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BaeR Is Required for Upregulation of Expression of *acrD* in *Escherichia* coli Following Treatment with Subinhibitory Concentrations of Kanamycin

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The AcrAD-TolC efflux pump of *Escherichia coli* K-12 expels aminoglycosides such as kanamycin from the cell. The expression of genes encoding for proteins which make up the efflux pump are known to be regulated by the two-component systems BaeSR. In *E. coli* BaeSR has been shown to regulate the expression of *acrD* in response to treatment with subinhibitory concentrations of kanamycin but the direction of its regulation of *acrD* expression has not been established. One study's findings suggested that BaeR functions to upregulate *acrD* expression while another study suggested that BaeR downregulates *acrD* expression in response to subinhibitory concentrations of kanamycin. To resolve these conflicting results, this study explored the direction of BaeR's regulation of *acrD* expression. Reverse Transcription-qPCR was used to measure levels of *acrD* expression following exposure to subinhibitory concentrations of kanamycin in both wild type and *baeR* deletion mutant strains. In the wild type strain, a three-fold increase in *acrD* expression levels was observed post kanamycin treatment. In the *baeR* deletion mutant, no statistically significant change in expression levels was detected suggesting that BaeR is required for induction of *acrD* expression. These findings suggest that BaeR positively regulates expression of *acrD* in *E. coli* following treatment with subinhibitory concentrations of kanamycin treatment.

Escherichia coli are Gram-negative bacteria present in the gut of all humans (1). Pathogenic strains of *E. coli* can cause a variety of diseases including diarrhea and urinary tract infections (1). Antibiotics are an important option for treatment of such diseases, but the prevalence of multidrug-resistant *E. coli* has complicated treatment strategies (1). Multidrug resistance has been linked to efflux pumps capable of expelling a broad range of cell stressors such as antibiotics out of the cell, thereby reducing the intracellular concentration of antibiotic (2).

The AcrAD-TolC efflux pump in *E. coli* consists of a periplasmic linker AcrA which connects the outer membrane channel TolC to the cytoplasmic membrane pump AcrD (3). This efflux pump has been shown to expel aminoglycoside antibiotics from the cell, thus enhancing cell survival and rendering the antibiotic ineffective (3,5). Exposure to subinhibitory concentrations of the aminoglycoside kanamycin has been shown to induce the expression of *acrD* in *E. coli* (4,5).

The induction of *acrD* by both kanamycin and indole occurs via the two-component systems CpxAR and BaeSR. The BaeSR two component response regulatory system is composed of the BaeS protein situated in the plasma membrane and the cytosolic BaeR (7). BaeS has a periplasmic sensing domain which detects the presence of various environmental envelope stressors; this leads to the phosphorylation and activation of BaeR (7). Phosphorylated BaeR can then affect the expression of various genes by binding upstream of their promoters (7). CpxAR is another 2 component system in *E. coli* with a membrane sensory component, CpxA, and a response regulator, CpxR (7).

Several studies have attempted to elucidate the effects of knockouts of the individual protein components of the CpxAR and BaeSR two component systems on the induction of the *acrD* gene (4,5). Our research focused on BaeR's individual effect because it has been shown to play a key role in *acrD* regulation in response to indole, which triggers similar regulatory responses through CpxAR and BaeSR as does kanamycin (5,6,13). Furthermore, there have been conflicting results regarding the direction of BaeR's regulation of *acrD* expression (4,5).

BaeR's direct role in *acrD* regulation was shown by Hirakawa *et al.* (2005) who found that induction of *acrD* expression in response to indole pretreatment was still observed in CpxAR deletion mutants but was absent in BaeSR deletion mutants (6). Indole is a bacterial compound produced by *E. coli* that functions in cell-to-cell communication and acts a stressor to initiate signaling mechanisms in the cell that alter gene expression and lead to changes such as inhibition of cell division (14). Therefore, they hypothesized a direct role for the BaeSR system in upregulation of *acrD* and an indirect role for CpxAR that functions via the BaeSR system. Additionally, using an electrophoretic mobility shift assay, they found that BaeR binds directly to *acrD*'s promoter region (6). These findings imply that BaeR is the direct transcriptional regulator through which CpxAR and BaeSR regulate *acrD* expression in response to indole.

