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Deletion of AcrS Results in Increased Expression of *acrE* and Confers an Increase in Kanamycin Resistance in *Escherichia coli* BW25113

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***Escherichia coli* bacteria contain numerous efflux pumps that are responsible for intrinsic resistance to antimicrobial compounds. Previous studies have shown that the efflux pumps AcrAB and AcrEF are similar in structure and may be regulated with enzymes that have homologous functions. AcrR is a known repressor of the *acrAB* operon. It has been suggested that regulatory proteins AcrR and AcrS have analogous functions due to their encoding sequences being located upstream of *acrAB* and *acrEF*, respectively. Therefore, we hypothesized that deletion of the predicted regulatory gene *acrS* in *E. coli* BW25113 would result in increased expression of efflux pump protein *acrE*, and increased intrinsic kanamycin resistance. To test this hypothesis, we used a Δ*acrE*Δ*kan* and a Δ*acrS*Δ*kan* deletion mutant. Using an MIC assay, we found that the wild-type strain (BW25113), Δ*acrS*Δ*kan* and Δ*acrE*Δ*kan* had kanamycin MICs of 3.1 μg/ml, 12.5 μg/ml, and 3.1 μg/ml, respectively. Consistent with our MIC assay results, we showed using a quantitative polymerase chain reaction assay that the expression of *acrE* in Δ*acrS*Δ*kan* increased when cultured in the presence of sub-inhibitory concentrations of kanamycin. This effect was almost doubled when 6.3 μg/ml kanamycin was used, which may indicate that *acrE* expression increases in response to higher concentrations of antibiotic. In contrast to our expectations, *acrA* expression levels remained unchanged in all three of the strains, indicating that the AcrAB efflux pump may not be involved in acquiring kanamycin resistance in *E. coli*. These results support that AcrS may negatively regulate *acrEF*, and AcrEF participates in the intrinsic kanamycin resistance in *E. coli* BW25113.**

Escherichia coli bacteria use various protein efflux pumps as a mechanism to acquire intrinsic resistance to dyes, detergents and antibiotics (1). These pumps are part of the resistance-nodulation-cell division (RND) superfamily, which are multicomponent bacterial efflux pumps located in the cytoplasmic membrane (2). RND pumps are found in Gram-negative bacteria and include the pumps AcrAB and AcrEF (3). These pumps use energy-dependent transport to pump out a wide variety of compounds (1).

TolC is an outer-membrane channel used in numerous efflux pumps in *E. coli* (4). Both AcrAB and AcrEF require TolC to function (5). AcrB and AcrF are inner membrane secondary transporters, and AcrA and AcrE are the periplasmic proteins that connect TolC and AcrB or TolC and AcrF, respectively (Fig. S1) (3, 5). *acrA* and *acrB* are located on the *acrAB* operon while *acrE* and *acrF* are located on the *acrEF* operon (3). Transcribed divergently upstream of *acrAB* is the gene for the regulatory protein *acrR* and transcribed divergently upstream of *acrEF* is the gene for the potential repressor *acrS* (formerly known as *envR*) (5).

Globally, RND efflux pumps are regulated by stress-inducing conditions such as the presence of an antibiotic (6). AcrR is a local repressor of *acrAB* and an autoregulator of its own transcription as well (3). AcrR only plays a role in modulating and preventing the overexpression of *acrAB* when it is induced (7).

AcrB and AcrF were shown to have high sequence homology and are similar in their functions to pump out a diverse group of substrates including various antibiotics (novobiocin), dyes (crystal violet), and detergents (SDS) (8). Because AcrEF and AcrAB have homologous functions as both are multidrug exporters, it is proposed that AcrS and AcrR are also homologues (8). Work by Nishino and Yamaguchi shows that AcrS serves as a repressor of *acrEF* (8).

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Hirakawa *et al.* found that in addition to the negative regulation by AcrR, AcrS also negatively regulates *acrAB* (5). AcrS was shown to bind with a greater affinity to the same 24-bp palindromic sequence as the repressor AcrR, in the *acrA* promoter (5). Overexpressing *acrS* resulted in the decreased expression of both *acrA* and *acrB*, indicating the repressive effect of AcrS on AcrAB (5). Further, the overexpression of *acrS* was found to decrease the intrinsic resistance to various compounds including chloramphenicol, tetracycline and erythromycin (5).

