 1D, for all four *E. coli* K-12 strains, the pretreated strains had significantly lower normalized growth rates than the respective non-pretreated strains upon treatment with a lethal concentration of the two aminoglycosides, suggesting that kanamycin pretreatment appears to have caused all four *E. coli* K-12 strains to become more susceptible to aminoglycosides. This was unexpected for *E. coli* BW25113 as kanamycin pretreatment should induce transient adaptive resistance to aminoglycosides in wild type *E. coli* (2). In general, the trends between pretreated and non-pretreated cultures within each strain, for each antibiotic, were consistently observed at the upper range of antibiotic concentrations tested in the transient antibiotic resistance assay (data not shown for other upper range antibiotic concentrations).

Pretreatment with sub-inhibitory levels of kanamycin did not result in any long-term adaptive resistance in any of the *E. coli* K-12 strains. There were no differences in the MICs of kanamycin or streptomycin between the non-pretreated and the respective pretreated strain, for any of the four *E. coli* K-12 strains (Table 1). While this was expected for *E. coli* STVV11W-1, the lack of change in the MIC for the two aminoglycosides was unexpected for *E. coli* BW25113 as there should have been an increase in the two MICs for *E. coli* BW25113 (2, 3). These results indicate that the kanamycin pretreatment did not induce adaptive resistance to aminoglycosides that lasted for a long-term period in any of the strains.

DISCUSSION

A previous study conducted by Chen *et al.* (2) showed that treatment with a sub-inhibitory concentration of kanamycin in *E. coli* B23 induces transient (short-term) resistance to both aminoglycosides and non-aminoglycosides. Our results were, however, only partially consistent with their findings: while there was transient adaptive resistance to the non-aminoglycosides, ampicillin and tetracycline, upon kanamycin pretreatment, there was actually an increase in susceptibility to aminoglycosides, as seen in Fig. 1. As a competitive inhibitor of transpeptidase, ampicillin only needs to cross the outer membrane of *E. coli* because their target site is in the periplasm (9). Tetracycline, on the other hand, needs to cross both the outer membrane and the cell membrane to access the ribosomes to inhibit protein synthesis (8).

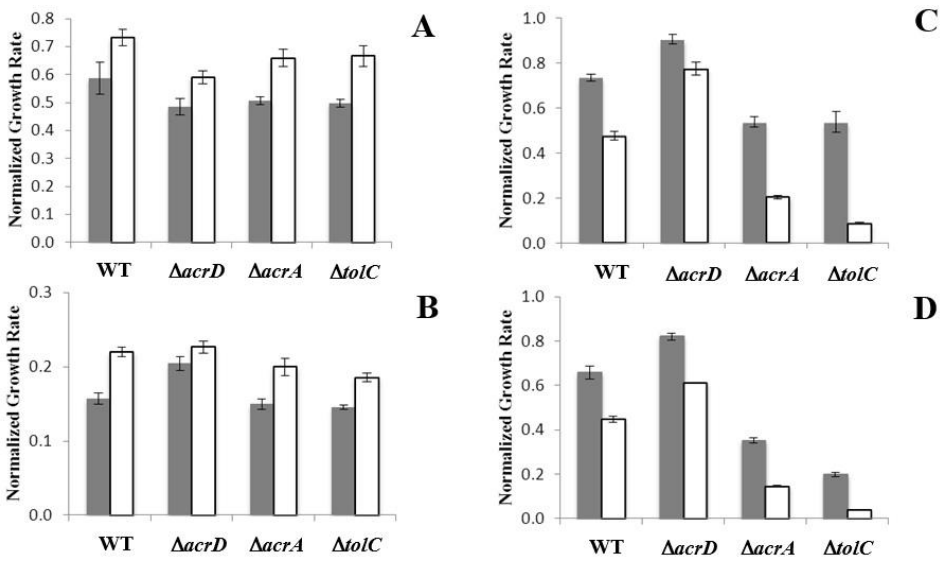

FIG 1 Short-term adaptive resistance in *E. coli* K-12 to tetracycline and ampicillin does not require *acrD*, *acrA* or *tolC*. (A) displays ampicillin challenge data, with *E. coli* WT and *E. coli* Δ*acrD* challenged with 4 µg/ml and *E. coli* Δ*acrA* and *E.coli* Δ*tolC* challenged with 8 µg/ml. (B) shows tetracycline challenge data, with *E. coli* WT, *E. coli* Δ*acrA* and *E. coli* Δ*tolC* challenged with 8 µg/ml and Δ*acrD* challenged with 4 µg/ml. (C) shows kanamycin challenge data, with all four strains challenged with 16 µg/ml. (D) shows streptomycin challenge data, with all four strains challenged with 32 µg/ml. Growth rates were normalized to the growth rate of strains grown in the absence of antibiotics. Presented antibiotic concentrations represent lethal concentrations (concentrations above the MIC of each antibiotic specific for each strain). Grey bars represent the non-pretreated control strains and white bars represent pretreated strains.