Conflicting results regarding the direction of BaeR's regulation of *acrD* expression are found in two papers by Besse *et al.* and Chu *et al* (4,5). In a study by Besse *et al.* (2014), *baeR* deletion strains showed a slight decrease in *acrD* expression while wild type (WT) strains showed an increase in *acrD* expression in response to subinhibitory concentrations of kanamycin. This suggests that BaeR upregulates *acrD* expression (5). Conversely, Chu *et al.* (2013) found that *baeR* knockout mutants of *E. coli* demonstrated a greater increase in expression of *acrD* than their respective wild type strains in response to

subinhibitory concentrations of kanamycin implying that BaeR functions to negatively regulate *acrD* expression post treatment (4). In another study, Hirakawa *et al.* (2005) reported that a knockout of *baeR* in *E. coli* showed no significant change in *acrD* expression in response to indole exposure while the WT showed a significant increase in *acrD* expression in response to indole treatment, implying that BaeR functions to upregulate *acrD* expression in *E. coli* in response to indole treatment (6). Since kanamycin and indole both induce expression of *acrD* via the CpxAR and BaeSR systems, we hypothesized that, similar to the mechanism elucidated in response to indole, the BaeR protein functions to upregulate the expression of *acrD* in *E. coli* in response to treatment with subinhibitory concentrations of kanamycin.

In this study, the role of BaeR in *acrD* regulation was studied by comparing the levels of induction of *acrD* in wild type and *baeR* deletion *E. coli* strains in response to subinhibitory concentrations of kanamycin using RT-qPCR. First the kanamycin cassette used to knockout *baeR* was removed. Subsequently, minimum inhibitory concentrations of kanamycin for wild type and *baeR* deletion strains were determined by MIC assay, from which the subinhibitory concentration of kanamycin was calculated. The *E. coli* cells were then treated with kanamycin at this subinhibitory concentration prior to qPCR quantification of *acrD* expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* BW25113 (subsequently referred to as WT) and JW2064-3, the *baeR* deletion mutant of the parent strain BW25113 (Table 1), were obtained from the University of British Columbia's MICB 421 bacterial strain collection. These strains were originally described in the Keio collection (9). While the WT is kanamycin sensitive, JW2064-3 carries a kanamycin cassette introduced in order to remove *baeR* (9). All strains were streaked on Lysogeny Broth (LB) agar (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl and 1.5% agar) and grown at 37°C to obtain single colonies. Overnight cultures were prepared by inoculating 10 mL of LB and incubating overnight at 37°C at 180 RPM. Cultures used for expression analysis were prepared by diluting the overnight culture 1:100 in LB and incubating on the shaker until the desired OD_{600} was achieved.

Preparation of competent JW2064-3. The *baeR* deletion mutant JW2064-3 was grown to OD_{600} of 0.5 and pelleted via centrifugation at 1000 x g for 10 mins at 4°C. Cell pellets were resuspended in 1:10 of the original culture volume in ice-cold TSS (85% LB broth v/v, 10% Polyethylene Glycol 3350 w/v, 5% Dimethyl Sulfoxide v/v and 20 mM of $MgCl_2$) (10). 150 µl aliquots of competent cells were transferred to microcentrifuge tubes and either stored on ice for short-term use or stored at -80°C.

Removal of kanamycin resistance gene in JW2064-3. The kanamycin resistance cassette found in the *baeR* deletion mutant JW2064-3 was removed by transformation with pCP20. This plasmid carries genes encoding for ampicillin resistance and FLP recombinase. 150 µL of competent JW2064-3 were transformed with 50 ng of pCP20 using previously described methods (10). Next, 0.8 mL of TSS was added to the transformed cells which were incubated at 30°C for 60 minutes on the shaker. 100 µl of recovered cells were plated on LB + ampicillin (100 µg/ml) and incubated at 30°C overnight to allow recombination. Ampicillin-resistant colonies were streaked onto LB agar and incubated at 42°C overnight to inhibit replication of pCP20. This was repeated a second time to ensure curing of the plasmid. Colonies were then grid plated onto LB and LB plates containing ampicillin (100 µg/ml) and kanamycin (100 µg/ml) which were incubated at 37°C overnight. Colonies sensitive to both kanamycin and ampicillin were selected and renamed HBFM15W-2, subsequently referred to as Δ*baeR*.