Another study by Belmans *et al.* found that the deletion of *acrS* resulted in an increase in kanamycin resistance in *E. coli* (9). However, the levels of *acrA* and *acrE* expression were not measured and therefore the increase in antibiotic resistance could not be attributed to either or both of AcrAB and AcrEF.

Multidrug efflux pumps are not simultaneously expressed under resting conditions, potentially preventing the unnecessary expenditure of resources through the production of excess proteins (5). AcrAB is constitutively expressed as it plays a major role in the intrinsic resistance of *E. coli* (6). Therefore, it is proposed that AcrS may regulate expression between *acrEF* and *acrAB* (5). The objective of this study was to investigate the relationship between AcrS and *acrE* expression in the presence of kanamycin to further our understanding of Belmans *et al.*'s study. We hypothesized that the deletion of *acrS* would result in increased expression of *acrE* and increased kanamycin resistance.

MATERIALS AND METHODS

Bacterial strains. *E. coli* BW25113, and mutant JW3232-1 (Δ*acrS*Δ*kan,* hereinafter referred to as *E. coli* Δ*acrS*Δ*kan*) was obtained from the Microbiology and Immunology Department at the University of British Columbia. *E. coli* JW3233-2 (Δ*acrE*::*kan*) was purchased from the Coli Genetic Stock Center at Yale University, and used to make *E.coli* Δ*acrE*Δ*kan. E. coli* BW25113, Δ*acrE*Δ*kan,* and Δ*acrS*Δ*kan* were streaked on LB agar with 50 µg/ml kanamycin to screen for kanamycin sensitivity.

Removal of kanamycin resistance cassette from JW3233-2. The kanamycin resistance cassette in the Keio collection strain was removed by site-directed recombination by flippase (FLP) recombinase (10). This was possible due to the FLP recognition targets that flank the kanamycin resistance cassette. $CaCl_2$ competent cells of JW3233-2 were prepared as described previously by Sambrook and Russel (11). The pCP20 plasmid, provided by the Microbiology and Immunology Department at the University of British Columbia, contained a FLP recombinase and an ampicillin resistance cassette used as a selection marker. 5 ml of LB media with 100 µg/ml ampicillin was inoculated with an isolated colony of pCP20 overnight shaking at 37 ˚C, and plasmid purification was performed using Invitrogen™ PureLink® Quick Plasmid Miniprep Kit, as per the manufacturer's instructions. The plasmid concentration was quantified using a Nanodrop3000 spectrophotometer, and 1 ng of pCP20 was transformed into 50 µl of $CaCl_2$ competent JW3233-2 cells, recovered for 1 hour at 37 ˚C shaking, and plated on LB agar with 100 µg/ml ampicillin overnight. A negative control was included by transforming competent JW3233-3 cells with water, and plated on LB agar with 100 µg/ml ampicillin. To destroy the heat-sensitive pCP20 plasmid, a colony from the pCP20 transformation plate was streaked on LB agar and incubated overnight at 43 ˚C. To screen for the absence of antibiotic resistance, colonies from the overnight 43 ˚C plate were streaked on three LB plates, one with no antibiotic, one that contained 50 µg/ml kanamycin, and a third that contained 100 µg/ml ampicillin.

Genotypic confirmation. All primers used for genotypic confirmation were designed using the genomic sequence of parent strain BW25113 (NCBI accession number CP009273) and Snapgene Viewer, and ordered from Integrated DNA Technologies, as shown in Table 1. Genomic DNA was isolated from 1 ml overnight cultures of *E. coli* BW25113, Δ*acrS*Δ*kan*, JW3233-2 and Δ*acrE*Δ*kan* with PureLink® Genomic DNA Mini Kit (Invitrogen) as per the manufacturer's instructions. DNA concentration was quantified using a Nanodrop3000 spectrophotometer. *E. coli* BW25113, Δ*acrS*Δ*kan*, JW3233-2 and Δ*acrE*Δ*kan* genomic DNA were used as DNA templates using each of the primer sets. PCR reactions were performed in 25 µl volumes containing 2.5 µl of 10X PCR Buffer - Mg, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 0.1 µl of Platinum® Taq DNA Polymerase (Invitrogen), 0.5 µl of DNA template and 19.65 µl of sterile water. Negative controls were performed by substituting the DNA template volume with sterile water, and positive controls using *gapA* primers with each genomic DNA sample. Thermo-cycler conditions consisted of an initial denaturation at 94 °C for 2 minutes, then 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 53 °C for 30 seconds, and extension at 72 °C for 90 seconds. This was followed by a final extension at 72 °C for 5 minutes. PCR products were loaded on a 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain in 0.5X TBE (Invitrogen) and run in 1X TBE buffer at 90V for 45 minutes on a 1.5% agarose. Amplicons were visualized and imaged by UV light using a FluorChem R system (Proteinsimple).