However, once tetracycline crosses the outer membrane, it is able to passively diffuse through the cell membrane without the use of a protein channel (13). The increase in short-term resistance to these two antibiotics observed in *E. coli* as a result of kanamycin pretreatment must, therefore, be preventing these two antibiotics from crossing the outer membrane: one possibility is altered outer membrane permeability. OmpC and OmpF are outer membrane porins that allow for the passive diffusion of small hydrophilic molecules, with larger molecules (excluded by OmpC) using OmpF to move across the outer membrane due to its larger diameter (14). Both ampicillin and tetracycline are believed to preferentially use OmpF as their method of crossing the outer membrane (15). A previous study conducted by Hu *et al.* found that kanamycin pretreatment of *E. coli* B23 induced an increase in the level of OmpC relative to OmpF (16). Since OmpC and OmpF are reciprocally expressed (17), even though Hu *et al*. were not able to show a decrease in the expression level of OmpF, the increased expression of OmpC should theoretically result in a corresponding decrease in the expression of OmpF, resulting in decreased permeability of the outer membrane to ampicillin and tetracycline (16). This could explain why there was an increased resistance to these non-aminoglycosides in the short run.

Like tetracycline, as inhibitors of protein synthesis, kanamycin and streptomycin also need to cross both the outer membrane and the cell membrane in order to reach their target site: the ribosomes. Though, because of the large size of aminoglycosides, they are unlikely to use outer membrane porins to cross the outer membrane in *E. coli* (18), and instead, they use a process known as self-promoted uptake (19). In self-promoted uptake, the cationic aminoglycosides competitively displace Mg^{2+} bridges that exist between the lipopolysaccharide molecules that make up the lipid bilayer, causing a destabilization in the outer membrane to allow for uptake of the aminoglycosides (19). Since they do not use the outer membrane porins for passage across the outer membrane, the permeability of the outer membrane to the aminoglycosides would not be affected by the aforementioned proposed mechanism of short-term adaptive resistance.

The mechanism of aminoglycoside transport, and therefore kanamycin transport, across the cell membrane could explain why kanamycin pretreatment resulted in increased susceptibility to both aminoglycosides in the short-term. Once inside the periplasm, the aminoglycosides are subsequently transported across the cell membrane in a process that involves electron transport and the electron transport chain (ETC), a process called energy-dependent phase I (EDPI) (19). Inside the cytoplasm, the aminoglycosides bind to the 30S ribosomal subunit, taking part in another process called energy-dependent phase II (19). In doing so, the aminoglycosides disrupt the elongation of the growing polypeptide chain by impairing the proofreading process in translation, resulting in an increased rate of error during synthesis of the polypeptides and premature termination (19). While this mechanism does inhibit polypeptide synthesis by producing non-functional proteins, it could also produce truncated or misread, yet functional, proteins similar to the proteins that are produced as a result of missense mutations (19). One possibility is that the wild type form

of these proteins are normally involved in the ETC, and therefore, EDPI. When the aberrant versions of these proteins insert themselves in the cell membrane, they could potentially alter the EDPI, increasing cell membrane permeability to allow more aminoglycosides to enter the cell. Thus, exposure to kanamycin during the pretreatment could have resulted in mutated proteins that increased the uptake of aminoglycosides, leading to an increased short-term susceptibility to both aminoglycosides observed in all four *E. coli* K-12 strains.

These aforementioned effects of kanamycin pretreatment — mainly increased resistance to non-aminoglycosides and increased susceptibility to aminoglycoside — in *E. coli*, however, were only transient. Over time, OmpF levels could be restored to their normal levels after removal of the kanamycin pretreatment (2) while new ribosomes could be made that have not been affected by the pretreatment, allowing wild type functional proteins to replace the aberrant proteins in the cell membrane as part of regular protein turnover. This recovery could explain why increased susceptibility to aminoglycosides was not observed in the long-term (Table 1). Nevertheless, given that the pattern of transient effects of kanamycin pretreatment was consistently the same between *E. coli* BW25113 and the three deletion strains across all four antibiotics, collectively, the results of Fig. 1 indicate that *acrD*, *acrA* and *tolC* were not necessary in the previously observed transient adaptive resistance in *E. coli* upon kanamycin pretreatment (2).

The fact that short-term adaptive resistance to aminoglycosides (Figs. 1C and 1D) was not observed in our study was inconsistent with previous studies (2). One possible explanation for the conflicting results could be the fact that different laboratory strains of *E. coli* were used by Chen *et al*. (2). In our study, the *E. coli* K-12 strain BW25113 was used, whereas Chen *et al.* used an *E. coli* B23 strain in their experiments (2). Although related, these two commonly used laboratory *E. coli* strains are not identical (20). For example, B strains of *E. coli* are known to grow faster in minimal medium than K-12 strains (20). Furthermore, *E. coli* K-12 strains express both OmpC and OmpF, whereas B strains only express OmpF (20). Both of these examples illustrate that the *E. coli* strains are not the same, and as the mechanism(s) mediating adaptive resistance are not fully understood, we cannot be sure that the inherent differences in the *E. coli* strains used are not the reason for the observed inconsistencies.