Minimum Inhibitory Concentration assay (MIC). Working cultures of kanamycin sensitive WT and Δ*baeR* were grown to an OD_{600} of 0.125 and diluted 1:100 in LB. Equal number of cells from both cultures were then added in duplicate to a 96-well polypropylene plate containing LB and kanamycin (0, 0.08, 0.17, 0.33, 0.66, 1.33, 2.67, 5.33, 10.67, 21.33 and 42.67 µg/mL) in a final volume of 100 µL (12). Sterile LB was added to wells as a negative control, and LB without kanamycin was inoculated to serve as a positive control for growth. The plate was incubated overnight at 37°C and the MIC was read as the lowest concentration of antibiotic at which there was no visible turbidity (12). The subinhibitory concentration of kanamycin was taken to be one third the concentration of the lowest MIC (4,5).

Total RNA extraction and cDNA conversion. Working cultures of WT and Δ*baeR* were grown to an OD600 of 0.6 and 0.7 respectively. Two 5 mL samples were taken from each culture, one before and one after 30 minutes of exposure to kanamycin at a final concentration of 3 µg/mL. Samples were kept on ice while an RNase-free zone was set up in the biosafety cabinet using RNase*Zap*® (Thermofisher™, catalogue number: AM9780) on the surfaces, and handling all equipment with gloves on. RNA was extracted from approximately 10^9 cells using the RNAqueous® Total RNA Isolation Kit (Thermofisher™, catalogue number: AM1914) and eluted in a final volume of 100 µL of the elution buffer found in the kit. SuperScript® II Reverse Transcriptase kit (Thermofisher™, catalogue number: 18064014) was used to synthesize cDNA from 3 µL of eluted RNA, followed by storage of any unused RNA and cDNA at -80°C.

Analysis of *acrD* expression using qPCR. qPCR was performed on cDNA samples using primers specific for *acrD* and *tatA*. RNA was converted to cDNA using the SsoAdvanced™ SYBR® Green Supermix kit (Life Technologies™, catalogue number: 172-5279). Primer design (Table 2) was carried out using Primer3 online tool with suggested settings described in the manual.

Parent Strain	Deletion	Resistance	Strain Name	Reference
BW25113	N/A	N/A	BW25113	5
BW25113	Δ*baeR*	kanamycin	JW2064-3	5
BW25113	Δ*baeR*	N/A	HBFM15W-2	This study

TABLE 1. *E. coli* strains used in the experiments

Target Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Amplicon Size (bp)
acrD	CTGATGCCATTGAGTCACAG	GATATCGCGGAACTGTTCTG	137
tatA	GCAGGATAAAACCAGTCAGG	GCCTGTTCCTGATTCGTATC	81

TABLE 2. RT-qPCR primer sets.

cDNA samples from kanamycin untreated and treated WT and Δ*baeR E. coli* strains were diluted 1:40, and 2 µL was used to set up triplicates of 20 µL qPCR reactions containing 250 nM forward and reverse primers. Negative controls included reactions set up with no template, and with 2 µL of RNA sample from WT and Δ*baeR*. qPCR parameters were then set up as per instructions manual, using 55°C as the annealing/extension temperature. A melt curve step was also included. This entire procedure was repeated for each of the WT and Δ*baeR* samples. Expression results were normalized using reference *tatA* to account for variable amounts of template loaded between samples, and relative expression graphs were produced by CFX Connect (Bio-Rad™) computer software which uses a delta-delta Ct method for analysis (11).