Minimum inhibitory concentration of kanamycin. The method for the MIC assay was adapted from Belmans *et al.*'s protocol (9). The three strains BW25114 Δ*acrS*Δ*kan* and Δ*acrE*Δ*kan* were grown overnight in 5 ml of LB broth and then diluted to a concentration between 10^4 to 10^5 cfu/ml. 2-fold serial dilutions of kanamycin were used from 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, and 0.4 µg/ml. Four biological replicates were performed for each strain, each plated in triplicate in a 96 well-plate. 5 µl of culture was added to each well. One column of LB broth without culture was added for each dilution of kanamycin as a negative control. The plates were grown overnight between 18 - 36 hours at 37 ˚C. Growth was detected by visual confirmation.

Growth curve. Overnight cultures of *E. coli* BW25113, Δ*acrS*Δ*kan* and Δ*acrE*Δ*kan* were used to inoculate 100 ml of LB media (diluted 1:100). Kanamycin was added to a final concentration of 1.6 µg/ml in each of the inoculated flasks and incubated on a shaking platform at 37 ˚C until OD_{600} readings plateaued indicating stationary phase had been achieved. OD_{600} were read every 30 minutes using a spectrophotometer.

RNA extraction. Overnight cultures of *E. coli* BW25113, Δ*acrS*Δ*kan* and Δ*acrE*Δ*kan* were used to inoculate 5 ml LB media with varying concentrations of kanamycin, and incubated at 37 ˚C

TABLE 1 Primer sequences used for genotypic confirmation by PCR for the presence or absence of the *acrS*, *acrE* and *gapA* in the BW25113, Δ*acrS*Δ*kan*, JW3233-2 and Δ*acrE*Δ*kan*. Amplicon sizes based on the genome of BW25113.

Gene	Direction	Sequence (5′ to 3′)	Gene Size (bp)	Amplicon Size (bp)
gapA	Forward	CCGTATCGGT CGCATTGTTT	996	708
	Reverse	AGACGAACG GTCAGGTCAA C		
acrE (flanking)	Forward	GTTTATGTGC CTTGAGATGC CT	1158	1442
	Reverse	GGCGGTGCAA TTGTTGGAT		
acrE (nested)	Forward	CGCCGGTTGT AACGATAAGG	1158	1026
	Reverse	AGGCCGCTGA CAATGACT		
acrS (flanking)	Forward	GCACAGGCTC AGGTAATGAT T	663	1129
	Reverse	AGGCATCTCA AGGCACATAA AC		
acrS (nested)	Forward	CAGAGCGGG GGCTTGTTTA	663	352
	Reverse	CTTGACGGCT GGATTAGAGC		

TABLE 2 Primer sequences used for qPCR to measure the relative abundance of *acrA*, *acrS*, *acrE* and *gapA* in the BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan*.

Gene	Direction	Sequence (5′ to 3′)	Gene Size (bp)	Amplicon Size (bp)
gapA	Forward	GCAAACTGAC TGGTATGGCG	996	70
	Reverse	AGACGAACG GTCAGGTCAA C		
acrE	Forward	AAGTCAGGTT GAAGCGCG	1158	105
	Reverse	AGGCCGCTGA CAATGACT		
acrS	Forward	CAGAGCGGG GGCTTGTTTA	663	78
	Reverse	GGTGCCTTCA GCGGAATT		
acrA	Forward	CTGGACACCA GGACGCAC	1194	111
	Reverse	CCGATCGTTG CAAGCCAG		

shaking until the OD_{600} reached 0.5. Kanamycin concentrations were chosen based on the highest common concentration of kanamycin capable of growth for all three strains, and the highest concentration for the Δ*acrS*Δ*kan* strain. RNA extraction was performed using the RNeasy Mini Kit (Qiagen), with directions adapted from RNAprotect® Bacteria Reagent Handbook (Qiagen), as described by Hay *et al*. (12). RNAse-free DNAse (Qiagen) and TURBO DNA-free™ Kit (Ambion) were used following the manufacturer's instructions to degrade DNA in the RNA extraction.

