Sub-Inhibitory Concentrations of Kanamycin May Induce Expression of the Aminoglycoside Efflux Pump acrD Through the Two-Component Systems CpxAR and BaeSR in Escherichia coli K-12

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The AcrAD-TolC multidrug efflux complex of Escherichia coli K-12 mediates efflux of aminoglycoside antibiotics. Previous studies have shown that pretreatment of E. coli K-12 with sub-inhibitory concentrations of kanamycin induces adaptive resistance to aminoglycoside antibiotics and that acrD expression is upregulated in the presence of kanamycin through a process that involves two-component systems CpxAR and BaeSR. The exact interactions between the components of the systems are still largely unknown. In this study, the interactions between the various components and their role in transient adaptive resistance against kanamycin was further investigated. E. coli K-12 ΔacrD, ΔbaeS, ΔbaeR, ΔcpxA, and ΔcpxR single-deletion mutants were examined in order to elucidate the mechanism of acrD regulation. A transient adaptive resistant assay of the E. coli K-12 mutant strains using kanamycin was performed in conjunction with measurements of acrD expression levels using quantitative reverse transcriptase PCR (qRT-PCR) in order to determine the role of each component in the regulation of acrD expression. Our results provide support for a model where acrD transcription and adaptive resistance to kanamycin decrease in the ΔbaeS and ΔbaeR strains, suggesting a role for these genes as positive regulators of acrD. Also, an increase of acrD level was observed in the ΔcpxA strain, suggesting a negative regulatory role for this sensor. Wild type, ΔcpxA, and ΔcpxR strains displayed similar levels of adaptive resistance to kanamycin, indicating that BaeSR can function independent of CpxAR.

Antibiotic resistance is becoming a serious clinical problem due to the widespread misuse of antibiotics. Efflux pumps are of serious concern due to their ability to export multiple classes of antibiotics (1). Efflux pumps in Gram-negative bacteria consist of a cytoplasmic membrane pump connected to an outer membrane channel by a periplasmic linker protein (1). In Escherichia coli, two efflux pump systems are the subject of intense study: the multidrug efflux system AcrAB-TolC, and the aminoglycoside efflux system AcrAD-TolC (2). In both systems, the cytoplasmic linker (AcrA) connects the outer membrane channel (TolC) to the cytoplasmic membrane pump (AcrB or AcrD) (2).

It is known that bacteria which possess these systems increase expression of the cytoplasmic membrane pumps in response to incubation in sub-inhibitory concentrations of kanamycin; such pretreatment also causes an increase in the minimum inhibitory concentration (MIC) to kanamycin (2). Regulation of acrB is governed by the repressor AcrR but the expression of acrD is not regulated by AcrR (2, 3). Regulation of acrD is not well characterized, but it has been shown that the two-component systems BaeSR and CpxAR are involved (4, 5). Deletion strains of these regulatory systems indicate that BaeSR is required for differential expression of acrD in response to high concentrations of indole (a naturally biosynthesized compound that acts as an extracellular signal for many bacteria and has been shown to induce expression of acrD), and that CpxAR appears to positively modulate the effect of BaeR (5). Separate studies have shown that the same regulatory mechanism does not occur in response to the presence of kanamycin, though these studies did not include deletion mutants of the sensors BaeS and CpxA and therefore are incomplete (4).

In this study, the mechanism of acrD regulation was further investigated by comparing the differential expression levels of acrD in response to kanamycin pretreatment of E. coli K-12 mutants bearing deletions of genes encoding cpxA, cpxR, baeS, and baeR. In addition, the effect of kanamycin pretreatment on the ability to grow and survive in the presence of inhibitory levels of kanamycin was investigated. These observations were then compared in order to evaluate the effect of AcrD in adaptive kanamycin resistance.

MATERIALS AND METHODS
Bacterial strains. E. coli BW25113 (an E. coli K-12 derivative), STVW11W-1 (ΔacrD), CFLP12W-1 (ΔcpxR), and CFLP12W-2 (ΔbaeR) were obtained from the culture collection of the Department of Microbiology and Immunology at the University of British Columbia. E. coli JW3882-1 (ΔcpxA) and JW2063-3 (ΔbaeS) were obtained from the Coli Genetic Stock Center at Yale University (CGSC) (6). Indole-induced wild type cells were used as a positive control for the qPCR analysis. The strains used in this study are summarized in Table 1.