RESULTS

Kanamycin gene in JW2064-3 was removed by transformation with pCP20. In order to generate a Δ*baeR* strain sensitive to kanamycin, we transformed plasmid pCP20 into JW2064-3, an *E. coli* strain in which the *baeR* gene sequence is replaced with a kanamycin resistance cassette (9). Transformants were grown at 30°C on plates containing ampicillin to select for pCP20. Transformant colonies were then re-streaked and grown at 42°C in the absence of the ampicillin to cure the strain of pCP20. Cells cured of the plasmid did not grow on plates containing kanamycin indicating removal of the kanamycin resistance cassette from JW2064-3. The new strain was termed HBFM15W-2 and referred to as Δ*baeR* (Table 1).

The sub inhibitory concentration of kanamycin for WT and Δ*baeR E. coli* strains is 3 µg/mL. An MIC assay was carried out to determine the minimum inhibitory concentration of kanamycin for the WT and Δ*baeR* strains. The MIC of the WT and Δ*baeR* was 10.67 µg/mL and 21.33 µg/mL respectively. No turbidity was observed in the negative control containing only LB, ruling out outside contamination of the wells in the MIC assay. We defined the subinhibitory concentration of kanamycin as one third the concentration of the lowest MIC for either strain. This value was calculated to be 3 µg/mL of kanamycin.

***acrD* expression increased in the WT *E. coli* strain but was unchanged in the Δ*baeR E. coli* strain following kanamycin treatment.** To determine whether BaeR regulates *acrD* expression following treatment with subinhibitory concentrations of kanamycin, RT-qPCR was used to measure *acrD* transcript levels in the WT and Δ*baeR* strains of *E. coli* before and after exposure to kanamycin at 3 µg/mL. Fig. 1 shows the normalized relative expression of acrD before and after kanamycin treatment for each strain. In WT *E. coli acrD* expression increased 3 fold following kanamycin treatment (p-value <0.05) (Fig. 1A). By comparison, the Δ*baeR E. coli* strain did not show a statistically significant change (p-value > 0.05) in expression following treatment with kanamycin (Fig. 1B). qPCR of extracted RNA template using *acrD* primers showed no amplification, verifying lack of DNA contamination during the extraction process and qPCR set-up. Similarly, reactions set up without template did not show amplification indicating that cross contamination had not occurred. Melt curve analysis of *acrD* and *tatA* amplified products showed a single peak at 86.5 and 84.5 °C respectively, suggesting presence of only the desired DNA product for each primer set. Taken together, these results suggest that BaeR positively regulates the expression of *acrD* in response to subinhibitory concentrations of kanamycin.

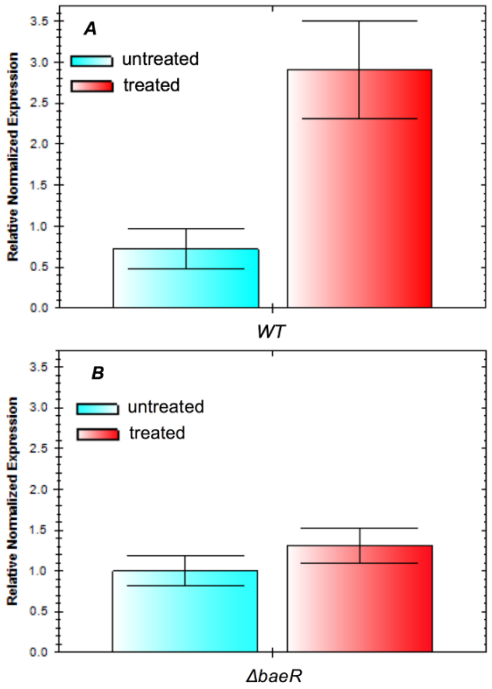

FIG 1. The effect of a *baeR* knockout on expression of *acrD* in response to treatment with subinhibitory levels of kanamycin. *acrD* expression levels were measured in WT and Δ*baeR* samples grown to exponential phase, before and after treatment with 3 µg/mL of kanamycin for 30 minutes. The effects of kanamycin treatment in (A) WT (n=1) and (B) Δ*baeR* (n=1) were monitored as fold increase in the mean of triplicate isolates +/- Standard Error of the Mean (SEM) normalized with respect to untreated samples.