RT-qPCR. cDNA was reverse transcribed from RNA extracts using SuperScript™ II Reverse Transcriptase (Invitrogen), as per the manufacturer's instructions using random primers and 2 µl of RNA extract. qPCR primers were designed for *gapA*, *acrA*, *acrE*, and *acrS* using Snapgene viewer and *E. coli* BW25113 genome (NCBI accession number CP009273) and ordered from Integrated DNA Technologies, as listed in Table 2. *E. coli* BW25113, Δ*acrS*Δ*kan* and Δ*acrE*Δ*kan* cDNA were used as templates for each of the primer sets. RNA extracts before and after treatment with TURBO DNA-free™ Kit (Ambion) were also used as templates to check for DNA contamination in the RNA extracts. For each primer set, *E. coli* BW25113 genomic DNA was used as the template for a positive control, and sterile water was used as the template for the negative control. *gapA*, *acrA*, *acrE* and *acrS* transcript levels were measured by qPCR with SsoAdvanced™ Universal SYBR® Green Supermix, as per the manufacturer's instructions. 25 µl reactions were performed with 12.5 µl SsoAdvanced™ Universal SYBR® Green Supermix, 11 µl of dH_20, 0.5 µl of cDNA, 0.5 µl of 10 µM forward primer and 0.5 µl of 10 µM reverse primer. Cycling and fluorescence was measured in Bio-Rad CFX Connect™ Real-Time PCR Detection System. Thermo-cycler conditions consisted of an initial denaturation at 95 °C for 10 minutes, then 40 cycles of denaturation at 94 °C for 30 seconds, annealing and extension at 53 °C for 30 seconds. This was followed by a melting temperature analysis cycle, which began at 95 ˚C for 10 seconds before lowering to 65 ˚C and increasing in 0.5 ˚C increments every 30 seconds before ending at 95 ˚C.

RESULTS

Strains BW25113, Δ*acrS*Δ*kan* and Δ*acrE*Δ*kan* are sensitive to kanamycin. In order to compare intrinsic kanamycin resistance to BW25113, the kanamycin resistant cassette from JW3232-1 (Δ*acrS::kan*) and JW3233-2 (Δ*acrE::kan*) needed to be removed if it was still present (6). To check for kanamycin sensitivity, BW25113, JW3232-1 and JW3233-2 were plated on LB agar with 50 µg/ml kanamycin. BW25113 and JW3232-1 had no growth whereas JW3233-2 contained colonies. No growth matched the phenotype with kanamycin sensitivity and indicated that the kanamycin resistant cassette in JW3232-1 was removed. JW3232-1 was renamed to Δ*acrS*Δ*kan*. Removal of the kanamycin resistance cassette from JW3233-2 was accomplished using the FLP recombinase through homologous recombination. After transformation using the pCP20 plasmid and induction of the FLP recombinase, we found that JW3233-2 grew on LB plates with 100 µg/ml ampicillin. Our transformed negative control showed no growth on LB plates with ampicillin, indicating that JW3233-2 $CaCl_2$ competent cells do not have ampicillin resistance. Following heat inactivation of pCP20 in JW3233-2 cells on antibiotic-free LB agar, we observed no growth on LB plates containing 50 µg/ml kanamycin or 100 µg/ml ampicillin. JW3233-2 was renamed to Δ*acrE*Δ*kan*. Our results were consistent among the

replicates. These results demonstrate that BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* are sensitive to 50 µg/ml kanamycin.

Strains BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* matched the expected genotypes. To investigate the effects of an *acrS* deletion, confirmation of the correct presence or absence of target genes in BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* was necessary. PCR was used to amplify *acrS* and *acrE* in the three strains and visualized through gel electrophoresis. Figure 1 shows the 1.5% agarose gel image of the PCR amplicons. Negative controls for each primer set showed no bands, indicating that positive results were not due to contaminations. Positive controls with *gapA* primers and each genomic DNA sample had the expected band at around 700 bp, which demonstrated the proper functioning of the PCR and gel electrophoresis materials and equipment.