Stock solutions. Kanamycin monosulphate ( Gibco®) was dissolved in sterile distilled water to a final concentration of 5 mg/ml. Ampicillin sodium salt (Sigma-Aldrich®) was dissolved in sterile distilled water to a final concentration of 10 mg/ml. Indole (Sigma-Aldrich®) was dissolved in sterile H2O at 60°C to a stock concentration of 20 mM. Stock solutions were sterilized using a 0.22 µl nitrocellulose filter (EMD Millipore™). Sterile antibiotic solutions were stored at -20°C and sterile indole was stored at room temperature.
TABLE 1 Kanamycin-sensitive E. coli K-12 strain names

<table>
<thead>
<tr>
<th>Parent Strain</th>
<th>Deletion</th>
<th>Strain Name</th>
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<tr>
<td>BW25113</td>
<td>N/A</td>
<td>BW25113</td>
</tr>
<tr>
<td>JW2454-1</td>
<td>ΔacrD</td>
<td>STVV11W-1</td>
</tr>
<tr>
<td>JW3882-1</td>
<td>ΔcppxA</td>
<td>BRTT13W-1</td>
</tr>
<tr>
<td>JW3883-1</td>
<td>ΔcppxR</td>
<td>CFLP12W-2</td>
</tr>
<tr>
<td>JW2063-3</td>
<td>ΔbaeS</td>
<td>BRTT13W-2</td>
</tr>
<tr>
<td>JW2064-3</td>
<td>ΔbaeR</td>
<td>CFLP12W-3</td>
</tr>
</tbody>
</table>

Culture methods. All strains were grown in lysogeny broth (LB) (2% tryptone, 0.5% yeast extract, 1% NaCl) liquid medium in a shaking air incubator at 100 rpm at 30°C. Overnight cultures were prepared by inoculating 0.1 ml LB with a loopful of bacteria and incubating for 18 hours. Working cultures were prepared by diluting overnight cultures 1:20 in LB and incubating until an OD600 was achieved. Indole-induced cells were prepared by supplementing control or pretreated wild type cultures at a final concentration of 2 mM and incubating for 2 hours.

Preparation of competent E. coli JW3882-1 and JW2063-3 cells. E. coli JW3882-1 and JW2063-3 cultures were grown to an OD600 of 0.6 in 10 ml LB at 30°C. 1.5 ml of culture was centrifuged at 5000 x g at 4°C to pellet cells. The pellets were resuspended and washed twice in 100 µl cold H2O followed by one wash in 100 µl cold 10% glycerol. After the final centrifugation, the cell pellets were resuspended in 40 µl cold 10% glycerol and stored on ice until electroporation.

Removal of the kanamycin resistance gene in E. coli JW3882-1 and JW2063-3. The kanamycin resistance gene present in the genomes of E. coli JW3882-1 and JW2063-3 were removed by transforming the cells with pCP20. pCP20 contains genes encoding FLP recombinase and ampicillin resistance. 1 µl of 520 ng/µl pCP20 was mixed on ice with 40 µl of competent cells and electroporated with the Bio-Rad MicroPulser using the Ec2 setting. 1 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) was added to electroporated cells, which were recovered at 30°C for 1 h. 100 µl of recovered cells were plated on LB + ampicillin (100 µg/ml) agar plates and incubated at 30°C overnight. Ampicillin-resistant colonies were streaked on LB agar plates and incubated at 42°C overnight to inhibit pCP20 replication. Colonies were then re-streaked on LB agar plates and incubated again at 42°C overnight in order to ensure curing of pCP20. Colonies were then grid plated onto LB + ampicillin (100 µg/ml) and LB + kanamycin (100 µg/ml) agar plates and incubated at 30°C overnight. Double-sensitive colonies of JW3882-1 and JW2063-3 were selected and renamed BRTT13W-1 and BRTT13W-2, respectively.