This potential difference in the two *E. coli* wild type strains used could also be a possible reason for why our results, as seen in Table 1, seemed to contradict the other results by Chen *et al.* (2). Chen *et al.* showed that pretreatment of *E. coli* B23 with a sub-inhibitory concentration of kanamycin induces long-term adaptive resistance to aminoglycosides while our results (Table 1) suggest that pretreatment of *E. coli* BW25113 with a sub-inhibitory concentration of kanamycin did not offer long-term protection to a subsequent challenge to lethal levels of kanamycin or streptomycin. In addition, the increase in MIC of kanamycin and streptomycin that they observed as a result of pretreatment was very small, increasing only from 4 μg/ml to 6 μg/ml, and thus, may not be significant (2). Similarly, Sidhu *et al.* also showed that long-term adaptive resistance to aminoglycosides was induced in *E. coli* BW25113 upon kanamycin pretreatment; however, they assessed long-term adaptive resistance by comparing ratios of OD_{595} values, calculated by taking the OD_{595} value at the final time point (18 hours) and dividing by the OD_{595} value at an earlier time point (6 hours) (3). Adaptive resistance was then defined as an increase in this ratio for the pretreated cultures relative to the untreated cultures (3). MIC assays may be able to detect a smaller drop in the MIC if the concentration range that was used was not separated by a two-fold difference, the way MIC are usually performed. In the literature, it has also been noted that the number of passages through which a strain has gone may affect laboratory results (21). Although unlikely, we cannot rule out the possibility that the *E. coli* strain BW25113 used in our study is inherently different than the strain used by Sidhu *et al* (3).

We had originally hypothesized that all three genes (*acrA*, *acrD* and *tolC*) were necessary for long-term adaptive resistance to aminoglycosides, but as this long-term adaptive resistance was not observed in the wild-type *E. coli* BW25113, we are unable to fully assess the involvement of *acrA* and *tolC* in the previously observed phenomenon of adaptive resistance. Hence, while it appears that *acrD*, *acrA* and *tolC* are all necessary in the long-term adaptive resistance to aminoglycosides upon kanamycin pretreatment, given that the MICs of kanamycin and streptomycin for all 3 deletion strains did not increase with pretreatment (Table 1), this is still inconclusive.

Our findings indicate that the kanamycin pretreatment induced an increased transient resistance to non-aminoglycosides but an increased transient susceptibility to aminoglycosides in all four strains of *E. coli* K-12 used. In the long-term, the pretreatment did not induce resistance to aminoglycosides in any of the four *E. coli* K-12 strains used, including the wild type *E. coli* K-12 control strain. These results indicate that the three genes —*acrD*, *acrA* and *tolC*— are not necessary for short-term adaptive resistance, and while it appears that these three genes may be involved in long-term adaptive resistance, the involvement of *acrA* and *tolC* in long-term adaptive resistance cannot be fully concluded. As such, it is still inconclusive as to whether these three genes are necessary for the physiological phenomenon of adaptive resistance, and therefore, further research is needed to elucidate this uncertainty.

FUTURE DIRECTIONS

The *acrA, acrD* and *tolC E. coli* deletion mutants used in this study were obtained from the Keio collection, and therefore derived from a parental *E. coli K-12* strain; however, the previously observed short-term adaptive resistance to all four of the tested antibiotics was seen in *E. coli B23*. Therefore, we suggest that the same deletion mutants be derived from the *E. coli* B23 strain, so that

future research can be more comparable to past studies by accounting for the inherent genetic differences in the *E. coli* wild type strains.

To test if the increase in transient susceptibility to aminoglycosides was, in fact, due to increased cell membrane permeability to these aminoglycosides, radioactively labeled (^{14}C) kanamycin or streptomycin could be used to compare the uptake of the aminoglycosides into the pretreated (with non-radioactively labeled kanamycin) or non-pretreated cells over the 2 hour period. If the specific incorporation of the radioactively labeled aminoglycosides is higher in the pretreated cells than in the non-pretreated cells, then the increase in transient susceptibility to aminoglycosides would be due to a greater uptake of the aminoglycosides into the pretreated cells. If there is no significant difference in the specific incorporation of the radioactively labeled aminoglycosides, then another mechanism is responsible for the increased susceptibility.

Because there seems to be varying degrees of reproducibility between the currently used assays for determining adaptive resistance in *E. coli*, another way of measuring increased cell viability—and, therefore, adaptive resistance —could be used to give more reproducible results. One such example would be to construct a green fluorescent protein (GFP) reporter plasmid containing the promoter/regulatory region of a household gene (like *ftsZ*) that is upstream of a GFP reporter gene. This reporter plasmid would subsequently be stably transformed into the *E. coli* K-12 strains. This reporter plasmid would enable the monitoring of the expression levels of this household gene by measuring the reporter gene activity, which would be reflective of viability of the cells. While the expression level per viable cell would not change, assuming there were the same number of transformed cells used initially, a higher overall expression level in the pretreated cells (compared to non-pretreated cells) that are subsequently treated with lethal levels of antibiotics would indicate there was increased resistance to the antibiotics, and therefore, adaptive resistance.

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