PCR amplicons using BW25113 genomic DNA as template with *acrE* flanking and nested primers, and *acrS* flanking and nested primers matched the expected band sizes (as listed in Table 1). Genomic DNA of Δ*acrS*Δ*kan* as the template was expected to have the same amplicon band sizes using the *acrE* nested and flanking primers as BW25113. As there is a deletion of *acrS* in Δ*acrS*Δ*kan*, a PCR product about 500 bp in size is expected using the *acrS* flanking primers, and no product is expected using the *acrS* nested primers. Figure 1 showing PCR amplicons using Δ*acrS*Δ*kan* genomic DNA match the expected results.

Genomic DNA of JW3233-3 and Δ*acrE*Δ*kan* as the template was expected to have the same amplicon band sizes as BW25113 using the *acrS* nested and flanking primers. JW3233-2 has a kanamycin resistance cassette and FRT regions in place of *acrE*, and thus *acrE* flanking primers are expected to have an amplicon size around 1200 bp in length. As there is a deletion of *acrE* and kanamycin resistance cassette in Δ*acrE*Δ*kan*, a PCR product about 300 bp in size is expected using the *acrE* flanking primers. No product is expected using *acrE* nested primers for JW3233-2 and Δ*acrE*Δ*kan* genomic DNA. Figure 1 shows the bands that match the expected band sizes or the expected absence of bands. However, there is an unexpected band using the *acrE* nested primers and JW3233-2 genomic DNA that ran higher than bands in the other samples.

Together these results indicate that the strains BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* contain the expected genes or deletion of genes.

Δ*acrS*Δ*kan* has a higher MIC of kanamycin than BW25113 and Δ*acrE*Δ*kan*. Once the correct phenotypes and genotypes of the three strains were confirmed, we measured the MIC of kanamycin to determine the effect of an *acrS* or *acrE* deletion on the kanamycin resistance in BW25113. Four MIC assays of kanamycin were used to provide support for the change in kanamycin resistance in the three strains. The MICs showed that BW25113 and

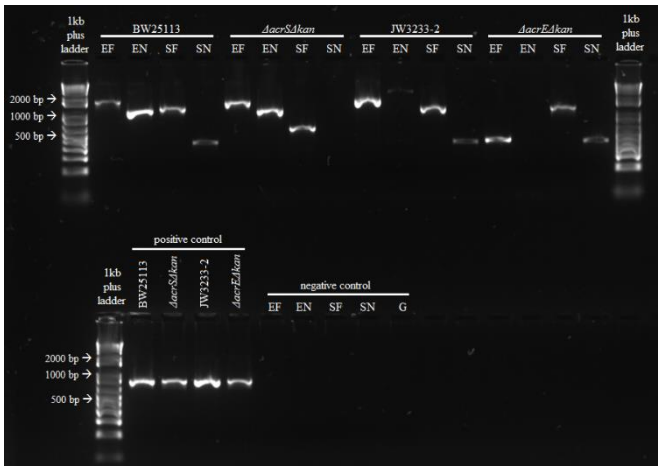

FIG 1 PCR products of various strains run on a 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain run in 1X TBE for 45 minutes at 90V. Primer acronyms are as follows: EF = *acrE* (flanking), EN = *acrE* (nested), SF = *acrS* (flanking), SN = *acrS* (nested), G = *gapA*. Positive control performed using *gapA* primer set, and negative control performed with each primer set, substituting template volume with sterile water. The unexpected band using the *acrE* nested primers and JW3233-2 genomic DNA was considered erroneous data as the following experiments focused on Δ*acrE*Δ*kan*.

Δ*acrE*Δ*kan* had a MIC of 3.1 µg/ml, and Δ*acrS*Δ*kan* had a higher MIC of 12.5 µg/ml, which was as expected. However, in some of the Δ*acrE*Δ*kan* replicates, the MIC appeared to be 6.3 µg/ml. results demonstrate that the strain Δ*acrS*Δ*kan* has a higher intrinsic kanamycin resistance than BW25113 and Δ*acrE*Δ*kan*.

Growth rates among BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* were all similar. We next aimed to determine if the different observed MICs of kanamycin were the result of varying growth rates in BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan*. This was done by culturing the three strains individually in LB with 1.6 µg/ml kanamycin, and constructing a growth curve. As shown in Supplemental Figure S3, we observed a distinct lag, exponential and stationary phase over a 5-hour period in all three strains. We found that all three strains grew similarly as they had matching growth phases.