Determination of the minimum inhibitory concentration for kanamycin. Dilutions of kanamycin were made in LB to give final concentrations of 0, 4, 8, 12, 14, 16, 18, 20, 22, 24, 28, and 56 µg/ml. 100 µl of each dilution was added to 96-well plates (Fischer Scientific™) in triplicate for each E. coli strain. Working cultures of all strains were diluted to an OD600 of 0.01 and 100 µl were added to the kanamycin dilutions. Plates were incubated at 30°C for 18 hours and visually inspected. The minimum inhibitory concentration (MIC) was estimated as the lowest concentration of kanamycin at which no visible growth occurred. The sub-inhibitory concentration of kanamycin was taken to be 1/4 of the lowest MIC.

Transient kanamycin resistance assay. Dilutions of kanamycin were made in LB to give final concentrations of 6, 12, 18, 24 µg/ml. 100 µl of the dilutions were added to 96-well plates (Fischer Scientific™). Working cultures of all strains were prepared and diluted 1:20 in fresh LB. Cultures were pretreated by adding kanamycin to a final concentration of 2.5 µg/ml. Indole-treated BW25113 cultures were supplemented with indole to a final concentration of 2 mM. Non-pretreated control cultures and pretreated cultures were incubated at 30°C for 2 hours. The cells were washed following pretreatment to remove kanamycin by centrifuging all cultures for 10 min at 5000 x g. Cells were resuspended in 10 ml LB, re-centrifuged, and resuspended in 10 ml LB. Triplicate aliquots of 100 µl of culture were added to the kanamycin dilutions in 96-well plates and were incubated at 30°C. Growth was monitored by measuring the OD600 using a microplate reader (Bio-Rad™). Readings were taken at 0, 30, 60, 90 minutes before incubation overnight and a final reading at 18 hours. Relative growth was calculated as the ratio of final turbidity and initial turbidity.

Total RNA extraction and cDNA conversion. Control and pretreated cultures of BW25113, indole-induced BW25113, STVV11W-1, CFLP12W-2, CFLP12W-3, BRTT13W-1, and BRTT13W-2 were prepared as described above without washing after incubation. Approximately 10⁶ cells were taken from each culture and used for RNA extraction with a modified TRIzol® (Life Technologies™) and PureLink® RNA Mini Kit (Life Technologies™) protocol. In brief, pelleted cells were resuspended in 400 µg/ml lysozyme in TE, TRIzol® reagent was added and incubated for 5 minutes at room temperature. Chloroform was added and mixtures were centrifuged at 12,000 x g for 15 minutes. The upper RNA-containing aqueous phase was removed, avoiding the interface, and added to 75% ethanol in sterile RNAse-free H2O. This solution was then added to a PureLink® column and RNA was isolated according to the manufacturer’s instructions. Extracted RNA was purified with DNase I and tested for DNA contamination with PCR and gel electrophoresis; results showed no amplification of the reference gene mdoG after 35 cycles. RNA integrity was assessed by running samples on a 1.5% agarose gel and examining the intensity and purity of the banding patterns. 0.5 µg of RNA from each sample was used for first strand cDNA synthesis with the SuperScript® VIVO™ cDNA Synthesis Kit (Life Technologies™) according to the manufacturer’s instructions.

Analysis of acrD expression. qPCR was performed using primers specific for target gene (acrD) and the reference gene (mdoG) (7) with the SsoAdvanced™ SYBR® Green Supermix (Life Technologies™) following the manufacturer’s instructions and 100 ng cDNA template (Table 2). The reaction was held at 95°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds, using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad™). Standard curves for both acrD and mdoG were prepared by diluting PCR products in 10-fold increments. 8-dilutions were run in triplicate simultaneously with the cDNA samples and the absolute amount of transcripts for each gene were determined using the standard curve. Melt-curve analysis was performed over 65-95°C in 0.5°C increments with 3 seconds per step; the resulting curve had a single maximum at 84.5°C.

Statistical analysis of results. Transient kanamycin resistance data was analyzed by averaging triplicate values and comparing ratios of final and initial OD600 values. Statistical significance (P<0.05) was determined by performing an unpaired t-test which compared control and pretreatments within each strain at each concentration of kanamycin. Analysis of qPCR data was performed by determining the absolute quantity of both the target and reference genes in all samples using standard curves; the ratio of acrD/mdoG expression for all samples was determined by dividing the acrD copy number by the mdoG copy number (9).
These ratios were then normalized to the wild type control sample.