DISCUSSION

The objective of this study was to test whether or not BaeR regulates *acrD* expression in *E. coli* in response to treatment with subinhibitory concentrations of kanamycin. After determining the subinhibitory

concentration of kanamycin for the WT and Δ*baeR* strains, *acrD* expression levels were measured using RT-qPCR before and after treatment with kanamycin.

RT-qPCR results showed a 3 fold increase in *acrD* expression levels in treated WT *E. coli* compared to the untreated samples. An increase in *acrD* expression levels was expected in accordance with previous studies by Chu *et al.* (2013) and Besse *et al.* (2014) which showed a 1.5 and 1.7-fold increase in *acrD* expression respectively (4,5). Additionally, this is also in agreement with a previous study by Nishino *et al.* (2005) which found a 2.3-fold increase in the expression of *acrD* in response to overproduction of BaeR using a DNA microarray analysis (13). This further supports the previous findings in the literature that *acrD* expression is induced in response to treatment with subinhibitory concentrations of kanamycin.

While the WT *E. coli* showed an increase in *acrD* expression in response to kanamycin treatment, the Δ*baeR* strain did not show any statistically significant change between the treated and untreated samples. Similarly, Besse *et al.* (2014) observed lower levels of *acrD* in the Δ*baeR* strain than the WT in response to kanamycin treatment (5). This loss of *acrD* induction when *baeR* is knocked out suggests that BaeR positively regulates *acrD* expression in response to subinhibitory concentrations of kanamycin. These findings contradict observations of Chu *et al.* (2013) who saw a 4.5-fold increase in *acrD* expression in Δ*baeR* compared to the WT in response to subinhibitory concentrations of kanamycin (4).

Suggesting a similar role for BaeR in regulation of *acrD* expression in response to indole and kanamycin, Hirakawa *et al.* (2005) found that in response to treatment with indole, WT *E. coli* demonstrated a 5-fold increase in *acrD* expression in WT strains and no statistically significant increase in *acrD* expression in *baeSR* mutant strains (6). These observations suggest that BaeR positively regulates *acrD* expression in response to indole. While these findings cannot be used to directly support our findings in response to kanamycin, they suggest parallels between the responses to the two stressors adding strength to the validity of our conclusions which is that BaeR upregulates expression of *acrD* in response to kanamycin treatment.

In conclusion, it has previously been shown that BaeSR two component system is responsible for controlling the expression of *acrD* under kanamycin-induced stress but there were conflicting reports regarding its negative or positive regulatory role (4,5). Our data further supported the positive regulatory effect of BaeR on *acrD* in response to subinhibitory concentrations of kanamycin, refuting findings by Chu *et al.* (2013) to the contrary and allowing future studies to focus on elucidating the roles of other components in this regulatory pathway.

FUTURE DIRECTIONS

Although this study supports the hypothesis that BaeR has a direct regulatory effect on *acrD* expression and its findings are in accordance with findings by other papers in the literature, these results could be further supported using an alternative quantification assay. For instance, the *acrD* promoter could be ligated into a plasmid carrying *a* reporter gene whose expression can be easily measured. A lower amount of reporter gene expression in kanamycin-treated Δ*baeR* compared to the WT would further confirm our results, suggesting a positive regulatory role for BaeR. This alternate quantification assay would support the validity of the qPCR results, ensuring that the variables and assumptions affecting the RT-qPCR protocol did not significantly alter the results.

Additionally, future studies could determine if there is a relationship between the subinhibitory concentration of kanamycin used and the levels of *acrD* expressed in the cell. The WT strain can be exposed to incrementally increasing sub-inhibitory concentrations of kanamycin, followed by *acrD* directed RT-qPCR of samples. To further validate obtained results the reporter assay mentioned previously can also be used to measure and compare relative amounts of *acrD* expressed in response to various concentrations of kanamycin.

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