RT-qPCR demonstrated that Δ*acrS*Δ*kan* has a higher level of *acrE* expression than BW25113 and Δ*acrE*Δ*kan*, and *acrA* expression does not vary among the three strains. In order to test if the deletion of *acrS* leads to an increase in *acrE* and *acrA* expression, the RNA expression levels of the three strains were measured by RT-qPCR. RNA extraction was performed on two biological replicates of *E. coli* BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* cultures with 1.6 µg/ml, and 1 biological replicate of *E. coli* Δ*acrS*Δ*kan* grown in 6.3 µg/ml.

We expected that a Ct value below 30 would represent successful amplification. The positive controls using *E. coli* BW25113 genomic DNA as template showed that the

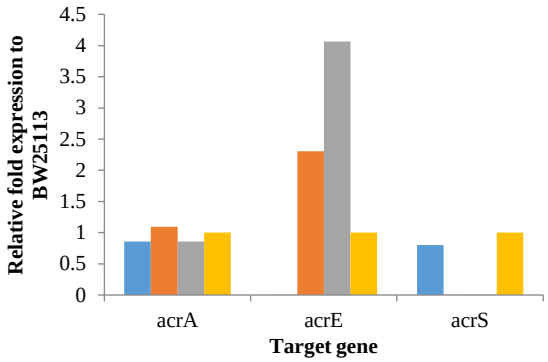

FIG. 2 acrA, acrE and acrS expression levels of Δ*acrS*Δ*kan* cultured in 1.6 µg/ml or 6.3 µg/ml kanamycin and Δ*acrE*Δ*kan* expressed as fold changes relative to the parent strain, BW25113, as measured by qPCR. From left to right (blue to yellow) within each section: Δ*acrE*Δ*kan*, Δ*acrS*Δ*kan* with 1.6 µg/ml kan, Δ*acrS*Δ*kan* with 6.3 µg/ml kan, BW25113.

primers targeting *gapA*, *acrA*, *acrE*, and *acrS* were amplifying as expected, with Ct values around 15. The melt curve of each primer set showed a single peak indicating that a single qPCR product was made. The negative controls using sterile water as the template for each primer set had Ct values above 33, providing a threshold value for a qPCR sample containing no template. RNA extracts prior to the TURBO DNA-free™ Kit (Ambion) had Ct values around 25, but Ct values greater than 33 after TURBO DNA-free™ Kit (Ambion), indicating removal of genomic DNA contamination. The positive control demonstrated that the equipment and materials used were functioning properly, and the negative control allowed for confirmation that there would not be any false positives produced in the samples due to defective reagents.

To compare the gene expression between *E. coli* BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan*, RT-qPCR was performed using primers designed for the genes of interest, as shown in Table 2. Two independent qPCR experiments were performed in triplicate. A summary of the Ct values is shown in Table S1. qPCR products of the same size were made, as shown by a single peak in the melt curve analysis (Figure S4). The melting temperatures of all samples per gene were as follows: *acrA* 84.5 ˚C, *acrE* 84 ˚C, *acrS* 78.5 ˚C, and *gapA* 82.5 ˚C. These results demonstrate that the RNA isolation and RT-qPCR were effective in the conversion of pure RNA to cDNA and amplification of the expected genes.

We next analyzed our qPCR data to compare the expression of *acrA*, *acrE*, and *acrS* using the gene study function in CFX Manager™ Software (BioRad). BW25113 cDNA was used as the control for the baseline expression levels, and the *gapA* primer was used as an internal positive control gene (9). The results of the normalized expression with respect to BW25113 and *gapA* expression levels are shown in Figure 2. The expression of *acrA* is similar across all three strains. As expected, expression of *acrE* in Δ*acrE*Δ*kan* and *acrS* in Δ*acrS*Δ*kan* was not detected. Compared to *E. coli* BW25113 *acrE* expression levels, there was a two-fold increase when the Δ*acrS*Δ*kan* strain was grown in 1.6 µg/ml kanamycin and a four-fold increase when the Δ*acrS*Δ*kan* strain was grown in 6.3 µg/ml kanamycin. There was not a large difference seen in the *acrS* expression levels between BW25113 and Δ*acrS*Δ*kan*. In summary, these results show that *acrE* expression in Δ*acrS*Δ*kan* is higher than BW25113 and Δ*acrE*Δ*kan*, but there is no difference in *acrA* expression levels among the three strains.