### TABLE 2 Target and amplicon information for primers used in qPCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrD</td>
<td>Fwd ATCTTTTTTTTTTTTGACAT 132</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev GCTCTTGTTTGGCCTTTTGC</td>
<td></td>
</tr>
<tr>
<td>mdoG</td>
<td>Fwd TCTCCTCAGGGTTGTTGCG    123</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev CCGCAGACTGATTTTCAGGG</td>
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</table>

### RESULTS

The MIC to kanamycin is increased in acrD regulatory deletion mutants. The MIC assay was performed to determine the appropriate sub-inhibitory Kanamycin concentration for the transient adaptive resistance assay. The MIC of the ΔcpxA, ΔbaeS, and ΔbaeR strains (28, 28, and 20 μg/ml, respectively) were higher than that of the wild type strain (13 μg/ml) (Table 3). The MIC for the ΔacrD and ΔcpxR strains (10 μg/ml and 11.5 μg/ml) were marginally lower than that of the wild type strain. In order to allow for accurate comparisons across all strains, the sub-inhibitory concentration of kanamycin used for pretreatment was taken as ¾ of the lowest MIC (2.5 μg/ml).

### TABLE 3 Minimum Inhibitory Concentration (MIC) of growth in kanamycin treated E. coli strains

<table>
<thead>
<tr>
<th>KAN</th>
<th>WT</th>
<th>ΔacrD</th>
<th>ΔcpxA</th>
<th>ΔcpxR</th>
<th>ΔbaeS</th>
<th>ΔbaeR</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>13</td>
<td>10</td>
<td>28</td>
<td>11.5</td>
<td>28</td>
<td>20</td>
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</table>

Pretreatment with sub-inhibitory kanamycin concentrations resulted in increased resistance to kanamycin in wild type and indole-induced samples. This assay was performed on the E. coli deletion strains in order to determine the importance of the regulatory systems with respect to survival in inhibitory concentrations of kanamycin. The wild type E. coli strain showed a 1.26 fold increase (p-value < 0.05) in growth after pretreatment with kanamycin. The indole-induced sample had a similar, yet marginally higher fold increase of 1.34 (p-value < 0.01) after pretreatment (Fig 1). The ΔacrD, ΔcpxR, and ΔcpxA mutants showed no significant (p-value > 0.05) change in growth in the pretreated sample compared to the control condition. The growth of the ΔbaeS and ΔbaeR mutants was 1.34 and 1.22 fold greater in the control condition than the pretreated condition respectively. No significant differences were obtained from samples incubated in 18 μg/ml and 24 μg/ml (data not shown). These data indicate that the deletion of acrD regulatory elements prevent the induction of adaptive kanamycin resistance.

Basal levels of acrD transcription were unchanged across wild type, indole-induced, ΔcpxR, and ΔbaeR strains but increased in ΔcpxA and ΔbaeS strains. qPCR was utilized in order to compare the basal levels of acrD transcription between regulatory deletion strains. There was no significant difference in acrD transcription between wild type, indole-induced, ΔcpxR and ΔbaeR strains (p-value > 0.05) (Fig 2). ΔcpxA and ΔbaeS strains had increases of 1.90 and 2.37 fold over wild type, respectively (p-values of 0.015 and 0.005). acrD transcription levels in the ΔacrD strain were negligible (Fig 2). This indicates that CpxA acts to negatively regulate acrD expression in a non-induced state, resulting in a higher basal expression level when deleted. BaeS also appears to regulate basal levels of acrD expression in a similar manner.

acrD transcription increased after pretreatment in wild type, indole-induced, ΔcpxA, and ΔcpxR strains compared to untreated controls. qPCR was again utilized to assay for the level of acrD transcription in each of the regulatory deletion strains in both control and pretreated conditions. The pretreated wild type strain showed a 1.71 fold increase (p-value = 0.004) over the untreated control condition, while indole-induced showed a 1.92 fold increase (p-value = 0.007), ΔcpxA showed a 2.10 fold increase (p-value = 0.002), and ΔcpxR showed a 3.37 fold increase (p-value = 0.0448) (Fig 2). There were no statistically significant differences in acrD expression between pretreated and control treatments in ΔbaeS and ΔbaeR (p-values of 0.5540 and 0.1424, respectively). The ΔacrD strain showed an insignificant difference between pretreated and untreated samples (Fig 2). The data are consistent with the proposed model (Fig 3), as the ΔbaeS and ΔbaeR strains did not demonstrate differential acrD expression while the ΔcpxA and ΔcpxR strains retained their ability to increase acrD expression upon pretreatment.
FIG 2 effect of pretreatment with sub-inhibitory concentrations of kanamycin on the expression of acrD in regulatory deletion mutants. acrD standard curve had a slope of -3.663, an R² value of 0.990, and an enzyme efficiency of 97.4%. (A) Expression of acrD normalized to the expression of the control treatment wild type sample. Data is shown as the mean +/- SEM of 3 replicates. * denotes statistically significant differences (P<0.05). (B) Increase in acrD expression between kanamycin pre-treated and control treatments. Data is shown as the fold increase of the mean of triplicate isolates +/- SEM. Dashed line indicates identical acrD expression between control and pre-treated samples.