DISCUSSION

E. coli bacteria achieve multidrug resistance through the stress-induced regulation of multiple efflux pumps, including AcrE (6). In this study, we investigated whether AcrS was involved in negatively regulating *acrE* expression and therefore impacting kanamycin resistance. This was done with the initial acquisition of the necessary strains lacking a kanamycin resistant cassette (BW25113, Δ*acrS*Δ*kan* and Δ*acrE*Δ*kan*) and followed by PCR confirmation of the presence or absence of *acrE, acrS,* and *gapA* in the three strains. A MIC of kanamycin was used to determine how the deletion of *acrS* would affect the intrinsic kanamycin resistance in BW25113, and RT-qPCR was used to demonstrate the effect of Δ*acrS*Δ*kan* on *acrE* levels. We hypothesized that an *acrS* deletion would lead to an increase in *acrE* expression and kanamycin resistance.

Kanamycin MIC assays were done to determine the level of kanamycin resistance in the three strains BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan*. Δ*acrS*Δ*kan* displayed a higher kanamycin resistance than BW25113, thereby confirming results obtained by Belmans *et al.* Δ*acrS*Δ*kan* bacteria also demonstrated an elevated kanamycin resistance compared to the Δ*acrE*Δ*kan* strain as expected. In the absence of the potential repressor AcrS, it is possible that the *acrAB* and *acrEF* operons are not inhibited by this repressor, leading to an inflated expression of *acrAB* and *acrEF.* A greater production of these efflux pumps would create the components to pump out more of the substrates present, specifically kanamycin in this study. Deletion of another RND efflux pump other than the main efflux pump AcrAB should not affect the antibiotic resistance in *E. coli* because other pumps are not constitutively expressed (6). It was predicted that Δ*acrE*Δ*kan* should have the same level of kanamycin resistance as the parent strain. Moreover, the method used for the MIC had a range of starting concentrations of culture from 10^4 to 10^5 cfu/ml, which could have affected the resulting MIC values.

This may have led to some discrepancies in the detected MIC values in the Δ*acrE*Δ*kan* strain, which ranged from 3.1 to 6.3 μg/ml and if repeated should have a closer range in starting concentrations. A potential explanation for these differences could be that *acrS* regulation may function in the same way as *acrR* regulation in which *acrR* negatively regulates itself. Overexpression of *acrAB* has been observed to increase the levels of *acrR* as well implying a feedback loop from *acrAB* to *acrR* (7). AcrS may also be part of a negative feedback loop in which the lack of *acrE* allows for the downregulation of *acrS* as it may no longer be needed for *acrE* repression. Therefore, the downregulation of *acrS* may show a phenotype similar to that of Δ*acrS*Δ*kan* with increased kanamycin resistance. This result also indicates that there may be more factors that contribute to *acrEF* regulation and are affected by the absence of *acrE*. Also, because AcrB and AcrF are homologues, functional AcrF may still be produced in the *acrE* mutant. It has been reported that AcrF may complement an *acrB* mutant and function as an efflux pump with AcrA, AcrF, and TolC (13). Consequently, if AcrF is produced and necessary for acquiring kanamycin resistance in Δ*acrE*Δ*kan*, it is possible that is was combined with AcrA and TolC creating the slight increase in kanamycin resistance. Further research is required to unravel the interplay between these systems.

To explain the observed increase in kanamycin resistance in the MIC, RT-qPCR was performed to quantitatively measure the levels of *acrE, acrA,* and *acrS* in each strain. The role of AcrS as a possible repressor of both AcrAB and AcrEF allowed us to reason that an *acrS* deletion would result in an increased expression in *acrE* and *acrA*. In Figure 2 it is shown that in Δ*acrS*Δ*kan* strains, the *acrE* levels are elevated with respect to BW25113, while the *acrA* levels are similar to the parent strain. These results support the notion that AcrS is a repressor of *acrEF* as the absence of AcrS should result in this increase of *acrE*. The lack of an increase in *acrA* levels in the Δ*acrS*Δ*kan* strain grown at two different kanamycin concentrations however was unexpected because of the previously reported role of AcrS in the repression of *acrAB* (5).

E. coli bacteria use numerous RND efflux pumps that are not expressed at the same time, and therefore our results provide support for the idea that the AcrAB efflux pump may not be required for intrinsic kanamycin resistance. If AcrAB does not participate in pumping out kanamycin, it would not be induced in the presence of this particular stressor. Further, other antibiotics may induce the upregulation of the efflux pump necessary for resistance and downregulate the other pumps to prevent excessive protein production (5). The present results suggest the increased MIC in Δ*acrS*Δ*kan* may be due to the increased *acrE* levels leading to greater amounts of AcrEF and not a contribution from AcrAB.

Figure 2 also shows that *acrE* expression increases in Δ*acrS*Δ*kan* with increasing concentrations of kanamycin. The larger amount of stressor (kanamycin) present may upregulate *acrE* so that the cells can survive. In the Δ*acrE*Δ*kan* strain, the levels of *acrA* and *acrS* were relatively close to the levels detected in the parent strain suggesting that AcrE does not greatly affect *acrA* and *acrS* expression (Figure 2). This study contributes to further the understanding of intrinsic resistance to a broad substrate range in which different efflux pumps play a role in the resistance to distinct antibiotics in Gram-negative bacteria. However, because the qPCR analysis only had one or two biological replicates in each condition, a significant effect on gene expression cannot be determined.

In conclusion, we show that *E. coli* strain BW25113 containing an *acrS* deletion grown in the presence of 1.6 μg/ml kanamycin results in increased expression of *acrE* as measured by qPCR. Moreover, *acrE* expression appears to be elevated when a higher concentration of kanamycin is used, indicating that *E. coli* may be upregulating efflux pump production in response to more kanamycin in the cell. However, more biological replicates would need to be performed to validate our results. Our gene expression data show only the relative amount of *acrE* in each strain with respect to the parent strain, which means we cannot infer the absolute level of *acrE* necessary to confer resistance to kanamycin in *E. coli*. In further support of our hypothesis, kanamycin resistance appears to be higher in the Δ*acrS*Δ*kan* mutant than the Δ*acrE*Δ*kan* mutant and the parent strain. We believe that the absence of the negative regulator AcrS allows *acrE* expression to be upregulated. However, AcrS may not be the only regulator of *acrE*, so we cannot assume that the expression of this gene is uncontrolled in the Δ*acrS*Δ*kan* mutant.

FUTURE DIRECTIONS

In this study, we have shown that an *acrS* deletion mutant leads to an increase in the expression of *acrE* in the presence of kanamycin. Furthermore, kanamycin resistance is higher in these Δ*acrS*Δ*kan* mutants compared to the parent strain BW25113, and Δ*acrE*Δ*kan*. Future investigations could be performed to compare the relative

abundance of AcrE protein in BW25113, Δ*acrS*Δ*kan*, and Δ*acrE*Δ*kan* using a membrane extraction method followed by resolution of the products using Polyacrylamide Gel Electrophoresis (SDS-PAGE). If the protein band intensity of AcrE in Δ*acrS*Δ*kan* mutants grown in LB and kanamycin is greater than BW25113, this would support our current findings on AcrEF-mediated kanamycin resistance. As an alternative strategy to our original experimental design, an *acrS* overexpression *E. coli* mutant could also be used to support our findings by measuring *acrE* expression levels in these mutants after exposure to kanamycin. We would expect a lower *acrE* level compared to the parent strain because the increase in AcrS would act to further negatively regulate *acrE* expression.

Because the MIC assay results for our Δ*acrE*Δ*kan* strain were inconsistent, we propose using a disc diffusion assay to look at kanamycin resistance in our three strains instead. A larger zone of inhibition would potentially correlate with higher susceptibility to the antibiotic. Multiple concentrations of kanamycin diffusion discs could be tested to find a concentration at which growth of bacteria is not inhibited, which would provide an analogous finding to the MIC.

In the original study by Hirakawa *et al.*, resistance to various antibiotics such as tetracycline, ampicillin and erythromycin were shown to occur through the efflux pump AcrAB, which is negatively regulated by AcrS (5). However, they did not include any aminoglycoside-based drugs in their experiments. Therefore, we investigated the role of *acrS* in kanamycin resistance which serves as a follow-up study to work conducted by Belman *et al.* (9). Although in this study, we only tested one antibiotic in the aminoglycoside family. Therefore, aminoglycosides such as gentamicin and streptomycin could be further explored to better understand the role of AcrS on resistance to different antibiotics by regulation of the RND efflux pumps.

Finally, a double deletion mutant of *acrE* and *acrS* could be used to confirm if alternative mechanisms of kanamycin resistance exist in *E. coli* BW25113.

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