DISCUSSION

The goal of this study was to elucidate the mechanisms of acrD regulation by comparing the change in acrD expression with growth patterns after sub-inhibitory kanamycin treatment. First, the MIC of the regulatory deletion strains were established in order to determine a suitable sub-inhibitory concentration of kanamycin to induce acrD expression. All strains were then pretreated along with an untreated control and the effects of pretreatment were investigated in two ways. A transient adaptive resistance assay was performed in order to see how deleting regulatory genes impacted the ability of the strains to survive in inhibitory concentrations of kanamycin. qPCR was also performed in order to determine the effect of regulatory deletions on the expression of acrD. The results of these two assays were compared and subsequently used to develop a preliminary model of acrD expression in response to kanamycin-induced stress.

In the qPCR expression assay, the 1.71 fold increase in wild type (WT), 1.92 fold increase in the positive control (indole-induced) and the minimal levels of acrD in the ΔacrD mutant provided a background level for analysis (Fig 2). In accordance with the proposed mechanism, whereby the BaeSR two-component system is directly responsible for modifying acrD expression, the lack of significant change in acrD expression in both the baeS and baeR deletion strain after pretreatment (Fig 2) implies that the inducing effect requires both of these genes individually. The 3.37 fold increase in acrD expression in ΔcpxR (Fig 1) was unexpected but can be rationalized. While the BaeSR two-component system is purported to act directly on the acrD gene, the CpxAR two-component system acts as a modifier of BaeR activity. Thus with or without BaeR, there should still be sub-inhibitory kanamycin induction of acrD expression, similar to the wild type strain. While these data seem reasonable, conclusions must be drawn cautiously as only one biological replicate was performed due to time constraints.

Basal levels of acrD expression are expected to be similar among all conditions because acrD is not actively expressed under normal conditions (10). The controls among all conditions should therefore be similar as none have been exposed to kanamycin. This is the case among most of the strains, which do not have a significant difference (p-value > 0.05), yet unexpectedly the ΔcpxA and ΔbaeS strains both have higher basal expression than all other strains. One explanation is that, without the sensor component of the two-component systems in the ΔcpxA and ΔbaeS strains, there is compensatory cross-talk from other sensors leading to the increased basal levels. This hypothesis is further validated by cross-talk from EnvZ to CpxR in the absence of CpxA, as would be the case in the ΔcpxA strain (11, 12). This cross-talk may slightly raise the basal level of acrD expression as EnvZ could stimulate CpxR, resulting in increased acrD expression before induction. It is possible that the less studied BaeSR system is also stimulated in the absence of BaeR. It is also worth considering that these two strains were constructed during this study by recombinase-mediated excision of a kanamycin resistance cassette from the genome and therefore these novel strains have not yet been extensively characterized.

The increase in relative growth of the pre-treated WT E. coli in relation to the untreated control is an expected result. We see a statistically significant difference between both the control and pretreated cultures. Both of the two component systems are intact and functional, so it is reasonable to expect an increase in relative growth with the pre-treated cultures. We observe the same trend in our positive control, the indole-induced sample, and the result is statistically significant. Given what is already known about these regulatory systems and the cross-reactivity of indole with the kanamycin
FIG 3 The proposed mechanism for regulation of acrD expression in response to kanamycin-induced stress. OM = outer membrane; IM = inner cytoplasmic membrane.

sensors, this result provides some validation of our method. We see that the relative growth rate of the acrD deletion mutant is not significantly different, however the fact that growth occurred in this condition demonstrates that the bacteria were still able to grow in the presence of kanamycin. This result demonstrates the functional redundancy in the antibiotic efflux mechanism that is possible due to the presence of the AcrAB-ToLC system. We speculate that this system is able to compensate for the loss of acrD, which is why growth is observed here. This does not invalidate our results, however, because the two efflux systems have mutually exclusive regulatory systems. Considering that our study focuses on the regulation of the AcrAD-ToLC system, it is the effects of regulator knockouts on acrD expression that are relevant to this study. The absence of a statistically significant difference in relative growth between the control and pretreated samples for the ΔcpxR and ΔcpxA mutant strains is likely due to the redundancy in the kanamycin-sensing mechanism of E. coli. That is, both the BaeSR and CpxAR two-component systems respond to kanamycin-induced stress. With the removal of the CpxAR system, BaeSR continues to function and is able to compensate for the absence of the CpxAR system. It is known that there is notable overlap and cross-talk between two component regulatory systems (11), so it not unreasonable to assume compensatory effects by the BaeSR system in the absence of CpxAR. Our results with the ΔbaeS and ΔbaeR mutant strains demonstrate that there is a significant difference between the control and pretreated samples, but the pretreated samples show lower relative growth compared to the untreated control samples. A possible explanation for the observed result is that since both BaeS and BaeR are needed for inductive effects to have a protective effect for the cell, a deletion of either creates a scenario in which no pre-induction is possible and the CpxAR system in not able to compensate. When we expose the cells to sub-MIC kanamycin treatment, we are inducing a protective response. Instead, the toxic effect of the kanamycin negatively affects the cells, and when the high concentration of kanamycin is added, they are physiologically challenged and grow slowly relative to the untreated cells. This, in turn, results in the observed lower relative growth compared to the control cultures which were healthier at the start of incubation because they were not pretreated.

Based on the above results, we propose a regulatory mechanism for the expression of acrD (Fig 4). The mechanism involves the two sensor kinase systems CpxAR and BaeSR. Based on the increase in acrD expression in both control and pretreated conditions in the ΔcpxA strain, we postulate that CpxA represses CpxR and is deregulated under kanamycin-induced stress. Similarly, since no increase in acrD expression was observed in either ΔbaeS or ΔbaeR strains, we infer that the BaeSR system is required for kanamycin-induced upregulation of acrD. Further, we suggest that BaeS positively regulates BaeR under kanamycin-induced stressed due to lack of induction in the ΔbaeS strain.

In conclusion, it has been previously shown that BaeSR and CpxAR are two-component systems responsible for controlling the expression of acrD under kanamycin-induced stress; however, the actual interactions between the components were unclear. Our data further elucidate the interactions between components by suggesting necessity of the individual BaeSR components in the transcription of acrD, as well as providing data suggesting a role of CpxA as a repressor of CpxR function. In terms of adaptive resistance to kanamycin upon pretreatment, our results provided a few key findings. First, AcrD is not exclusively necessary for adaptive kanamycin resistance, since the AcrAB-ToLC system can compensate for the absence of AcrD. Secondly, while the CpxAR two-component system has been shown to be involved in acrD regulation, the data do not demonstrate that its contribution to pre-inductive protection is significant. Finally, the BaeSR system is required for adaptive protection, and its absence does not allow for any protective processes to begin, resulting in the pre-treated cells growing at a slower rate when subsequently treated with a higher concentration of antibiotic.

FUTURE DIRECTIONS

Since the AcrAB-ToLC efflux system also provides resistance against aminoglycosides such as kanamycin, it would be beneficial in future experiments to utilize baeSR and cpxAR deletion mutants that are also acrB deletion strains. This would remove the functional kanamycin redundancy and allow for more accurate comparisons of kanamycin sensitivity since the only form of kanamycin resistance will be due to the AcrAD-ToLC system.
A logical continuation of this experiment would be to investigate whether the transcriptional analysis performed is valid by determining how much AcrD is actually translated and functionally expressed. This would involve abundance-based western blots specifically looking for AcrD. If the levels of acrD transcription correspond to the levels of AcrD actually produced, this will indicate that another system, separate from AcrAD-TolC, is involved in adaptive kanamycin resistance.

Finally, in order to confirm the validity of the presented results, the deletion strains used in this experiment should be verified for the correct genotype using PCR specific for each gene.

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